Design and synthesis of inhibitors for N-myristoyltransferase, a promising treatment for parasitic diseases

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Abstract

*N*-myristoyltransferase (NMT) is a monomeric enzyme, which is ubiquitous in eukaryotes, catalysing an irreversible co-translational transfer of myristate to a particular set of proteins containing an *N*-terminal glycine. The work presented in this thesis illustrates the use of synthesised small molecules as a probe to investigate the role of NMT in parasites.

Chapter 1 looks at the two parasitic diseases that this project targets: malaria and visceral leishmaniasis, which are caused by *Plasmodium falciparum* (Pf) and *Leishmania donovani* (Ld) respectively. Additionally, biological evidence to show that NMT is a promising drug target in these parasites is presented.

Chapter 2 describes a piggy-back strategy, which was applied to discover a benzofuran compound with moderate inhibition against PfNMT. This molecule was re-synthesised and then validated as a hit by an optimised scintillant proximity assay.

Chapter 3 details the process of drug design based on the inhibition data against PfNMT and structural information of the related enzymes, in which a library of 150 benzofuran analogues was generated. Importantly, the cell inhibition results provided strong evidence to chemically validate NMT as a drug target in *P. falciparum*, leading to the identification of a lead candidate.

Chapter 4 gives the initial structure-activity relationships for LdNMT inhibitors, whereby the whole library of the PfNMT inhibitors was screened against LdNMT. In addition, combined with the parasite inhibition assay, two compounds were determined to be the early lead candidates for LdNMT inhibitors.

Chapter 5 provides an overview of the work detailed in this thesis and suggests the future directions that will continue the advancement of this project.
Acknowledgements

First and foremost a big thanks to my supervisor Robin (Prof. Robin Leatherbarrow) for the support and guidance during the PhD period. I thoroughly enjoyed the experience with all the ups and downs and learned so much from the project, thank you for offering me this opportunity! Ed (Dr. Ed Tate), you are so smart, always sharing your wisdom with me, watching out for the progress of the project. Thank you both for your great help.

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## Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>3D-QSAR</td>
<td>3D-quantitive structure-activity relationship</td>
</tr>
<tr>
<td>ACT</td>
<td>artemisinin-based combination therapy</td>
</tr>
<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>Ar</td>
<td>aromatic ring</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ARL</td>
<td>ARF-like</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>BMIDM</td>
<td>bone marrow derived macrophage</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAN</td>
<td>ceric ammonium nitrate</td>
</tr>
<tr>
<td>CAP</td>
<td>cytoskeletal-associated protein</td>
</tr>
<tr>
<td>Cbz</td>
<td>carbobenzyloxy</td>
</tr>
<tr>
<td>CDPK</td>
<td>calcium-dependent protein kinase</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIAD</td>
<td>diisopropyl azodicarboxylate</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDCI</td>
<td>1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>eq.</td>
<td>equivalent(s)</td>
</tr>
<tr>
<td>ESI</td>
<td>electronspray ionisation</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
</tr>
<tr>
<td>etc.</td>
<td>et cetera</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescent-activated cell sorting</td>
</tr>
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Design and synthesis of inhibitors for N-myristoyltransferase

FCS  fetal calf serum
Fmoc fluorenylmethyloxycarbonyl
GAP45 45kDa gliding-associated protein
GRASP golgi re-assembly stacking protein
HASP hydrophilic acylated surface protein
HE hydroethidine
HOBt N-Hydroxybenzotriazole
HPLC High performance liquid chromatography
hr hour
HTS high throughput screen
IC₅₀ concentration of a drug that is required for 50% inhibition in vitro
i-Pr iso-propyl
J coupling constant
Kₐᵥ app apparent Michaelis constant
LC-MS Liquid chromatography-mass spectrometry
m-CPBA meta-Chloroperoxybenzoic acid
Me methyl
MMV Medicines for Malaria Venture
MTIP myosin A tail domain-interacting protein
Mw molecular weight
Myr myristate
m/z mass to charge ratio
NADPH nicotinamide adenine dinucleotide phosphate
n.d. not determined
NIMR National Institute of Medical Research
NMR Nuclear magnetic resonance
NMT N-myristoyltransferase
  PfNMT - *Plasmodium falciparum* N-myristoyltransferase
  LdNMT - *Leishmania donovani* N-myristoyltransferase
  HsNMT1 - *Homo sapiens* N-myristoyltransferase 1
  HsNMT2 - *Homo sapiens* N-myristoyltransferase 2
  PvNMT - *Plasmodium vivax* N-myristoyltransferase
  LmNMT - *Leishmania major* N-myristoyltransferase
  CaNMT - *Candida albicans* N-myristoyltransferase
  TbNMT - *Trypanosoma brucei* N-myristoyltransferase
  ScNMT - *Saccharomyces cerevisiae* N-myristoyltransferase
PDB Protein Data Bank
PFT protein farnesyltransferase
PG protecting group
Ph phenyl
PIP5K phosphatidyl-inositol 4-phosphate 5-kinase
Design and synthesis of inhibitors for N-myristoyltransferase

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PK</td>
<td>pharmacokinetics</td>
</tr>
<tr>
<td>PPEF</td>
<td>protein phosphatase with EF-hand</td>
</tr>
<tr>
<td>ppm</td>
<td>part(s) per million</td>
</tr>
<tr>
<td>PyBOP</td>
<td>benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>r.t.</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SMP</td>
<td>small myristoylated protein</td>
</tr>
<tr>
<td>SPA</td>
<td>scintillation proximity assay</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>half life</td>
</tr>
<tr>
<td>t-Bu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>tₕ</td>
<td>retention time</td>
</tr>
<tr>
<td>Tr</td>
<td>trityl</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>VL</td>
<td>visceral leishmaniasis</td>
</tr>
<tr>
<td>Vₘₐₓ</td>
<td>Maximum rate of reaction</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
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Nomenclature and symbolism for amino acids were adopted from the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature 1972).
1 Introduction and overview

1.1 Parasitic diseases

Parasitic diseases are infectious diseases caused by parasites, which affect all living organisms and cause mild discomfort or even death\(^1\). Approximately 240 parasitic diseases are reported to be spread from insects to humans\(^2\); among them, malaria and visceral leishmaniasis are most prevalent and deadly\(^3\).

1.1.1 Malaria

Malaria, a tropical disease, remains one of the most severe global health problems of our time, with 3 billion people at risk, approximately 243 million cases and 863,000 attributed deaths reported globally in 2008\(^4\). It is caused by micro-organisms of the genus *Plasmodium* that are transmitted to human hosts via female *Anopheles* mosquitoes. There are five different species that can infect humans: *P. vivax*, *P. ovale*, *P. malariae*, *P. Knowlesi* and *P. falciparum*; of which *falciparum* malaria causes most mortality (93%)\(^4\), mainly in children below the age of five.

1.1.1.1 Global distribution

Malaria has a broad distribution in both the tropics and subtropics. The countries of sub-Saharan Africa account for the majority of all malaria cases, with the remainder mostly clustered in India, Brazil, Afghanistan, Sri Lanka, Thailand, Indonesia, Vietnam and Cambodia\(^5\) (Figure 1.1).
1.1.1.2 Life cycle of malaria parasites

The life cycle of malaria parasites involves two hosts, the human and mosquito. Beginning with a bite of an infected female *Anopheles* mosquito, sporozoites are transmitted to a human host. The sporozoites migrate to the liver in ca. 30 minutes (in the case of *P. falciparum*) and invade hepatocytes, maturing into schizonts (ca. 5 days in *P. falciparum*; *P. vivax* forms a dormant stage, which lasts for weeks or even years before continuing to schizonts). Schizonts then replicate, leading to the rupture of hepatocytes and the release of merozoites. After initial replication in the liver (exo-erythrocytic cycle), the extracellular merozoites rapidly infect red blood cells, where the parasites mature and replicate in a ca. 48 hour cycle (in the case of *P. falciparum*), through immature and mature trophozoites, followed by schizonts which then rupture, releasing merozoites to infect more erythrocytes (asexual erythrocytic cycle). Blood stage parasites are mainly responsible for the clinical symptoms of malaria, such as fever, diarrhoea and vomiting; therefore most chemotherapeutic treatments of the disease target this stage. Some parasites differentiate into gametocytes, which in turn infect a healthy mosquito during its blood meal from an infected human host. For the period of

Figure 1.1 Distribution of malaria risk areas in the world in 2007

![Figure 1.1 Distribution of malaria risk areas in the world in 2007](image)
the sexual reproduction of parasites in a mosquito (sporogonic cycle), male and female gametes yield zygotes, which then develop into ookinetes and mature into oocysts. The oocysts grow and rupture to release sporozoites that migrate to the mosquito’s salivary glands, which can be delivered to a new human host to start another life cycle\(^8\) (Figure 1.2).

\[\text{Figure 1.2 Life cycle of malaria parasite}\]

\((\text{The Picture is from http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Malaria_il.htm})\)

\(\text{During a blood meal, the parasites (sporozoite form) from an infected mosquito are delivered into the bloodstream of the human host. After rapid invasion of the liver cells, the parasites develop and replicate in the exo-erythrocytic cycle. The rupture of schizonts releases merozoites which further infect red blood cells to undergo the erythrocytic cycle, a phase of asexual reproduction producing merozoites to infect more erythrocytes which accounts for the most clinical symptoms of malaria. Some merozoites differentiate into gametocytes which are ingested as a part of a healthy mosquito’s blood meal, to initiate the sexual reproduction called sporogonic cycle. The male and female gamates form a zygote and subsequently mature to oocyst. Sporozoites, once ruptured from oocysts, migrate to the mosquito’s salivary glands from where sporozoites return to another human host during a new bite.}\)
1.1.1.3 Development of antimalarial drugs

Attempts to develop vaccines for malaria are in their infancy because of the complexity of malaria parasites and lack of funding\(^\text{[9]}\). In 2010, GSK Biologicals launched a phase III trial of its most promising vaccine candidate, known as RTS,S, in seven African countries along with the local leading research institutions\(^\text{[10]}\). Currently however, chemotherapy remains the only option for the treatment.

Chloroquine (Figure 1.3) was used extensively in the treatment of all types of malaria for several decades as it was safe, highly effective and cheap. However, since chloroquine resistance was first reported in Thailand in 1957, resistance spread so fast that today chloroquine has lost its efficacy in most epidemic areas\(^\text{[11]}\). Systematic exploration of chemical space around the 4-aminoquinoline scaffold of chloroquine discovered a promising derivative AQ-13 (Figure 1.3), showing excellent inhibition against chloroquine-resistant *P. falciparum* parasites and satisfactory pharmacokinetics\(^\text{[12, 13]}\). This molecule is currently in a phase II clinical trial\(^\text{[14]}\). Amodiaquine (Figure 1.3) is another effective agent for chloroquine-resistant parasites\(^\text{[15]}\). However, its \(p\)-hydroxyanilino moiety was found to be oxidised by cytochrome P450 to a chemically reactive quinoneimine species in rats, thereby accounting for the observed toxicity in humans\(^\text{[16]}\). Regioisomers of amodiaquine, proposed and developed by O’Neil\(^\text{[17]}\), successfully bypassed the formation of the toxic metabolite, resulting in the discovery of \(N\)-t-butyl isoquine\(^\text{[18]}\) (Figure 1.3). Unfortunately, this molecule suffered from an unexpected side effect at the high dose regimen and discontinued in the phase I trial\(^\text{[19]}\).

Artemisinin (Figure 1.3), a plant-derived natural product initially identified by Chinese researchers\(^\text{[20, 21]}\), was shown to be highly potent for multi-drug resistant malaria parasites. To overcome its poor bioavailability, subsequent work concentrated on the modification of the C-10 moiety, leading to the identification of artemisinin’s semisynthetic derivatives artemether, artesunate and dihydroartemisinin (Figure 1.3). At present, artemisinin-based combination therapies (ACT) are recommended by World Health Organization for malaria in regions where drug resistance against other
antimalarials is prevalent. Artemisone is a single-drug therapy, displaying less toxicity than current ACTs, now in a phase II clinical trial\[^6\]. However, high costs could prevent it from being widely used in the future. The supply of artemisinin is a major drawback of this therapy category since purely chemical synthesis is generally too expensive\[^{22-25}\] and sole extraction from natural plants is not ecologically friendly. With the rapid development of biotechnology, a combination of bioengineering and chemical synthesis becomes feasible: Keasling’s lab successfully transplanted the plant biosynthetic genes into yeasts to allow the production of artemisinic acid\[^{26}\] (Figure 1.3), which can be simply converted to artemisinin in 5 steps\[^{27}\].

Artemisinin’s unusual endoperoxide bridge is essential to its mechanism of action, which involves a complexation with haem ($\text{Fe}^{2+}$) by the coordination of the peroxide bridge with iron. This binding interrupts the haemin detoxification process used by the parasite and thus frees the radical species which can kill the parasite\[^{28}\]. Inspired by this mechanism, totally synthetic peroxide-containing compounds were designed and synthesised. Encouragingly, they were found to mimic the behaviour of artemisinin in parasites\[^{29}\]. Among them, 1,2,4-trioxane OZ277 (Figure 1.3) exhibited comparable in-vivo activity with current ACTs\[^{30}\]. However, a phase II clinical study revealed that this compound had a 3-fold lower exposure in malaria patients compared with healthy volunteers\[^{31}\]. Subsequent study found that its analogue or OZ439\[^{32}\] (Figure 1.3) showed a longer half-life with enhanced exposure than OZ277 in malaria patients\[^{31}\] and the clinical evaluation of OZ439 is underway\[^{6}\]. Given the stability concerns of the 1,2,4-trioxolane moiety, O’Neill proposed a class of 1,2,4,5-tetraoxane compounds\[^{33}\] which showed comparable inhibition and better stability than the 1,2,4-trioxolane series, leading to the discovery of another clinical candidate RKA182 (Figure 1.3). All the above drugs and clinical candidates are supposed to inhibit the growth of hemozoin, a key target for the development of the antimalarial drugs\[^{34-36}\].
Artemisinin resistance was first recorded in western Cambodia in 2009\cite{37}. Therefore, it is urgent to develop new drugs, especially considering that no novel class of antimalarials has been introduced into clinical practice since 1996\cite{38}. Funded by MMV (Medicines for Malaria Venture) and in cooperation with the pharmaceutical industry, more than five million compounds (natural products and synthetic molecules) have been screened for activity against malaria parasites\cite{39, 40}; among them, Norvatis identified a synthetic compound related to the spiroindolone class and successfully optimised this molecule to NITD609 (Figure 1.3), possessing sub-nM parasite inhibition and satisfactory pharmacokinetics\cite{41}. More excitingly, the compound was suggested to adopt a novel mechanism of action different from those of artemisinin and chloroquine\cite{42}. Therefore, this molecule is viewed as a promising alternative to artemisinin if artemisinin resistance were to spread and is currently in a phase I clinical trial\cite{43}.

Figure 1.3 Structures of antimalarial drugs in clinical use

Artemisinin resistance was first recorded in western Cambodia in 2009\cite{37}. Therefore, it is urgent to develop new drugs, especially considering that no novel class of antimalarials has been introduced into clinical practice since 1996\cite{38}. Funded by MMV (Medicines for Malaria Venture) and in cooperation with the pharmaceutical industry, more than five million compounds (natural products and synthetic molecules) have been screened for activity against malaria parasites\cite{39, 40}; among them, Norvatis identified a synthetic compound related to the spiroindolone class and successfully optimised this molecule to NITD609 (Figure 1.3), possessing sub-nM parasite inhibition and satisfactory pharmacokinetics\cite{41}. More excitingly, the compound was suggested to adopt a novel mechanism of action different from those of artemisinin and chloroquine\cite{42}. Therefore, this molecule is viewed as a promising alternative to artemisinin if artemisinin resistance were to spread and is currently in a phase I clinical trial\cite{43}.
1.1.2 Visceral leishmaniasis

Visceral leishmaniasis (VL, also known as kala-azar) is another lethal parasitic disease, characterised by fever, weight loss, anemia and depression of the immune system, threatening about 200 million people with an estimated 500,000 cases annually and greater than 50,000 deaths reported in 2004\textsuperscript{[44]}. The real burden of VL might be much higher than the number of reported cases because of serious underreporting\textsuperscript{[45]} and poor access to diagnosis and treatment\textsuperscript{[46]} in some developing countries. The disease is transmitted by a female \textit{Phlebotomine} sandfly and caused by different species of \textit{Leishmania} parasites.

1.1.2.1 Global distribution

90\% of reported VL cases occur in just five countries: Bangladesh, Brazil, India, Nepal and Sudan\textsuperscript{[47, 48]} (Figure 1.4). The interesting problem associated with the geography affects the choice of treatment. In South Asia and East Africa (Old World) where drug resistance is a major concern, humans are the main reservoir for disease transmission and \textit{L. donovani} is the causative agent\textsuperscript{[49]}; in contrast, in South America (New World), domestic dog infection is the principal route to transmit the disease and drug resistance is no longer a problem\textsuperscript{[48]}.

**Figure 1.4 Global distribution of visceral leishmaniasis in 2009\textsuperscript{[48]}**
1.1.2.2 Life cycle of leishmania parasites

Beginning with a bite of a female *Phlebotomine* sandfly, promastigote parasites (the infective stage) are transmitted into a human host and phagocytised by macrophages to form amastigotes, the causative stage of leishmaniasis. After that, the amastigotes replicate inside cells, responsible for a variety of clinical symptoms, such as fever and weight loss. During another blood meal from an infected human, a healthy sandfly takes up the infected macrophages which are then converted back to promastigotes in fly’s midgut. The resulting promastigotes multiply and migrate to fly’s proboscis, which can be delivered to another human host to start a new life cycle (Figure 1.5).

![Diagram of leishmania parasit‌e life cycle](http://www.uni-tuebingen.de/modeling/Mod_Leish_Cycle_en.html)

**Figure 1.5 Life cycle of leishmania parasites**

*(The picture is from http://www.uni-tuebingen.de/modeling/Mod_Leish_Cycle_en.html)*

During a blood meal, promastigotes (infective stage) are transmitted from a female sandfly into a human host, where they are phagocytised and transformed into amastigotes (causative stage). Amastigotes continue to replicate inside cells, which are then taken up by a healthy sandfly during its blood meal. In fly’s midgut, amastigotes are converted back to promastigotes, which undergo a further replication and migrate to fly’s proboscis, where the parasites return to another human host during a new bite.
1.1.2.3 Development of antileishmanial drugs

Although VL is one of the most severe tropical diseases in the world, its research into new drugs has stalled for a long time\textsuperscript{49}. Despite the toxicity associated with gastrointestinal symptoms\textsuperscript{51} and emerging clinical resistance\textsuperscript{52, 53}, antimony-based therapy (sodium stibogluconate, meglumine antimoniate, Figure 1.6), developed nearly 70 years ago, is still widely used as the first-line antileishmanial treatment because it requires minimal monitoring. Miltefosine (Figure 1.6), originally used as an anti-cancer drug, was later identified to be the first oral drug for VL\textsuperscript{54}. However, rat models demonstrated that miltefosine induced testicular changes\textsuperscript{55}, raising concerns of potential teratogenicity, thereby requiring careful use and monitoring especially for women in child-bearing age\textsuperscript{56}. Amphotericin B (Figure 1.6), especially its liposomal formulation, is a breakthrough for the treatment of VL since it is safe and effective, requiring a low administrable dose\textsuperscript{57}. However, the high cost of US $300 per patient is still prohibitive for most VL-epidemic countries\textsuperscript{49}. Paromomycin (Figure 1.6), originally used as a broad spectrum antibiotic against bacteria and intestinal protozoa, showed good cure rates for the VL patients with minor toxicity\textsuperscript{58}. In addition, the low cost (US $15 per treatment\textsuperscript{49}) is a big advantage over other available drugs. However, resistance was observed \textit{in vitro}\textsuperscript{59}. Therefore, paromomycin is suggested for use in combination with other therapies to avoid future problems with clinical failure\textsuperscript{60}.

Combination regimens have been intensively and successfully used for the treatment of malaria, thereby forming the rationale for the same approach applied to VL. Currently, clinical trials of a variety of combinations based on the five available antileishmanial drugs are underway\textsuperscript{61} (data from \url{http://clinicaltrials.gov/ct2/home}). In terms of the novel agents, sitamaquine (Figure 1.6), an oral treatment of VL, was developed by the Walter Reed Army Institute and GSK\textsuperscript{62}, receiving promising efficacy and acceptable safety profile from a phase II clinical trial\textsuperscript{63}; Institute Pasteur Korea screened a 26,500-compound library with diverse structures, leading to the identification of CH872 (excellent inhibition and toxicity tolerance \textit{in vitro}, Figure 1.6) as a good candidate to the VL drug discovery pipeline\textsuperscript{64}; a therapeutic vaccine for VL via
(re-) activation of CD8+ T-cell responses is currently progressing through the final pre-clinical stages, with the aim to conduct a phase I clinical trial in early 2011 (personal communication from Prof. D. Smith, York University).

Figure 1.6 Structures of antileishmanial drugs in clinical use

1.1.3 Summary

Malaria and VL affect hundreds of millions of people worldwide and result in significant mortality and devastating social and economic consequences, especially in poor third world countries (Table 1.1). However, most available drugs that were developed decades ago, frequently suffer from resistance or severe toxicities. In terms of the antimalarials, ACT is currently heavily relied on to treat the disease and new treatments are still based on the structure of artemisinin or its key peroxide moiety (OZ series and RKA182), raising potential resistance concerns (Table 1.1). In fact, signs of artemisinin resistance were observed in western Cambodia in 2009 although it is not...
yet a significant clinical problem. Nevertheless, it is still urgent to discover drug candidates with novel structures and inhibition mechanisms. The situation for VL is even more challenging, whereby high supply costs prevent amphotericin from being widely used and other available drugs are associated with severe toxicities or resistance (Table 1.1). No novel compounds nor vaccines are expected to be approved until 2014 at the earliest\cite{65, 31}. Thus, new strategies of chemotherapy are desperately needed.

Table 1.1 Current situation of malaria and visceral leishmaniasis

<table>
<thead>
<tr>
<th></th>
<th>Malaria</th>
<th>Visceral leishmaniasis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Global burden</strong></td>
<td>Billion people at risk</td>
<td>250 million people in threat</td>
</tr>
<tr>
<td></td>
<td>243 million cases</td>
<td>500,000 cases</td>
</tr>
<tr>
<td></td>
<td>86300 deaths</td>
<td>&gt;50,000 deaths</td>
</tr>
<tr>
<td><strong>Global distribution</strong></td>
<td>Sub-Saharan Africa, India, Brazil, Afghanistan, Sri Lanka, Thailand,</td>
<td>Bangladesh, Brazil, India, Nepal</td>
</tr>
<tr>
<td></td>
<td>Indonesia, Vietnam, Cambodia</td>
<td>Sudan</td>
</tr>
<tr>
<td><strong>Transmission vector</strong></td>
<td>Female <em>Anopheles</em> mosquito and human host</td>
<td>Female <em>Phlebotomine</em> sandfly, human</td>
</tr>
<tr>
<td></td>
<td></td>
<td>host in south Asia, east Africa or dog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>host in South America</td>
</tr>
<tr>
<td><strong>Causative parasites</strong></td>
<td><em>P. falciparum</em> (most deadly)</td>
<td><em>L. donovani</em> (infests human)</td>
</tr>
<tr>
<td></td>
<td><em>P. vivax</em> (most widespread, latent stage)</td>
<td><em>L. infantum</em> and <em>L. chagasi</em> (infect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dog)</td>
</tr>
<tr>
<td><strong>Available drugs</strong></td>
<td>Artemisinin-based combination therapy (cost, supply shortage, potential</td>
<td>Antimonials, Miltesofine (toxicity)</td>
</tr>
<tr>
<td>(their weakness)</td>
<td>resistance)</td>
<td>Paromomycin (rapid drug resistance)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amphotericin B (cost)</td>
</tr>
<tr>
<td><strong>Novel drugs in</strong></td>
<td>Artemisone</td>
<td>Sitamaquine</td>
</tr>
<tr>
<td>clinical trial**</td>
<td>OZ series (1,2,4-trioxane series)</td>
<td>CD8⁺ T-cell vaccine</td>
</tr>
<tr>
<td></td>
<td>RKA182 (1,2,4,5-tetraoxane series)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NITD609 (spiroindolone series)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RTS,S (Vaccine)</td>
<td></td>
</tr>
<tr>
<td><strong>Therapeutic challenges</strong></td>
<td>Circumventing drug resistance</td>
<td>Cheap, safe, orally bioavailable drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with minimal monitoring</td>
</tr>
</tbody>
</table>
1.2 N-myristoyltransferase

N-myristoyltransferase (NMT) is a monomeric enzyme, ubiquitous in eukaryotes. NMT catalyses a co-translational transfer of a saturated 14-carbon acyl or myristate from myristoyl-Coenzyme A (myr-CoA) to a protein substrate containing an N-terminal glycine via an amide bond\[^{66}\]. N-myristoylation plays a key role in protein trafficking, mediating protein-protein interactions and stabilising protein structures\[^{67}\].

1.2.1 Mechanism of NMT

N-myristoylation by NMT follows an ordered Bi-Bi mechanism, in which the binding of myr-CoA to NMT occurs prior to the binding of a protein substrate\[^{68}\]; bound myr-CoA facilitates the opening of a second binding pocket for a protein substrate, followed by a transfer of myristate to the protein substrate via a nucleophilic addition-elimination reaction, finally with the release of CoA and the myristoylated protein.

![Figure 1.7 The catalytic cycle of NMT](image)

The enzyme (shown in yellow) first binds myr-CoA, inducing a conformational change which is to accommodate the binding of a peptide substrate (coloured in green). Then the amide bond (drawn in cyan) is formed via a nucleophilic addition-elimination reaction, followed by the release of CoA and the myristoylated protein\[^{68}\].
1.2.2 NMT is a promising drug target

Lipid modification of cellular proteins in eukaryotes, which includes myristoylation, palmitoylation and prenylation, plays important roles in protein trafficking and signal transduction in living systems\cite{69}. In general, myristoylation or prenylation produces transient interactions of proteins with membranes, a prerequisite for palmitoylation to yield dually lipidated proteins that have long-lived associations with cellular membranes\cite{70}. Therefore, inhibition of myristoylation has the potential to disrupt multi-protein pathways, an attractive characteristic to prevent resistance.

1.2.2.1 NMT is an anti-fungal target

NMTs are well characterised in *Saccharomyces cerevisiae*\cite{71}, *Candida albicans*\cite{72}, *Cryptococcus neoformans*\cite{73} and mammalian cells (NMT1\cite{74} and NMT2\cite{75}). Disruption of the yeast NMT gene was reported to cause recessive lethality in *S. cerevisiae*\cite{71} and genetic studies revealed that NMT was essential for the viability of *C. neoformans*\cite{76} and *C. albicans*\cite{77}. Researchers at Roche further showed that a lack of NMT resulted in a loss of ability of *C. albicans* to infect mice\cite{78}. In addition, biochemical analysis showed high conservation of myr-CoA binding sites, but divergent peptide binding specificities between human and fungal NMTs\cite{67}, indicating a possibility to gain selectivity when targeting the peptide binding pocket\cite{79}. As a consequence, NMT was viewed as a novel anti-fungal target by a number of pharmaceutical companies (Pfizer, Roche and SSP, merged into Boehringer Ingelheim in 2010), with the aim of developing selective non-peptidic inhibitors. Several lead candidates were identified from these projects, showing both excellent potency and selectivity over human NMTs *in vitro* (Pfizer, unpublished data; Roche\cite{80-83}; SSP\cite{84}). Unfortunately, given a narrow spectrum of antifungal activity of these molecules, these projects were terminated; nevertheless this data proves the concept that targeting the peptide binding pocket of NMT to develop selective inhibitors is feasible.
1.2.2.2 Essentiality of NMT in parasites

NMTs from parasitic organisms have been well characterised during the past decade, which include *Plasmodium falciparum* (Pf, most deadly species in malaria)[85], *Plasmodium vivax* (Pv, a latent but highly prevalent form of *Plasmodium* species), *Leishmania major* (Lm, causative agent of cutaneous leishmaniasis)[86], *Leishmania donovani* (Ld, causative agent of VL)[87] and *Trypanosoma brucei* (Tb, causative agent of African sleeping sickness)[86]. Myr-CoA analogues, the non-specific competitive NMT inhibitors, were reported to inhibit the growth of promastigotes of *L. major* parasites[86], indicating a correlation between the loss of parasite viability and reduced N-myristoylation. In addition, disruption of the LmNMT gene resulted in the demise of promastigotes[86]. The same approach was applied to *L. donovani* promastigotes and the results revealed that the parasites required at least one allele of the NMT gene for successful propagation in culture[87]. However, current difficulties in transfecting and knocking down the NMT gene selectively in intracellular amastigotes prevent direct evidence of essentiality of NMT in this causative stage[87, 86]. As in *T. brucei*, NMT was shown to be crucial for the viability of both host bloodstream and insect procyclic forms, using the RNA interference technique[86]. Further studies demonstrated that after silencing the NMT gene, bloodstream form *T. brucei* parasites were not able to establish an infection in a mouse model, genetically validating NMT as an anti-*trypanosomatid* target[88]. Several PfNMT inhibitors were reported to be anti-parasitic[89, 85], but it is not conclusive to show that NMT is critical for the growth of *Plasmodium* parasites. Additionally, full cycle cultivation of the asexual blood stage in *P. vivax* is currently not available, limiting the biological research into *vivax* malaria[90].

1.2.2.3 Myristoylated proteins in parasites

Aside from the chemical approach leading to effective drugs, identification of downstream myristoylated proteins and study of their biological functions is equally important to validate NMT as a drug target for parasites. Table 1.2 summarises the
known myristoylated proteins in parasites and their corresponding functions determined so far. These proteins are also found to be palmitoylated in vivo except for ARF (ADP-ribosylation factor) and ARL (ARF-like) proteins where there is no cysteine residue existing in the N-terminal motif to allow palmitoylation to occur. In *Plasmodium*, ARF<sup>1</sup><sup>[92]</sup> was shown to activate PIP5K (Phosphatidyl-inositol 4-phosphate 5-kinase), a key component to regulate cell motility and signalling<sup>[92]</sup>; GRASP1 (Golgi re-assembly stacking protein 1), as the name suggests, maintains the function of Golgi to transport proteins although recent research reported that its analogue, GRASP2, did not actually need myristoylation to mediate protein trafficking in *Plasmodium*<sup>[93]</sup>; two members of the calcium-dependent protein kinase (CDPK) family: CDPK1<sup>[94]</sup> was shown to form a motor complex with GAP45 (45kDa gliding-associated protein) and MTIP (Myosin A tail domain-interacting protein) to regulate parasite invasion<sup>[95]</sup> and CDPK4 was determined to be critical for the gametocyte differentiation<sup>[96]</sup>. In *Leishmania*, dual acylation of HASPB (hydrophilic acylated surface protein B) protein was found to allow the exposure of this variable antigen on the external surface of infective stages for immune recognition in vivo<sup>[97-99]</sup> and the same protein was later described to be essential for the differentiation of *L. major* parasites in their sand fly vector<sup>[100]</sup>; SMP1 (small myristoylated protein 1), initially characterised in a Triton X-100 insoluble membrane fraction<sup>[101]</sup>, was thought to maintain a normal flagellum function in *Leishmania*<sup>[102]</sup>. In *Trypanosome*, both ARF1 and ARL1 proteins were proven to be critical for the viability of bloodstream trypanosomes<sup>[103-105]</sup>. All the above proteins and the processes in which they are involved suggest that *N*-myristoylation plays an important role in the biology of parasites.
Table 1.2 Characterised myristoylated proteins in parasites and their biological functions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Biological function</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em> ARF1</td>
<td>Activates PIP5K, a key regulator of cell motility, secretion and cell signalling</td>
<td>Only myristoylated, but not palmitoylated</td>
<td>[92, 91]</td>
</tr>
<tr>
<td><em>P. falciparum</em> GRASP1</td>
<td>Associated with Golgi assembly stacking</td>
<td>Existence of PfGRASP2, which is not myristoylated, via a lower-eukaryote-like mechanism</td>
<td>[93]</td>
</tr>
<tr>
<td><em>P. falciparum</em> CDPK1</td>
<td>Regulates merozoites invasion</td>
<td>Unusual kinase family in <em>Plasmodium</em> and involves three N-terminal membrane anchor motifs</td>
<td>[95, 106, 94]</td>
</tr>
<tr>
<td><em>P. falciparum</em> GAP45</td>
<td>Regulates gamete formation and mosquito transmission</td>
<td>Unusual kinase family in <em>Plasmodium</em></td>
<td>[96, 107]</td>
</tr>
<tr>
<td><em>P. falciparum</em> AK2</td>
<td>Important in adenine nucleotide metabolism</td>
<td>-</td>
<td>[109]</td>
</tr>
<tr>
<td><em>L. major</em> HASPB</td>
<td>Essential for the differentiation of parasites in the sand fly vector</td>
<td>Unusual transportation behaviour</td>
<td>[97, 100, 98, 99]</td>
</tr>
<tr>
<td><em>L. major</em> SMP1</td>
<td>Vital for normal flagellum function</td>
<td>Marker of flagellum elongation during amastigote to promastigote differentiation</td>
<td>[102, 101]</td>
</tr>
<tr>
<td><em>T. brucei</em> ARF1</td>
<td>Essential in endocytosis and Golgi-lysosome trafficking</td>
<td>Only myristoylated, but not palmitoylated</td>
<td>[105]</td>
</tr>
<tr>
<td><em>T. brucei</em> ARL1</td>
<td>Critical for the viability of bloodstream trypanosomes</td>
<td>Only myristoylated, but not palmitoylated</td>
<td>[103, 104]</td>
</tr>
<tr>
<td><em>T. brucei</em> CAP5.5</td>
<td>-</td>
<td>Strictly life-cycle-regulated and only expressed in the procyclic form trypanosomes</td>
<td>[114]</td>
</tr>
</tbody>
</table>

**Abbreviations used:** ARF, ADP ribosylation factor; PIP5K, Phosphatidylinositol 4-phosphate 5-kinase; GRASP, Golgi re-assembly stacking protein; CDPK, calcium-dependent protein kinase; GAP45, 45kDa gliding-associated protein; AK, adenylate kinase; HASP, hydrophilic acylated surface protein; PPEF, protein phosphatase with EF-hand; SMP, small myristoylated protein; ARL, ARF-like; CAP5.5, a novel cytoskeletal-associated protein
1.2.3 Current NMT inhibitors

As described in Section 1.2.1, there are two binding pockets in NMT. One is the myr-CoA binding pocket and the other is the peptide binding pocket. Most reported NMT inhibitors so far target the peptide binding pocket.

1.2.3.1 Peptidomimetic CaNMT inhibitors

As mentioned in Section 1.2.2.1, CaNMT was viewed as a promising anti-fungal target. Searle (merged into Pfizer in 2000) started from the synthesis of an octapeptide (GLYASKLS-NH₂, N-terminal octapeptide from a protein substrate), undergoing a partial replacement by ω-aminoalkyl and phenyl groups at the left side, to produce dipeptide inhibitor Sc-01 with high inhibition, but moderate selectivity of 18-fold over human NMT1[115]. Swapping ω-aminoalkyl by imidazole provided Sc-58272, showing comparable potency but much improved selectivity[116]. Although little antifungal activity was observed possibly because of the biological instability of the peptidomimetic in cells, Sc-58272 was successfully co-crystalised with CaNMT to map the peptide binding pocket (PDB code: 1IYK)[117].

![Figure 1.8 Structures of peptidomimetic CaNMT inhibitors](image)

1.2.3.2 Non-peptidic CaNMT inhibitors

Two main series of non-peptidic inhibitors have been documented using either benzofuran or benzothiazole as the scaffold. The benzofuran series was developed by identifying a hit compound or RO-01 (Figure 1.9) from a random screening[83]. The hit
was then modified to give RO-09-4609 (Figure 1.9) showing sub-μM enzyme inhibition and high selectivity over human NMT1. The secondary amine (highlighted with a red circle in Figure 1.9) in the C-4 side chain was found to interact with the C-terminal Leu of CaNMT[83]. To improve the anti-fungal activity of RO-09-4609, subsequent efforts focused on the modification of the C-2 side chain. With the aid of 3D-quantitative structure-activity relationship studies (3D-QSAR)[118, 119], RO-09-4746 and RO-02 (Figure 1.9) were discovered, showing much improved enzyme inhibition and antifungal activity[80-82]. However, poor pharmacokinetic (PK) profiles such as a short half life (t₁/₂) and low area under curve (AUC)[80, 81], indicated that these compounds might not be stable in vivo, responsible for the weak in-vivo efficacy observed in rats[81]. The project was later ceased given the narrow antifungal spectrum of this compound series.

Figure 1.9 The evolution of the benzofuran CaNMT inhibitors

The secondary amine highlighted with a red circle was observed to involve a hydrogen bond with the C-terminal Leu of CaNMT.

A benzothiazole series was developed by identifying CP-123457 (Figure 1.10) as a hit from a high throughput screening (HTS). The ester group was then replaced to enhance the antifungal activity, followed by the exploration of different linkers and N-substitutions on the right hand side of the scaffold, resulting in the identification of UK-362091 with IC₅₀ value of 11 nM and excellent selectivity over human NMT1 (Figure 1.10). An interaction between the secondary amine (highlighted with a red circle in Figure 1.10) and the C-terminal Leu of CaNMT was observed (Mr. A. Bell, Pfizer, unpublished data). Interestingly, a new cavity induced by naphthyl (highlighted in blue, Figure 1.10) in UK-362091 upon binding was found from the co-crystal structure, compared with its free amine analogue UK-370485 (Figure 1.11, Mr. A. Bell, Pfizer,
unpublished results). Inspired by the structure of UK-362091, SSP, a Japanese pharmaceutical company which is now a part of Boehringer Ingelheim, adopted combinatorial chemistry to discover FTR1335 (Figure 1.10) as their best candidate. Furthermore, FTR1335 was shown to be competitive with the peptide substrate and non-competitive with myr-CoA using an enzyme kinetic assay. However, the resulting antifungal activities were not high enough for the further optimisation.

![Figure 1.10 Development of the benzothiazole CaNMT inhibitors](image1)

**Figure 1.10** Development of the benzothiazole CaNMT inhibitors

The secondary amine highlighted with a red circle was observed to involve a hydrogen bond with the C-terminal Leu of CaNMT. Blue-coloured naphthyl was found to open up a new cavity on binding with CaNMT.

![Figure 1.11 Creation a new cavity by naphthyl in benzothiazole analogue UK-362091](image2)

**Figure 1.11** Creation a new cavity by naphthyl in benzothiazole analogue UK-362091

On comparison of the binding modes of the two structurally similar compounds (light green and dark green for UK-370485 and UK-362091 respectively), an orientation change of nearby amino acids (Tyr 210 and Phe 420) in the peptide binding pocket was noticed, which generates a new cavity occupied by naphthyl in compound UK-362091 (Pfizer, unpublished data).
1.2.3.3 Benzothiazole series for PfNMT

Partnering with Pfizer, benzothiazole analogues were further researched against PfNMT in our group\cite{89,121}. Compound **UK-370509** (Figure 1.12) was initially identified as a hit and the following work concentrated on the systematic variation of the composition of the right hand side chain (highlighted in red, Figure 1.12). However, only a small improvement of enzyme potency was achieved, whereby compounds **FM-54** and **FM-72** (Figure 1.12) were found to be most active\cite{122}. Additionally, the low selectivity over both human NMTs and poor solubility significantly restricts the further investigation of this series.

![Figure 1.12 Study of benzothiazole series as PfNMT inhibitors](image)

The red-coloured moiety was the side chain being systematically studied in the previous project\cite{122}.

1.2.3.4 Sulphonamide series for TbNMT

Screening of a library with 60,000 drug-like compounds\cite{123} at Dundee University identified **DDD64558**, a pyrazole sulphonamide derivative, as a hit against TbNMT (Figure 1.13)\cite{124}. Following work was undertaken to explore phenyl substitutions, leading to the discovery of **DDD85602** (Figure 1.13), in which the terminal amine (highlighted with a red circle, Figure 1.13) was found to be essential to maintain the inhibitory activity. Subsequent rigidification of **DDD85602** provided **DDD85646** (Figure 1.13), the most active inhibitor, possessing low nano-molar range of potency against both the enzyme and parasites. More importantly, a tight correlation between the reduced activity of TbNMT and the inhibition of the *Trypanosoma* parasites was achieved, chemically
validating NMT as a target in *T. brucei*\(^{[124]}\). However, its low selectivity over human NMTs raises toxicity concerns although an *in-vivo* mouse model suggested that all mice were able to survive at the highest therapeutic dose (50 mg kg\(^{-1}\))\(^{[124]}\). The major drawback is that this molecule failed to cross the blood-brain barrier to kill the parasites in brain. Nevertheless it is still an exciting example of early-stage drug discovery conducted by an academic institution tackling tropical parasitic diseases, which are typically not viewed as a profitable market by the pharmaceutical industry.

Figure 1.13 Identification of sulphonamide TbNMT inhibitors

The terminal amine highlighted with a red circle was speculated to involve a hydrogen bond with the C-terminal Val of TbNMT. MRC-5 cell line is derived from a normal lung tissue of a 14-week-old male fetus\(^{[129]}\).

1.3 Design and synthesis of parasite NMT inhibitors – PhD Project

1.3.1 Target evaluation of PfNMT and LdNMT

As stated in section 1.2.2, NMT is an attractive anti-parasitic target. By the time this project started in 2007, NMT from *P. falciparum* was well characterised\(^{[85]}\). A traffic light system, first proposed by Fairlamb\(^{[126]}\), is shown below to evaluate the targetability of PfNMT at that stage (Table 1.3). Firstly, in terms of target validation, neither genetic (manipulation of the NMT gene in *Plasmodium* was not possible then) nor chemical validation had been achieved by then. However, research on the myristoylated proteins in *P. falciparum* suggested that *N*-myristoylation is important to keep parasites live (described in section 1.2.2.3). Secondly, it is essential for a medicinal chemistry project to have reliable biological assays in place so that compounds can be properly evaluated.
and the structure-activity relationships can be derived. A scintillation proximity assay (SPA, detailed in section 2.1.2) was developed “in house”\textsuperscript{[107, 89]} as a robust enzyme assay which was successfully employed in HTS by the Dundee Drug Discovery Unit to identify hits against TbNMT\textsuperscript{[124]}. Fluorescent-activated cell sorting assay (FACS, detailed in section 3.4.1) was also established to assess cell growth inhibition (Dr. A. Holder and Dr. D. Moss, National Institute for Medical Research). Thirdly, there are two NMTs characterised from human, known as HsNMT1 and HsNMT2, raising potential toxicity concerns although some evidence showed that selective inhibition is possible\textsuperscript{[107, 89]}. Fourthly, with respect to resistance potential, there has been no NMT isoform found in parasitic organisms so far. In addition, inhibition of myristoylation is thought to disrupt multi-protein pathways in parasites (described in section 1.2.2), thereby reducing the possibility of developing resistance. Finally, there was no crystal structure of \textit{Plasmodium} NMT when the project started; however, this is a desirable tool to direct rational inhibitor design as the project moves forward.

\textbf{Table 1.3 Traffic light system\textsuperscript{[126]} used to evaluate PfNMT as an anti-malarial target in 2007}

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target validation</td>
<td>No genetic or chemical validation; downstream proteins associated with biological pathways of parasites</td>
</tr>
<tr>
<td>Assay feasibility</td>
<td>Well developed enzyme\textsuperscript{[89]} and cell-based assays\textsuperscript{[127]}</td>
</tr>
<tr>
<td>Toxicity concern</td>
<td>Existence of human homologues and selective inhibition is possible\textsuperscript{[107, 89]}</td>
</tr>
<tr>
<td>Resistance potential</td>
<td>Single gene copy and the potential to avoid resistance</td>
</tr>
<tr>
<td>Structural information</td>
<td>No \textit{Plasmodium} NMT crystal structure</td>
</tr>
</tbody>
</table>

\textcolor{red}{Red} represents that no information has been obtained; \textcolor{amber}{Amber} indicates that only little information has been achieved and more progress is required; \textcolor{green}{Green} means that information or concept has been well understood and established.

NMT was characterised from \textit{L. donovani} in 2009\textsuperscript{[87]}, the causative agent of VL. The same target evaluation approach is applied to LdNMT (Table 1.4). Compared with PfNMT, the situation for LdNMT is more challenging. A major hurdle is the lack of a simple and reliable cell-based assay. In addition, there was little biological or chemical evidence at that time to show \textit{N}-myristoylation is important for \textit{Leishmania} parasites to
survive.

Table 1.4 Traffic light system\textsuperscript{[126]} used to evaluate LdNMT as an anti-leishmanial target in 2009

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target validation</td>
<td>weak genetic validation\textsuperscript{[87]}; no chemical validation; weak functional knowledge of the downstream proteins</td>
</tr>
<tr>
<td>Assay feasibility</td>
<td>Well developed enzyme assay; lack of a reliable cell-based assay</td>
</tr>
<tr>
<td>Toxicity concern</td>
<td>Existence of human homologues</td>
</tr>
<tr>
<td>Resistance potential</td>
<td>Single gene copy and the potential to avoid resistance</td>
</tr>
<tr>
<td>Structural information</td>
<td>Structure of LdNMT with myr-CoA analogue\textsuperscript{[87]}</td>
</tr>
</tbody>
</table>

Red represents that no information has been obtained; Amber indicates that only little information has been achieved and more progress is required; Green means that information or concept has been well understood and established.

1.3.2 Project aim

According to the results of target evaluation, the primary aim of this project is to identify potent and selective inhibitors for PfNMT and use small molecules to chemically investigate the targetability of NMT in \textit{P. falciparum}. The project primarily comprises the hit identification and hit-to-lead development in the typical process of drug discovery (Figure 1.14) and the work presented in this thesis aims to a progress towards discovering lead candidates.

![Drug Discovery Process](image)

**Figure 1.14 Process of typical drug discovery**

Once a hit was validated, an iterative process involving library synthesis, biological evaluation (collaboration with Dr. A. Holder and Dr. D. Moss, NIMR) and structural information (collaboration with Prof. A. Wilkinson and Dr. J. Brannigan, University of York) would be carried out to generate lead compounds (Figure 1.15). Specifically the requirements were to:
1. Find inhibitors with excellent enzyme inhibition (at least sub-µM range of IC₅₀s) and good selectivity (at least 100-fold) over human NMTs.

2. Obtain a co-crystal structure of an inhibitor bound to the enzyme to rationalise the experimental SARs and direct future inhibitor design.

3. Use small molecules as a probe to chemically validate whether NMT is a target in *P. falciparum*.

**Figure 1.15 Hit-to-lead development cycle**

In 2009, pursuing LdNMT inhibitors was included as a minor project. Given the high similarity of the primary sequence between PfNMT and LdNMT, a piggy-back strategy was adopted (detailed in Section 2.1). The library of PfNMT inhibitors generated from the development cycle (Figure 1.15) was screened against LdNMT, followed by the parasite growth inhibition tests (collaboration with Prof. D. Smith and Dr. D. Pappe, University of York), with the purpose to identify good hits or early lead candidates against LdNMT. Specifically the aims were to:

1. Screen the library of PfNMT inhibitors to yield the preliminary SARs for LdNMT inhibitors.

2. Select good hits or early lead candidates with the aid of antileishmanial activity tests.
2 Hit Identification of PfNMT inhibitors

2.1 Screen Strategy

Given a molecular target, which is PfNMT in this project, the hit identification frequently relies on two strategies: high throughput screening (HTS) and piggy-back approach. HTS is well known to be able to identify new chemical entities, both synthetic compounds and natural products. However, lack of access to HTS facilities, the high costs and difficulty of dealing with large numbers of resulting hits prevent this strategy being widely used in academia (Table 2.1). A piggy-back approach is most useful when a molecular target present in one indication has been pursued for other indications as it would accelerate the process to identify good hits\(^\text{[128]}\). Therefore, it is a cost efficient strategy to start medicinal chemistry projects, particular in academic environments\(^\text{[129]}\).

However, as the definition of “piggy-back” suggests, it is most likely to produce molecules that are closely related to the starting points. Moreover, the emerging structure-activity relationships (SARs) are unlikely to be the same as those observed for the original indication\(^\text{[130]}\).

Table 2.1 Comparison between HTS and piggy-back approach

<table>
<thead>
<tr>
<th>Examples in parasitic diseases</th>
<th>HTS</th>
<th>Piggy-back approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBNMT by Dundee University(^\text{[123, 124]})</td>
<td>Identifies new chemical class of inhibitors</td>
<td>PFPFT (protein farnesyltransferase) by Yale University(^\text{[129]})</td>
</tr>
<tr>
<td>antimalarial targets by Norvatis(^\text{[131, 40]}) and GSK(^\text{[39]})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Advantages | Cost efficient; accelerates the process of hit identification |

Disadvantages | Costly; limited availability outside industry | Starts with existing chemical entities; SARs might be disease-specific\(^\text{[130]}\) |

By the time this project started, CaNMT inhibitors had been intensively studied\(^\text{[82, 84]}\) and the Dundee group was developing TBNMT inhibitors (unpublished at that time).

Given the resources available to us, it was therefore the most feasible and economical to use a piggy-back strategy to identify hit compounds, where a molecular target present in \(C. albicans\) and \(T. brucei\) (NMT here) is being investigated in \(P. falciparum\). In addition,
sequence alignment among these three NMTs reveals high similarities (Figure 2.1), increasing the chances of finding good hits using this approach.

![Figure 2.1 Sequence alignments of CaNMT, PfNMT and TbNMT](image)

The sequences of various NMT proteins have been aligned with the program MultAlign (Version 5.4.1.(Corpet 1988)). The colour codes are as follows: Red = exact match, blue = AA of similar properties, black = no match, with a consensus level of 90% high and 50% low. The consensus symbols are as follows: ! = any of IV, $ = any of LM, % = any of FY and # = any of NDQEBZ.

A small library (25 compounds) of reported CaNMT and TbNMT inhibitors (either kindly supplied by Dundee Drug Discovery Unit or synthesised “in house”) was screened using a developed scintillation proximity assay (SPA, detailed in Section 2.3), resulting in the discovery of a hit or compound 1 which displayed moderate inhibition. Compound 1, also known as RO-09-4609, was initially developed by Roche for its antifungal project, exhibiting sub-µM inhibition against CaNMT and excellent selectivity over human NMT1.[82] (Figure 1.15).

![Figure 2.2 Structure of the hit compound](image)

Initially developed to target CaNMT known as RO-09-4609

IC₅₀ CaNMT: 0.1 µM

IC₅₀ PbNMT: 47 µM

IC₅₀ TbNMT: >500 µM

---

**Figure 2.2 Structure of the hit compound**
2.2 Re-synthesis of the hit

To validate compound 1 as a hit, the first step is to re-synthesise this compound. Retro-synthetic analysis breaks compound 1 into two parts: the benzofuran scaffold and the C-4 side chain, as shown in Scheme 2.1.

![Scheme 2.1 Retro-synthetic analysis of the hit compound](image)

2.2.1 Synthesis of the benzofuran scaffold

Although various methods have been developed to make benzofurans\textsuperscript{[132]}, the synthesis of ethyl benzofuran-2-carboxylates remains problematic due to the hydrolysis of the ester group. Ether 4, the precursor of benzofuran 3, could be easily obtained by reacting compound 5 with ethyl bromoacetate under basic conditions (Scheme 2.1). The following methods were attempted to achieve the benzofuran formation.

P(MeNCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}N, a non-ionic base, was applied to synthesise benzofuran derivatives without having the hydrolysis issue\textsuperscript{[133]}. However, it is not practicable to get the benzofuran scaffold in large quantities because of the high cost of this base.

Sodium hydride was reported to successfully convert a variety of 2-mercaptophenyl ketones to 2,3-disubstituted benzo[b]thiophenes in 40-80\% yields\textsuperscript{[134]}, implying that the strong base could act a similar manner to afford benzofuran. Disappointingly, no desired conversion was observed when ether 4 was reacted under such conditions.

The Belanger group developed an improved method to produce benzofuran 3 using sodium ethoxide in ethanol\textsuperscript{[135]}. The isolated by-products were also discussed in the same paper (Scheme 2.2): carboxylic acids 6 and 8 were the hydrolysis products; subsequent decarboxylation of acid 6 gave benzofuran 7; diketone 9 was formed via another mode of cyclisation, which was observed when toluene was used as the
Initial trials of Belanger’s protocol gave around 20% yields of benzofuran 3 and two by-products, benzofuran 7 and acid 8, were isolated. Study of the reaction parameters suggested that the temperature and amounts of NaOEt were two key factors to improve the yields.

Table 2.2A presents the yields of the benzofuran scaffolds at different temperatures. It is clear that low temperature is necessary to form benzofuran (Table 2.2A, entry 3). Although maintaining a low temperature throughout the reaction resulted in a high selectivity between the desired product 3 and its decarboxylated by-product 7, nearly half of the starting material was recovered (Table 2.2A, entry 4). Instead, such selectivity could be achieved by varying the amount of the base. Table 2.2B shows that 1.5 eq. of NaOEt gives an optimal result. In addition, the concentration of NaOEt was also found to affect the said selectivity, whereby a higher base concentration leads to a lower selectivity (Table 2.2B, entries 3 vs. 5 and 4 vs. 6).
Table 2.2 Comparison of different conditions used to make benzofuran

(A) 2.0 equivalents of NaOEt in ethanol

<table>
<thead>
<tr>
<th>Entry</th>
<th>Temperature</th>
<th>Time</th>
<th>Product(s)</th>
<th>Overall yields</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80 °C</td>
<td>18 h</td>
<td>4 + 7 + unknown</td>
<td>10%</td>
</tr>
<tr>
<td>2</td>
<td>25 °C</td>
<td>18 h</td>
<td>4 + unknown</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>-15 ~ 7 °C</td>
<td>18 h</td>
<td>8 + 3 + 7</td>
<td>69% (35% for 3; 34% for 7)</td>
</tr>
<tr>
<td>4</td>
<td>-15 °C</td>
<td>15 h</td>
<td>8 + 3 + 7</td>
<td>33% (30% for 3; 3% for 7)</td>
</tr>
</tbody>
</table>

(B) Defined temperature (-15 ~ 7 °C) in ethanol

<table>
<thead>
<tr>
<th>Entry</th>
<th>Equivalent(s)</th>
<th>Concentration (mol / L)</th>
<th>Overall yields</th>
<th>Ratio between 3 and 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.6</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>0.6</td>
<td>73%</td>
<td>10 : 1</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>0.6</td>
<td>70%</td>
<td>3 : 1</td>
</tr>
<tr>
<td>4</td>
<td>6.0</td>
<td>0.6</td>
<td>68%</td>
<td>1 : 1</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>1.2</td>
<td>70%</td>
<td>1 : 1</td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td>1.2</td>
<td>62%</td>
<td>1 : 12</td>
</tr>
</tbody>
</table>

a The products were separated via silica chromatography. b Overall isolated yields of benzofuran scaffolds, including 3 and 7.

The optimised conditions: 1.5 eq. and 0.6M of NaOEt in EtOH, -15 ~ 7 °C, overall yields: 73%.

2.2.2 Incorporation of the C-4 side chain

The process of adding 1,3-dibromo propane to the benzofuran core involves a $S_N2$ substitution under mild conditions. However, it suffered from both moderate yields even when a large excess of the reagents were used, and a difficult separation due to the similar polarities between the desired product 2 and the allyl by-product 10, which was unavoidable under basic conditions (Scheme 2.3).

Further amination on compound 2 was achieved in another straightforward $S_N2$ reaction. Initial trials in refluxing ethanol could only give reasonable yields of 50% even
when a large excess (100 eq.) of amine was used. Therefore, a range of solvents were screened and DMF was found to be best where only 4 eq. of amine was needed to ensure a full conversion (Scheme 2.4).

![Scheme 2.4 Amination on bromopropyl benzofuran 2](image)

### 2.2.3 Summary

Hit compound 1 was successfully made in a 4-step synthesis (Scheme 2.5): 2,6-dihydroxy acetophenone 5 was initially coupled with ethyl bromoacetate, followed by cyclisation under basic conditions to produce benzofuran 3. Treatment of an excess of 1,3-dibromopropane with benzofuran 3 in the presence of potassium carbonate in DMF gave O-bromopropyl compound 2, which was further aminated to yield compound 1.

![Scheme 2.5](image)

#### 2.3 Activity confirmation of the re-synthesised hit compound

The inhibitory activity of the re-synthesised hit or compound 1 was tested via a scintillation proximity assay (SPA), which was developed in our group[89].

#### 2.3.1 Principles of SPA

The basis of SPA is to monitor the transfer of a radiolabelled substrate to a second substrate with an affinity tag (biotin in this case) which is subsequently captured
by a coated bead (streptavidin in this case). The bead, to which is attached a scintillant, ensures that the radiolabel on the product is close enough to the scintillant to allow the detection by a scintillation counter. In addition, the use of a scintillant that is immobilised on a solid support prevents the need to separate products from reactants, which significantly simplifies the work-up procedure used in a normal scintillation assay[84].

The procedure of the assay is described in detail in Figure 2.3. NMT, tritium labelled myr-CoA and biotinylated peptide are incubated together, where they are expected to follow a Bi-Bi mechanism (Section 1.2.1) to form a radiolabelled myristoylated peptide. Then, both the myristoylated and un-reacted peptides are captured by the streptavidin-coated scintillation beads. Radiation from tritium in the myristoylated peptide is sufficiently close to the scintillant and the absorption from $^3$H-decay results in the emission of a detectable photon, which is quantified and displayed in a scintillation counter.

The existence of an inhibitor blocks the myristoylation to occur, thus decreasing the signal recorded by the scintillation counter. Therefore, a weak signal indicates a good inhibitor and vice versa (Figure 2.3). A plot of enzyme activity against an inhibitor’s concentration gives an inhibition curve from which an IC$_{50}$ value can be determined. The IC$_{50}$ is a measure of the effectiveness of a compound, which represents how much of this compound is needed to inhibit a given biological process (N-myristoylation in this case) by half.
Figure 2.3 Mechanism of SPA

The radiolabelled (coloured in red) myr-CoA, biotinylated (shown in green) peptide and the enzyme are added together to form a radiolabelled myristoylated peptide; A streptavidin-coated bead incorporating a scintillant is expected to bind both myristoylated and unreacted peptides via the biotin functional group; Radiation from $^3$H of the myristoylated peptide is then sufficiently close to the scintillant and the absorption from $^3$H-decay leads to the emission of a photon, which can be detected and quantified by a scintillation counter.$^{[107]}

2.3.2 Assay development

The buffer conditions, including pH value, percentage of DMSO, and the selection of peptide substrates had been well documented previously in our group$^{[107, 122]}$ and so were used without further modification. The purpose here was to optimise incubation time, the enzyme and substrate concentrations to develop a robust assay for the NMTs investigated in this project.

Enzyme concentration and incubation time

In general, larger amounts of enzyme are expected to give higher signal. However, upon increase of the enzyme concentration, substrate depletion might occur, represented by the amount of signal reaching a plateau (Figure 2.4A). This meant that
under the conditions used in these assays, linearity was maintained only up to a PfNMT concentration of 1.7 nM (Figure 2.4B). Time-course experiments, where PfNMT concentration was fixed at 1.7 nM, showed that the signal increased linearly with the time up to 40 min (Figure 2.4C and 2.4D). Under these conditions, the amount of enzyme was rate-limiting and the gradient could be used to provide the initial velocity of reaction, an accurate and effective way to determine the inhibitory activities of test molecules[137].

Figure 2.4 Signal dependence on enzyme concentration and incubation time

(A, B) After 30 min incubation, signal intensity was found to be dependent on the enzyme concentration and the signal linearity was maintained up to a PfNMT concentration of 1.7 nM. Above this concentration of enzyme, substrate depletion was thought to be responsible for the observed leveling off of the curve. (C, D) Signal intensity was dependent on the incubation time at a fixed enzyme concentration (1.7 nM of PfNMT), where a linear response was maintained up to 40 minutes.
The same approach was applied to determine the optimal enzyme concentration and incubation time for three other investigated enzymes (Ld, Hs1 and Hs2 NMTs) and the results are listed in Table 2.3. To simplify the assay conditions and allow better comparisons, the incubation time was uniformly set at 30 min for the four NMTs employed in this study.

Table 2.3 Optimal enzyme concentration and incubation time for the investigated NMTs

<table>
<thead>
<tr>
<th></th>
<th>PfNMT</th>
<th>LdNMT</th>
<th>HsNMT1</th>
<th>HsNMT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (nM)</td>
<td>1.7</td>
<td>1.0</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>40</td>
<td>30</td>
<td>30</td>
<td>60</td>
</tr>
</tbody>
</table>

* Upper limit of the enzyme concentration to maintain a linear curve; *b* Upper end of the incubation time to keep the signal curve as a straight line.

Concentration of myr-CoA and peptide

Targeting the peptide binding pocket to find inhibitors that are selective over human NMTs was achieved in previous CaNMT projects. Therefore, the enzyme assay was designed to find inhibitors which are competitive with the peptide substrate. In theory, myr-CoA concentration in the assay should be well above its $K_{M}^{app}$, ensuring a full occupancy of the myr-CoA binding pocket to efficiently open up the peptide binding pocket; peptide concentration should be ideally below or around its $K_{M}^{app}$ to find molecules which are competitive with the peptide substrate[138].

To verify the $K_{M}^{app}$ for each substrate, a kinetic analysis was performed for each enzyme and is illustrated in Figure 2.5 for PfNMT. These data showed that for PfNMT, the $K_{M}^{app}$ of myr-CoA was 19.3 nM and the $K_{M}^{app}$ for peptide was 1.04 µM, similar to the previous data[107, 122].
Figure 2.5 $K_{m}^{app}$ determinations for myr-CoA and peptide in PfNMT

The $K_{m}^{app}$ of myr-CoA was determined using 500 - 0.2 nM myr-CoA with 500 nM peptide substrate while the $K_{m}^{app}$ of the peptide was measured over the range of 10 µM - 14 nM with a fixed myr-CoA concentration of 62.5 nM. The initial rates of reaction (cpm/min) were fitted to the Michaelis Menten equation as a function of either myr-CoA or peptide concentration, allowing the $K_{m}$ and $V_{max}$ to be determined respectively. Each measurement was done once due to the safety and cost considerations and the obtained values were consistent with the reported data\[107, 122\].

The kinetic parameters of investigated NMTs are listed in Table 2.4. Given the use of radioactive myr-CoA in the assay, it is desirable to use the minimal amount of myr-CoA while at the same time ensuring the concentration well above its $K_{m}$. The concentration was thus chosen to be 62.5 nM, under which conditions approximately 80% of myr-CoA pocket in enzymes (PfNMT and LdNMT) would be occupied and sufficient signal intensity was obtained. In addition, the amount of streptavidin beads required to quench the assay was found to be proportional to the amount of peptide used\[107\]. Considering that the $K_{M}^{app}$ values of peptide for test NMTs were between 0.29 and 1.56 µM, combining with the cost considerations of the beads, the peptide concentration was uniformly set at 0.5 µM in the assay.
Chapter 2 – Hit identification

Table 2.4 Kinetic data of investigated NMTs

<table>
<thead>
<tr>
<th></th>
<th>PfNMT</th>
<th>LdNMT</th>
<th>HsNMT1</th>
<th>HsNMT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw</td>
<td>56 kDa</td>
<td>51 kDa</td>
<td>56 kDa</td>
<td>56 kDa</td>
</tr>
<tr>
<td>$K_{M_{app}}$ (myr-CoA)</td>
<td>19.3 nM</td>
<td>17.5 nM</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>$K_{M_{app}}$ (peptide)</td>
<td>1.04 µM</td>
<td>0.29 µM</td>
<td>1.56 µM</td>
<td>0.30 µM</td>
</tr>
</tbody>
</table>

Data was tested using the same method applied to PfNMT and each measurement was done only once due to safety and cost considerations; n.d., not determined

Optimised conditions

Based on the above discussion, the optimal conditions for the assay are listed in Table 2.5, providing not only a high signal-to-noise ratio (generally greater than 30), but also the potential to identify selective inhibitors.

Table 2.5 Optimised conditions of SPA for investigated NMTs

<table>
<thead>
<tr>
<th></th>
<th>PfNMT</th>
<th>LdNMT</th>
<th>HsNMT1</th>
<th>HsNMT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>[enzyme]</td>
<td>1.7 nM</td>
<td>1.0 nM</td>
<td>1.0 nM</td>
<td>4.0 nM</td>
</tr>
<tr>
<td>[myr-CoA]</td>
<td></td>
<td>62.5 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[peptide]</td>
<td></td>
<td>0.5 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation time</td>
<td>30 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

2.3.3 Activity confirmation of the hit compound

The inhibitory activity of the re-synthesised hit compound was tested by SPA and the IC$_{50}$ value was found to be 51.4 µM against PfNMT (Figure 2.6), which was in accordance with the result of the screening hit (IC$_{50}$: 47.0 µM). In addition, the molecule displayed nearly no inhibition against both human NMTs. Therefore, compound 1 was validated as a hit compound because of its promising inhibitory activity and selectivity over human NMTs.
### Compound Enzyme IC$_{50}$ (µM)

<table>
<thead>
<tr>
<th>Compound</th>
<th>PfNMT</th>
<th>HsNMT1</th>
<th>HsNMT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51.4</td>
<td>NI (0%)$^a$</td>
<td>&gt;1000 (1.3%)$^a$</td>
</tr>
</tbody>
</table>

$^a$ The number in parentheses was the percentage inhibition values of a compound at 100 µM in triplicate; the variation was generally <10%. The IC$_{50}$ value is estimated from these to give a comparative indication of potency. NI: no inhibition.

**Figure 2.6** Activity determination of the re-synthesised hit

#### 2.4 Summary

A piggy-back strategy was applied to screen a small library of reported NMT inhibitors against PfNMT, leading to the discovery of compound 1 that showed moderate inhibition. Compound 1 was re-synthesised in 4 steps and evaluated by a scintillant proximity assay, which was optimised “in house”. The resulting moderate inhibition against PfNMT and excellent selectivity over human NMTs validated compound 1 as a hit for PfNMT inhibitors (Figure 2.7).

![Chemical structure of compound 1](image)

Initial screening:
IC$_{50}$ PfNMT: 47 µM

Resynthesis and biological evaluation:
IC$_{50}$ PfNMT: 51.4 µM
IC$_{50}$ HsNMT1&2: >1 mM

**Figure 2.7** Structure of the validated hit
3 Design and synthesis of PfNMT inhibitors

Based on the structure of hit compound 1, two locations were identified as areas that were promising for modification: the C-4 trimethylene side chain and the C-2 ester. The aim was to discover inhibitors showing high potency against PfNMT and excellent selectivity over human NMTs (Scheme 3.1).

Scheme 3.1 Strategy to design PfNMT inhibitors based on the structure of the hit compound: synthetic elaboration of the C-4 and C-2 positions

3.1 The first generation of PfNMT inhibitors

3.1.1 Optimisation of the flexible chain at the C-4 position

Exploration of the C-4 side chain was first carried out by analysing side chains with varied length, different basicity of hetero atom X and a range of R\(^1\) groups (Scheme 3.2).
3.1.1.1 Synthesis

Scheme 3.3 outlines the general synthetic route to make benzofuran analogues 13-32 with different length, basicity and varied $R^1$ groups at the C-4 side chain. Compounds 13-31 were prepared using the method to synthesise hit compound 1 (Section 2.2) and compound 32 was obtained by the de-benzylation of compound 16 in good yields under conventional hydrogenation conditions.

Scheme 3.3 (a) various alkyl dibromides, $K_2CO_3$, DMF, r.t., 5 hr, 30-76%; (b) $R^1NH(H)$, DMF, 80°C, 3 hr, 45-97%; (c) 10% Pd/C, 1,4-cyclohexadiene, EtOH, 80°C, 1 hr, 67%.

For full structural information of compounds 13-31 with different $R^1$ groups, please refer to the following SAR part, Section 3.1.1.2 or Appendix. Only the synthetic route is described here.

Compounds 33 and 34 with diverse basicity of the C-4 side chain were then made to assess whether the inhibitory activity is dependent on the basicity (Scheme 3.4).

Scheme 3.4 (a) benzyl 3-bromopropyl ether, $K_2CO_3$, DMF, r.t., 5 hr, 80%; (b) $N,N$-dimethyl benzylamine, $CH_3CN$, 85°C, 16 hr, 90%.
3.1.1.2 Structure-activity relationships

Investigation of the C-4 side chain length

The inhibitory activities of compounds with varied length of the C-4 side chain are compared in Table 3.1. The results clearly demonstrate that a trimethylene linker between the ether attachment point and the nitrogen (1 and 15) is optimal.

Table 3.1 Optimal length of the C-4 side chain in the first generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>IC50 (µM)</th>
<th>Compound</th>
<th>n</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>2</td>
<td>~220 (31.0%)</td>
<td>14</td>
<td>2</td>
<td>NI (0%)</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>9.0</td>
<td>1</td>
<td>3</td>
<td>51.4</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>~130 (43.8%)</td>
<td>31</td>
<td>4</td>
<td>~300 (25.0%)</td>
</tr>
</tbody>
</table>

The IC50 values were averaged from two independent dose-response curves; the variation was generally <15%. The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC50 values are estimated from these to give a comparative indication of potency. NI: no inhibition.

The importance of X

The amino group on the reported inhibitor was found to interact with the C-terminal Leu of ScNMT\textsuperscript{[139]}, playing an important role in exerting inhibition; a similar interaction was also observed in the CaNMT project\textsuperscript{[83]}. Therefore, it was interesting to examine whether such interaction would occur in PfNMT. Four representative inhibitors, with the C-4 side chains ranging from basic, weakly basic, neutral to charged, were thus assessed (Table 3.2). Sufficient basicity of the C-4 side chain was found to be essential in maintaining the inhibitory activity since compound 16 showed far higher potency than other three compounds. However, whether such interaction with the C-terminal Leu of PfNMT is via an ionic or hydrogen bond is not clear\textsuperscript{[124]}. 
Table 3.2 Dependence of inhibition on the basicity of the C-4 side chain

<table>
<thead>
<tr>
<th>Compound</th>
<th>X-N</th>
<th>IC₅₀ (µM)</th>
<th>Compound</th>
<th>X-N</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td></td>
<td>8.5</td>
<td>20</td>
<td></td>
<td>~107 (48.4%)</td>
</tr>
<tr>
<td>33</td>
<td>O</td>
<td>~146 (40.7%)</td>
<td>34</td>
<td></td>
<td>~220 (31.2%)</td>
</tr>
</tbody>
</table>

The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency.

Variation of R¹ groups

The effects of different N-substituents were then studied (Table 3.3). Among the alkyl R¹ variants, t-Bu (15) was found to be the right size while smaller (19, 23 and 32) or bigger ones (18, 24 and 29) resulted in a significant drop in activity. In addition, insertion of an extra nitrogen atom (29) was not favoured by PfNMT. Of the different aromatic R¹ groups, benzyl (16) was the best. Electronic deficiency (1, 17, 21, 27 and 28) led to a loss of inhibition by at least 5-fold; addition of electron-rich groups on the phenyl ring, such as methyl (25 and 26), failed to improve the potency; β-naphthyl (22) might be too big to be accommodated by the binding pocket, accounting for a 20-fold loss of inhibition (22 vs. 16).
Table 3.3 Exploration of $R^1$ groups in the C-4 side chain of the first generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^1$</th>
<th>$IC_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^1$</th>
<th>$IC_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td></td>
<td>9.0</td>
<td>18</td>
<td></td>
<td>84.4</td>
</tr>
<tr>
<td>19</td>
<td>$\cdot$Me</td>
<td>~108 (48.1%)$^b$</td>
<td>23</td>
<td></td>
<td>102.0</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>51.0</td>
<td>29</td>
<td>$\cdot$NH</td>
<td>~490 (17.0%)$^b$</td>
</tr>
<tr>
<td>32</td>
<td>$\cdot$H</td>
<td>~143 (41.1%)$^b$</td>
<td>1</td>
<td></td>
<td>51.4</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>8.5</td>
<td>17</td>
<td></td>
<td>54.5</td>
</tr>
<tr>
<td>21</td>
<td>$\cdot$N</td>
<td>~370 (21.2%)$^b$</td>
<td>25</td>
<td>$\cdot$H$_3$C</td>
<td>13.0</td>
</tr>
<tr>
<td>26</td>
<td>$\cdot$CH$_3$</td>
<td>31.6</td>
<td>27</td>
<td>$\cdot$F</td>
<td>&gt;100</td>
</tr>
<tr>
<td>28</td>
<td>$\cdot$F</td>
<td>&gt;100</td>
<td>22</td>
<td></td>
<td>~180 (35.6%)$^b$</td>
</tr>
</tbody>
</table>

$^a$ The $IC_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%. $^b$ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. $IC_{50}$ values are estimated from these to give a comparative indication of potency.

3.1.2 Comparison of the C-4 versus C-5 attachment in benzofuran

Considering that the C-4 side chains of compounds 15 and 16 were determined to be optimal, it was interesting to study the influence of the PfNMT inhibition when the side chain was changed from the C-4 to C-5 position (Scheme 3.5).
3.1.2.1 Synthesis

2,5-Dihydroxy-acetophenone 37 was selectively benzylated to give compound 38, which was then coupled with ethyl bromoacetate to form ether 39, the precursor of benzofuran. Base-catalysed cyclisation of ether 39, followed by the deprotection under conventional hydrogenation conditions afforded benzofuran 41, which was then attached with a trimethylene linker and finally aminated to yield target compounds 35 and 36 (Scheme 3.6).

3.1.2.2 Activity evaluation

Surprisingly, reposition of the side chain from the C-4 to C-5 position of the benzofuran scaffold resulted in a total loss of the inhibitory activity (Table 3.4), indicating that the C-4 position of the side chain is critical.
Table 3.4 Inhibitory activity comparison between the C-4 and C-5 side chain of benzofuran

<table>
<thead>
<tr>
<th>R¹</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>R¹</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35</td>
<td>NI (0%)ᵇ</td>
<td></td>
<td>15</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>NI (0%)ᵇ</td>
<td></td>
<td>16</td>
<td>8.5</td>
</tr>
</tbody>
</table>

ᵃ The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. NI: no inhibition.

3.1.3 Preliminary studies of heterocyclic cores other than benzofuran

Given the fact that the furan oxygen of hit compound 1 involves a hydrogen bond with His227 in CaNMT⁸², it was therefore worthwhile to assess the importance of benzofuran in PfNMT inhibitors and preliminary studies of heterocyclic scaffolds other than benzofuran was conducted.

3.1.3.1 Synthesis

Apart from benzofuran, there are other heterocyclic scaffolds, reported as readily prepared in literature, such as benzoazole¹⁴⁰, benzothiazole¹⁴⁰, benzoimidazole¹⁴⁰ and benzothiophene¹³⁴. As stated before, the C-4 position of the side chain in benzofuran is crucial to maintain the inhibitory activity. However, because of the restriction in the commercial availability of starting material, there are no such desired sites to modify in the scaffolds 43 and 44 (illustrated as red eclipses, Scheme 3.7). In 2008, the Svete group developed a facile method to make 8-hydroxyimidazo[1,2-α]pyridine 45¹⁴¹ (Scheme 3.7), which is a good mimic to benzofuran containing the C-4 side chain.
Scheme 3.7 Heterocyclic scaffolds as alternatives to benzofuran

The arrow indicates the desired modified site.

Cyclocondensation of 2-aminopyridin-3-ol 46 with ethyl bromopyruvate under thermal conditions gave 8-hydroximidazo[1,2-α]pyridine 45 directly\[141\], which was then incorporated with the side chains to yield compounds 48 and 49 (Scheme 3.8).

Scheme 3.8 (a) ethyl bromopyruvate, THF, 65 °C, 24 hr, 15%; (b) 1,3-dibromopropane, K₂CO₃, DMF, r.t., 5 hr, 24%; (c) R¹NH₂, DMF, 80 °C, 3 hr, 90% for 48 and 70% for 49.

3.1.3.2 Activity evaluation

Imidazo[1,2-α]pyridine inhibitors (48 and 49) were far less potent than their benzofuran counterparts (Table 3.5), indicating that the benzofuran core might be important. Current thought is that the difference of either electronic flow or aromacity between benzofuran and imidazo[1,2-α]pyridine is responsible for this huge discrepancy of the inhibitory activities.
Table 3.5 Inhibitory activity comparison between imidazo[1,2-α]pyridine and benzofuran inhibitors

<table>
<thead>
<tr>
<th>R¹</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>R¹</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48</td>
<td>~180 (35.7%)ᵇ</td>
<td>15</td>
<td></td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>NI (0%)ᵇ</td>
<td>16</td>
<td></td>
<td>8.5</td>
</tr>
</tbody>
</table>

ᵃ The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency. NI: no inhibition.

3.1.4 Exploration of the C-2 side chain

So far, two optimal C-4 side chains had been determined and attention was thus turned to the study of the C-2 side chain (Scheme 3.9).

Scheme 3.9 Strategy to design the C-2 side chain as the first generation of PfNMT inhibitors

3.1.4.1 Essentiality of the C-2 side chain

To assess the importance of the C-2 side chain, compounds 51-53 were synthesised from benzofuran 7, a by-product from making the desired benzofuran scaffold, according to the method to make hit compound 1 (Scheme 3.10).
Scheme 3.10 (a) 1,3-dibromopropane, K₂CO₃, DMF, r.t., 5 hr, 30%; (b) R¹NH₂, DMF, 80 °C, 3 hr, 70% - 90%.

The comparison between ethyl ester and hydrogen at the C-2 position are listed in Table 3.6. There is no doubt that the presence of the C-2 side chain is necessary to keep the inhibition.

Table 3.6 Investigation of the necessity of the C-2 side chain

<table>
<thead>
<tr>
<th>R¹</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>9.0</td>
<td>51</td>
<td>~100 (50.0%)ᵇ</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>47.0</td>
<td>52</td>
<td>NI (0%)ᵇ</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>8.5</td>
<td>53</td>
<td>~163 (28.0%)ᵇ</td>
</tr>
</tbody>
</table>

ᵃ The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency. NI: no inhibition.

3.1.4.2 Synthesis of the C-2 side chains other than ester

Ester has the potential to be broken down by esterases in the body, thereby always considered as a problematic functional group by medicinal chemists. To overcome this problem, a variety of functional groups (Y) other than ester such as amide, ketone, ether, etc., were investigated (Scheme 3.11).
Amide series

Synthesis of the target compound is straightforward, with the formation of amide 54 as a key step (Scheme 3.12).

Scheme 3.12 Synthetic analysis of the amide series in the first generation of PfNMT inhibitors

Direct conversion from ester to amide was first reported by Weinreb\textsuperscript{[142]}, in which Lewis acid AlMe\textsubscript{3} was used to activate the carbonyl of ester. However, the dangers involved with handling extremely flammable reagent AlMe\textsubscript{3} and the resulting moderate yields, prevented this reaction from being a robust method to generate amide at the C-2 position (Scheme 3.13). Encouragingly, indirect transition via carboxylic acid 55, which could be readily synthesised from the hydrolysis of ester 2 by lithium hydroxide, gave both milder coupling conditions and improved yields (Scheme 3.13).

Scheme 3.13 Model study of amide formation at the C-2 position

The PyBOP/DIPEA (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate and N,N-diisopropylethylamine) system is used to activate the
carboxylic acid moiety. The carboxylic acid is firstly deprotonated by DIPEA to give the carboxylate anion, which then attacks PyBOP to form 57. After that, intermediate 57 is decomposed upon the involvement of benzotriazole anion to afford 58, the activated form of the carboxylic acid. Finally, intermediate 58 undergoes a substitution with amine to generate the desired amide (Scheme 3.14).

$$\text{R}^+\text{O}_2\text{C}^– \xrightarrow{\text{DIPEA}} \text{R}^’\text{O}_2\text{C}^- \xrightarrow{\text{PyBOP}} \text{R}^’\text{O}_{\text{PBOP}} \xrightarrow{\text{N}^–\text{O}} \text{R}^’\text{N}^-\text{R}^-$$

Scheme 3.14 Mechanism of carboxylic acid activation by PyBOP/DIPEA to form an amide

In summary, ester 2 was treated with LiOH to afford carboxylic acid 55, which was then reacted with amines under PyBOP/DIPEA conditions to form an amide bond, followed by the amination to yield a set of amide compounds 59-65 (Scheme 3.15).

$$\text{Br} \xrightarrow{\text{LiOH, THF/H}_2\text{O, r.t., 18 hr, 90%}} \text{COOEt} \xrightarrow{\text{PyBOP, DIPEA, R}^\text{N}^-\text{H(H), DMF/DCM, r.t., 3 hr, 50% - 70%}} \text{Br} \xrightarrow{\text{R}^\text{N}^-\text{H}, \text{DMF, 80} \degree\text{C, 3 hr, 70% - 90%}} \text{CON(H)R}^2$$

Scheme 3.15 (a) LiOH, THF/H₂O, r.t., 18 hr, 90%; (b) PyBOP, DIPEA, R¹NH(H), DMF/DCM, r.t., 3 hr, 50% - 70%; (c) R²NH₂, DMF, 80 °C, 3 hr, 70% - 90%;

For full structural information of compounds 59-65 with different R¹ and R² groups, please refer to the following SAR part, Section 3.1.4.3 or Appendix. Only the synthetic route is described here.

**Ketone series**

The key intermediate towards the desired ketone molecules is compound 66, which was proposed to be made by a base-catalysed cyclisation of compound 67, readily synthesised from starting material 5 (Scheme 3.16).
Interestingly, the reaction anticipated to provide ether 69 actually gave ketone 70 directly, indicating the possibility of one-pot reaction to make compound 70 (Scheme 3.17).

Scheme 3.17 One-pot reaction to make 2-ketone benzofuran 70

However, there was still half of starting material left even extending the reaction time or increasing the amount of base when acetone was used as the solvent. In addition, the polarities of compounds 5 and 70 were found to be extremely similar, increasing the difficulty of the purification. Therefore, several other solvents were screened and acetonitrile was shown to be optimal to drive such conversion into completion (data not shown here).

In summary, 2,6-dihydroxy acetophenone 5 was reacted with α-halogenated methyl ketones under basic conditions to form benzofurans 70 and 71, which were then functionalised with the side chains to generate ketone molecules 72-75 (Scheme 3.18).

Scheme 3.18 (a) R²COCH₂X (X=Cl or Br), K₂CO₃, CH₃CN, 80 °C, 24 hr, 20% - 40%; (b) 1,3-dibromopropane, K₂CO₃, DMF, r.t., 5 hr, 40% - 50%; (c) R¹NH₂, DMF, 80 °C, 3 hr, 80% - 90%.

For full structural information of compounds 72-75 with different R¹ and R² groups, please refer to the following SAR part, Section 3.1.4.3 or Appendix. Only the synthetic route is described here.
Reduction of ketone 70 was also attempted. However, neither conventional reducing agents (NaBH₄ and LiAlH₄) nor Lewis acid triggered environment was able to afford the reduced forms of ketone 70 (Scheme 3.19).

Scheme 3.19 Trials of reducing the C-2 ketone benzofuran

**Ether series**

Scheme 3.20 outlines the retro-synthetic analysis of the proposed ether compounds. Compound 78 required for a Mitsunobu reaction can be prepared in a simple two-step procedure involving reduction and S_N2 substitution. The Mitsunobu reaction to make the C-2 ether is considered to be the key step.

Scheme 3.20 Retro-synthetic analysis of the ether series in the first generation of PfNMT inhibitors

Unfortunately, this Mitsunobu reaction generally afforded yields of less than 20%, even screening a range of azo reagents and phosphine ligands. In most cases, the conditions of diisopropyl azodicarboxylate (DIAD) and triphenylphosphine (PPh₃) in THF were found to give the best yields (ca. 25%), thus employed in the following Mitsunobu reactions.

In addition, chemical instability is another hurdle to explore the ether series. Some of the ether compounds were observed to decompose under weakly acidic conditions (pH=3), which are not suitable for further study from a medicinal chemistry perspective. Scheme 3.21A suggests that electron rich phenyl substituents R³, such as...
methyl and methoxy, have detrimental effects on the stability of the resulting ethers. Aside from the electronic dependence, scheme 3.21B indicates that the size of R\(^1\) affects the stability as well, whereby bigger R\(^1\) (t-Bu and 4-Me benzyl in this case) contributes to the stable ethers.

Scheme 3.21 Chemical instability amongst the ether series

To sum up the synthesis, ester 3 was reduced to alcohol 79, which was then added to a trimethylene side chain to form compound 78. This compound underwent either a Mitsunobu reaction, followed by amination to generate ether compounds 80-85 or direct amination to yield compounds 86 and 87 (Scheme 3.22).

Scheme 3.22 (a) LiAlH\(_4\), THF, 0 °C - r.t., 70%; (b) 1,3-dibromo propane, K\(_2\)CO\(_3\), DMF, r.t., 5 hr, 40%; (c) DIAD, PPh\(_3\), ArOH, THF, r.t., 4 hr, 15% - 25%; (d) R\(^1\)NH\(_2\), DMF, 80 °C, 3 hr, 70% - 95%.

For full structural information of compounds 80-87 with different R\(^1\) and R\(^3\) groups, please refer to the following SAR part, Section 3.1.4.3 or Appendix. Only the synthetic route is described here.

Thio ether series

Thio ether was reported to be formed by reacting alcohol with aromatic thiol in TFA environment \(^{[80, 145]}\). When the same conditions were applied to compounds 86 and 87, only the former gave the desired product 88. The colour of the reaction containing compound 87 turned to dark blue immediately after the addition of TFA, suggesting the
existence of the carbocation, which is thought to complicate the reaction (Scheme 3.23).

![Scheme 3.23](image-url)

Scheme 3.23 (a) thiophenol, 20% TFA in DCM, r.t., 2.5 hr, 17%

The t-Bu moiety in compound 87 was speculated to be vulnerable under strong acidic conditions (20% TFA), with the tendency to form a t-Bu carbocation. To remove such potential effect, bromide 78 was used instead of compound 87 under the same TFA conditions. However, decomposition was still observed, indicating that it could be the C-2 alcohol rather than the t-Bu to be instable under TFA conditions (Scheme 3.24, method 1). The formation of compound 88 could be rationalised that the stabilising effect of the phenyl ring in the C-4 side chain of compound 86 would shield the C-2 alcohol from being attacked. To circumvent the strong acidic conditions and given that thiophenol (pKa = 8.2) is more acidic than phenol (pKa = 9.9), a Mitsunobu reaction was therefore attempted. Unfortunately, no desired conversion was observed (Scheme 3.24, method 2).

![Scheme 3.24](image-url)

Scheme 3.24 Trials of converting the C-2 alcohol to the C-2 thio ether

To sum up, only compound 88 was able to be generated from the thio ether series.

3.1.4.3 Structure-activity relationships

![Generic structure](image-url)

Generic structure

Y: CONH, CO, CH₂O, CH₂S
R¹, R²: alkyl or aromatic groups

Re-assess the suitable size of R¹

Interestingly, the results presented in Table 3.7a showed that compounds with
\( t\)-Bu (59, 72 and 80) in the C-4 side chain were much more potent than their benzyl counterparts (60, 73 and 81) when phenyl was used as \( R^2 \). Given that \( t\)-Bu is much smaller than benzyl, it is likely that bulky groups for both \( R^1 \) and \( R^2 \) are not tolerated by the enzyme. The hypothesis is further supported by two pieces of evidence: firstly, tuning aromatic \( R^1 \) (91-93) failed to display any inhibitory activity; secondly, replacing the phenyl ring by another bulky group cyclohexyl as \( R^2 \) (62) only marginally improved the potency (Table 3.7b).

**Table 3.7 Interdependence between the C-2 and the C-4 side chains in first generation of PfNMT inhibitors**

<table>
<thead>
<tr>
<th>( Y )</th>
<th>Compound</th>
<th>( IC_{50} ) (µM)(^a)</th>
<th>( R^1 )</th>
<th>Compound</th>
<th>( IC_{50} ) (µM)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CONH-</td>
<td>59</td>
<td>9.2</td>
<td>-</td>
<td>60 (( R^1=Me ))</td>
<td>NI (0%)(^b)</td>
</tr>
<tr>
<td>-CO-</td>
<td>72</td>
<td>10.6</td>
<td>-</td>
<td>73 (( R^1=H ))</td>
<td>NI (0%)(^b)</td>
</tr>
<tr>
<td>-CH(_2)O-</td>
<td>80</td>
<td>1.4</td>
<td>-</td>
<td>81 (( R^1=Me ))</td>
<td>98.3</td>
</tr>
<tr>
<td>-CH(_2)S-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>88 (( R^1=H ))</td>
<td>~170 (37.1%)(^b)</td>
</tr>
</tbody>
</table>

\( a\) The \( IC_{50} \) values were averaged from two independent dose-response curves; the variation was generally <15\%.

\( b\) The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10\%. \( IC_{50} \) values are estimated from these to give a comparative indication of potency. NI: no inhibition.

\( \text{\( a\) The \( IC_{50} \) values were averaged from two independent dose-response curves; the variation was generally <15\%. } \text{\( b\) The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10\%. } \text{\( IC_{50} \) values are estimated from these to give a comparative indication of potency. NI: no inhibition.} \)
Given the above findings, it was interesting to investigate the other three combinations of R₁ and R₂: “big R₁ – small R₂”, “small R₁ - big R₂” and “small R₁ – small R₂”, where any R groups with the physical size smaller than t-Bu were considered “small” (including t-Bu) and any R groups with the physical size bigger than Ph were considered “big” (including Ph).

**Big R₁ - Small R₂**

The inhibitory activities of this category of compounds (86, 75 and 16) were diverse (Table 3.8), but at least they were all more active than big R₁ – big R₂ combinations, where no inhibition could be observed.

**Table 3.8 Big R₁ – Small R₂ combination in the first generation of PfNMT inhibitors**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y-R²</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>Y-R²</th>
<th>IC₅₀ (µM)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>1/₂CH₂OH</td>
<td>~230 (30.0%)ᵇ</td>
<td>75</td>
<td>1/₂COCH₃</td>
<td>71.5</td>
</tr>
<tr>
<td>16</td>
<td>1/₃COOEt</td>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇ The number in parentheses was the percentage inhibition values of a compound at 100 µM in triplicate; the variation was generally <10%. The IC₅₀ value is estimated from these to give a comparative indication of potency.

**Small R₁ - Big R₂**

A set of compounds with t-Bu – Ph combination (59, 72 and 80) are listed in Table 3.7a and all of them showed IC₅₀s in the single-digit µM range. It was interesting to assess whether t-Bu would be the right size for R₁. Amide series was chosen because of synthetic accessibility. Amides 94-96 with various sizes of R₁ were prepared and their inhibitory activities were measured. The results showed that t-Bu derivative 72 was most active among compounds with small R₁ groups (Table 3.9).
Table 3.9 Small $R^1$ – Big $R^2$ combination in the first generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^1$</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^1$</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>$\frac{1}{2}$-Me</td>
<td>$\approx 182$ (45.4%)$^b$</td>
<td>95</td>
<td>$\frac{1}{2}$-Me</td>
<td>67.4</td>
</tr>
<tr>
<td>96</td>
<td>$\frac{1}{3}$-COOEt</td>
<td>$\approx 215$ (31.7%)$^b$</td>
<td>72</td>
<td>$\frac{1}{3}$-COOEt</td>
<td>10.6</td>
</tr>
</tbody>
</table>

$^a$ The IC$_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%. $^b$ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC$_{50}$ values are estimated from these to give a comparative indication of potency.

Small $R^1$ - Small $R^2$

Compounds of this combination were generally more active than the ones with big $R^1$ – small $R^2$ combination (87, 74 in Table 3.10 vs. 86, 75 in Table 3.8), suggesting that small $R^1$, especially t-Bu, is favoured by the enzyme.

Table 3.10 Small $R^1$ – Small $R^2$ combination in the first generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y-$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>Y-$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>$\frac{1}{2}$-CH$_2$OH</td>
<td>30.0</td>
<td>74</td>
<td>$\frac{1}{2}$-COCH$_3$</td>
<td>53.2</td>
</tr>
<tr>
<td>15</td>
<td>$\frac{1}{3}$-COOEt</td>
<td>9.0</td>
<td>63</td>
<td>$\frac{1}{3}$-CONH-t-Bu</td>
<td>63.6</td>
</tr>
</tbody>
</table>

$^a$ The IC$_{50}$ values were averaged from two independent dose-response curves.

Summary

To conclude, when incorporating variations of the C-2 side chain (except ethyl ester), a small $R^1$ group, especially t-Bu, is much more favourable to inhibit PfNMT than the big $R^1$ counterparts. Therefore this reduced the two optimal C-4 side chain identified in Section 3.1.1.2 to one, with t-Bu as $R^1$ group being more generally preferred. This was then used for the subsequent inhibitor development (Scheme 3.25).
Scheme 3.25 t-Bu was determined to be an optimal R\textsuperscript{1} group in the first generation of PfNMT inhibitors

Activity dependence on the size of R\textsuperscript{2}

Inhibitory activities of compounds with varied R\textsuperscript{2} substitutions are listed in Table 3.11. With regard to the amide series, activity peaked when R\textsuperscript{2} was phenyl (59); either a smaller R\textsuperscript{2} (63) or a bigger one (64) resulted in a loss of potency; addition of an extra methylene to phenyl provided slightly less activity (61 vs. 59); removing aromaticity of R\textsuperscript{2} was found to reduce the activity by 4-fold (59 vs. 62), indicating a possible π-π interaction with the enzyme. Of the ether series, compound 80 was the most active inhibitor identified so far, with an IC\textsubscript{50} value of 1.4 µM; either α or β naphthyl analogue (84 and 85) failed to improve the potency; interestingly, the α-isomer (84) was 4-fold more active than the β-isomer (85), suggesting that such R\textsuperscript{2} might sit in a shallow but relatively wide enzyme pocket. In the ketone series, it was clear that the bigger R\textsuperscript{2} was favoured by the enzyme (72 vs. 74).
Table 3.11  Exploration of $R^2$ in the C-2 side chain in the first generation PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^2$</th>
<th>$IC_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^2$</th>
<th>$IC_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td></td>
<td>63.6</td>
<td>62</td>
<td></td>
<td>30.0</td>
</tr>
<tr>
<td>59</td>
<td></td>
<td>9.2</td>
<td>61</td>
<td></td>
<td>15.5</td>
</tr>
<tr>
<td>64</td>
<td></td>
<td>56.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^2$</th>
<th>$IC_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^2$</th>
<th>$IC_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>$\text{H}$</td>
<td>30.0</td>
<td>80</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>84</td>
<td></td>
<td>2.1</td>
<td>85</td>
<td></td>
<td>8.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^2$</th>
<th>$IC_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^2$</th>
<th>$IC_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>$\text{Me}$</td>
<td>53.2</td>
<td>72</td>
<td></td>
<td>10.6</td>
</tr>
</tbody>
</table>

$^a$The $IC_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%.

To sum up, phenyl as $R^2$ was found to be generally optimal in the investigated amide, ether and ketone series in terms of its suitable size and a potential $\pi-\pi$ interaction with the enzyme (Scheme 3.26). And the next step was to study the suitable phenyl substituents.
A common scenario in drug design is to find an optimal phenyl substituent in an active compound for the potency maximisation. Given so many available substituents and different positions on the ring, the number of possible compounds is very large. Therefore, it is highly advantageous that compounds might be worth synthesising can be determined at an early stage. A rational approach based on the hydrophobic constant $\pi$ of the substituent (more positive the value, more hydrophobic the substituent) and Hammett constant $\sigma$ of the substituent (more positive the value, more electron-donating the substituent) was initially proposed and utilised by Topliss
\cite{146, 147}. The approach starts by selecting three substituents with good discrimination between $\pi$ and $\sigma$ (4-Cl, +$\pi$+$\sigma$; 4-Me, +$\pi$-$\sigma$; 4-OMe, -$\pi$-$\sigma$), aiming to determine the desired group of substituents. Further tuning based on Hansch’s aromatic substituent constants
\cite{148} leads to the discovery of the optimal substituent.

Given the synthetic ease, amide series was selected to fully investigate the optimal phenyl R$^3$ substituents. Compounds 97-101 were thus prepared and their inhibitory activities were measured (Table 3.12). It appeared that Cl or +$\pi$+$\sigma$ system, was not favoured in either para or meta-position (97, 100 vs. 59); 4-Me or +$\pi$-$\sigma$ system, had no effect on the inhibitory activity (99 vs. 59); but 4-OMe or -$\pi$-$\sigma$ system, was found to slightly increase the activity (98 vs. 59). However, further enhancement of -$\sigma$ effect to 4-NMe$_2$, failed to improve the potency (101 vs. 98), indicating that -$\pi$-$\sigma$ system possibly has only trivial influence over the inhibitory activity.
Table 3.12 Different phenyl R³ substitutions in the amide series in the first generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>R³</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>R³</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>H</td>
<td>9.2</td>
<td>97</td>
<td>4-Cl</td>
<td>17.2</td>
</tr>
<tr>
<td>98</td>
<td>4-OCH₃</td>
<td>6.8</td>
<td>99</td>
<td>4-Me</td>
<td>9.7</td>
</tr>
<tr>
<td>100</td>
<td>3-Cl</td>
<td>14.0</td>
<td>101</td>
<td>4-NMe₂</td>
<td>6.8</td>
</tr>
</tbody>
</table>

ᵃThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%.

Considering the stability issue in the ether series and the commercial availability of substituted 2-bromo acetophenones to form the ketone series, only chlorinated derivatives (82, 83 and 102) were synthesised. Introduction of chlorine on the phenyl group had a negative effect on inhibition (82 and 83 vs. 80; 102 vs. 72, Table 3.13), a similar trend to the results from the amide series.

Table 3.13 Exploration of phenyl R³ substitutions in the ether and ketone series in the first generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>R³</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>R³</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>H</td>
<td>1.4</td>
<td>82</td>
<td>4-Cl</td>
<td>2.3</td>
</tr>
<tr>
<td>83</td>
<td>3-Cl</td>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>R³</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>R³</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>H</td>
<td>10.6</td>
<td>102</td>
<td>4-Cl</td>
<td>28.9</td>
</tr>
</tbody>
</table>

ᵃThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%.
To sum up, phenyl with no substitution remained to be an optimal $R^2$ group (Scheme 3.27). No improved activity was achieved when $R^3$ substituents with varied $\pi$ and $\sigma$ were added, indicating that neither of these two parameters are critical to for excellent inhibition against the enzyme.

![Scheme 3.27 Phenyl with no substitution was determined to be an optimal $R^2$ in the first generation of PfNMT inhibitors](image)

$Y$: -CONH-, -CO-, -CH$_2$O-

$R^3$: substituents with varied $\pi$ and $\sigma$

3.1.5 Summary of the first generation of PfNMT inhibitors

Achievement

By optimising the C-4 side chain, compounds 15 and 16 were discovered. During the study of functional groups other than ester at the C-2 position, t-Bu in the C-4 side chain was found to give much more PfNMT inhibition than its benzyl counterpart. Among these inhibitors, ether 80 was the most active inhibitor with an IC$_{50}$ value of 1.4 $\mu$M, a 40-fold enhancement over the hit compound 1. In addition, compounds 59 and 72 were also identified as the best inhibitors from the amide and ketone series respectively (Scheme 3.28).
**Scheme 3.28** Summary of the development of the first generation of PfNMT inhibitors

**Problems to solve**

Aside from pursuing high potency against PfNMT, obtaining excellent selectivity over human NMTs (at least greater than 100-fold) is equally important. The three good inhibitors described in Scheme 3.28 were measured against both human NMTs (Table 3.14). However, all of these molecules displayed moderate selectivity: compound 80 showed 30-fold over HsNMT1 while compounds 59 and 72 gave only 8-fold over HsNMT2.

**Table 3.14** Moderate selectivity was observed in the first generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y-R²</th>
<th>Enzyme IC₅₀ (µM)a</th>
<th>PfNMT</th>
<th>HsNMT1</th>
<th>HsNMT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>-CH₂OPh</td>
<td>10.6</td>
<td>~148 (40.3%)b</td>
<td>81.9</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>-CONHPh</td>
<td>9.2</td>
<td>~446 (18.3%)b</td>
<td>~77 (56.6%)b</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>-COPh</td>
<td>1.4</td>
<td>~63 (61.4%)b</td>
<td>44.4</td>
<td></td>
</tr>
</tbody>
</table>

a The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. b The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency.
Given the highest inhibition and selectivity among the first generation of PfNMT inhibitors, the ether series is most promising. However, the Mitsunobu reaction required to form an ether bond suffered from low yields, significantly limiting the scale-up of the synthesis. In addition, the resulting ether was found to be acid labile, raising a big stability concern if this series would be progressed into the lead optimisation stage. Combined with the moderate selectivity described above, it was necessary to develop a second generation of PfNMT inhibitors, requiring higher potency, greater selectivity and more efficient synthesis.

3.2 The second generation of PfNMT inhibitors

3.2.1 Study of the rigid C-4 side chain

The first generation of PfNMT inhibitors is based on the flexible chain or trimethylene chain at the C-4 position of the benzofuran scaffold. The rigid chain at the same position was thus studied to examine whether it would lead to better inhibition or selectivity (Scheme 3.29).

Scheme 3.29 Strategy to develop an alternative C-4 side chain other than trimethylene one

3.2.1.1 Synthesis

Alternative C-4 side chains (compounds 109-111, including two rings and one constrained trimethylene) were constructed in excellent yields using the Mitsunobu reaction, followed by deprotection in 5% TFA. Different sizes of R¹ were then added to compound 109 via a reductive-amination reaction in good yields (Scheme 3.30).
Scheme 3.30 (a) DIAD, PPh₃, N-Boc-4-OH piperidine (103) or N-Boc-3-OH piperidine (104) or N-Boc-2,2-dimethyl propan-1-ol (105), THF, r.t., 4 hr, >95%; (b) 5% TFA in DCM, r.t., 2 hr, quantitative; (c) i. ketone or aldehyde, HOAc, THF/DMF, r.t., 2 hr; ii. NaBH(OAc)₃, THF/DMF, r.t., 12 hr, 55-87%.

For full structural information of compounds 112-116 with different R¹ groups, please refer to the following SAR part, Section 3.2.1.2 or Appendix. Only the synthetic route is described here.

3.2.1.2 Activity Evaluation

The inhibitory activities of compounds with various C-4 side chains are listed in Table 3.15. Encouragingly, the results showed that compound 109 with a piperidine moiety had the comparable potency with inhibitor 15 containing the optimal trimethylene side chain. Relocation of amino group in the piperidine resulted in a total loss of activity (110 vs. 109), supporting the previous SAR (Table 3.1) that a 3-carbon distance is an optimal length between the ether oxygen and the amino group. Direct comparison between the two primary amines (111 vs. 32) indicates that increasing the steric hindrance of the trimethylene side chain is harmful to the potency.
Table 3.15 Comparison of different C-4 side chains

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>R</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>O</td>
<td>9.0</td>
<td>109</td>
<td>O</td>
<td>16.5</td>
</tr>
<tr>
<td>110</td>
<td>O</td>
<td>NI (0%)ᵇ</td>
<td>111</td>
<td>O</td>
<td>&gt;1000 (7.1%)ᵇ</td>
</tr>
<tr>
<td>32</td>
<td>O</td>
<td>~220 (41.1%)ᵇ</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency. NI: no inhibition.

A range of R¹ groups were added to the amino group of piperidine to investigate their effects on inhibition (Table 3.16). The results demonstrate that a smaller R¹ gives better potency with the exception of i-Pr compound 114, which displayed slightly better activity than 109. However, the synthesis of i-Pr attachment was found to be highly dependent on the composition of the C-2 side chain (Section 3.2.2.1, ketone series). Therefore, 4-piperidinol was selected as an optimal rigid C-4 side chain, possessing comparable inhibitory activity with the previously optimal C-4 trimethylene side chain.
Chapter 3 – PfNMT inhibitors

Table 3.16 Examination of various $R^1$ on the 4-piperidinol side chain in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^1$</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^1$</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>H</td>
<td>16.5</td>
<td>112</td>
<td>Me</td>
<td>50.0</td>
</tr>
<tr>
<td>113</td>
<td></td>
<td>27.0</td>
<td>114</td>
<td></td>
<td>8.0</td>
</tr>
<tr>
<td>115</td>
<td></td>
<td>~208 (32.5%)$^b$</td>
<td>116</td>
<td>F</td>
<td>&gt;500 (14.7%)$^b$</td>
</tr>
</tbody>
</table>

$^a$ The IC$_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%.

$^b$ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC$_{50}$ values are estimated from these to give a comparative indication of potency.

3.2.1.3 Comparison between the 4-piperidinol and trimethylene side chains

The two optimal C-4 side chains identified so far are compared based on their molecular weight, rotatable bonds and synthetic ease (Table 3.17). With regard to the physiochemical properties, the 4-piperidinol side chain possesses lower molecular weight and fewer rotatable bonds. In addition, synthesis of the trimethylene side chain relies on chromatography to separate the desired product from its allyl by-product, while incorporation of the 4-piperidinol side chain involves a Mitsunobu reaction, which has been successfully performed in excellent yields with no need of chromatography (data not shown here)$^{[149]}$. To conclude, 4-piperidinol is more drug-like and easier to synthesise than its trimethylene counterpart, therefore selected as an optimal C-4 side chain in the second generation of PfNMT inhibitors.
Table 3.17 Comparison between piperidine and trimethylene side chains

<table>
<thead>
<tr>
<th>C-4 side chain</th>
<th>Molecular weight</th>
<th>Rotatable bonds</th>
<th>Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CH3][O][C]HN</td>
<td>114.1</td>
<td>4</td>
<td>Chromatography, moderate yields</td>
</tr>
<tr>
<td>[CH3][O][C]NH</td>
<td>84.1</td>
<td>1</td>
<td>No chromatography, excellent yields</td>
</tr>
</tbody>
</table>

3.2.2 Initial C-2 optimisation

Since 4-piperidinol was identified to be an optimal C-4 side chain in the second generation of PfNMT inhibitors, attention was then focused on the study of the C-2 side chains by starting with the non-ester series (Scheme 3.31).

Scheme 3.31 Strategy to develop the optimal C-2 side chain in the second generation of PfNMT inhibitors

3.2.2.1 Synthesis

Ketone series

4-Piperidinol was incorporated to benzofuran 70 in excellent yields using the Mitsunobu reaction, followed by the Boc removal to yield compound 117. Different sizes of R1 were then incorporated to compound 117 via a reductive-amination reaction (Scheme 3.32). However, the N-i-Pr derivative was not produced under these conditions, only compounds 118 and 119 being prepared at that stage.
Amide series

Ester 106 was hydrolysed in the presence of LiOH to give carboxylic acid 120, which was then reacted with various sizes of amines under PyBOP/DIPEA conditions to form an amide bond, followed by deprotection to generate compounds 121-132 (Scheme 3.33).

Ether series

Scheme 3.34 outlines the retro-synthetic route for the ether compounds. Compound 133 required for the Mitsunobu reaction or S_N2 substitution is proposed to be prepared in a two-step procedure (Scheme 3.34).
Selection of a suitable protecting group was found to be critical. Boc was initially used, towards the synthesis of the precursors of desired ethers, compounds 136 and 137 (Scheme 3.35). A range of deprotection conditions were then attempted; however, none of them worked: high concentration of acids (at least 1M) was required to remove Boc\textsuperscript{[150]}, under which conditions the ether bond was not able to survive; neutral conditions which involved 20% of ceric ammonium nitrate (CAN) in silica\textsuperscript{[151]} was not capable to achieve the conversion (Scheme 3.35).

A variety of other protecting groups were also screened. Fmoc (fluorenylmethyloxycarbonyl), a base labile group, was found not to work under Mitsunobu conditions to form the ether bond; Cbz (carbobenzyloxy), a neutral protecting group, could not tolerate the reducing agent which was used to convert ester 134 to alcohol 133 (Scheme 3.34); Tr (trityl), an acid labile group which is removed under weakly acidic conditions, under which the degradation of the C-2 ether bond was found not to occur, was finally utilised to provide the desired ether compounds.

Summarising the synthesis, key intermediate 140 was prepared in a straightforward 2-step reaction in good yields. Representative phenol and alkyl halides
were reacted with compound 140 under Mitsunobu and basic conditions respectively, to form the ether bond, followed by the removal of Tr under weakly acidic conditions to yield ether compounds 142-145. However, because of the steric hindrance of the Tr group, above ether formation experienced low to moderate yields. In addition, alcohol 141 was prepared under the same deprotection conditions (Scheme 3.36).

Scheme 3.36  (a) DIAD, PPh₃, N-Tr-4-OH piperidine (138), THF, r.t., 4 hr, 65%; (b) LiAlH₄, THF, 0°C - r.t., quantitative; (c) 0.1% TFA, 0.2% H₂O in DCM, r.t., 12 hr, 60%-90%; (d) DIAD, PPh₃, phenol, THF, r.t., 4 hr, 5%; (e) NaH, R₂X (X=Br or I), DMF, r.t., 15%-40%.

For full structural information of compounds 143-145 with different R² groups, please refer to the following SAR part, Section 3.2.2.2 or Appendix. Only the synthetic route is described here.

Amine series

Direct alkylation of amine by alcohol was reported by Zaragoza where phosphonium iodide 146, a readily synthesised agent, was used to furnish the conversion. The same conditions were applied to a model study in which alcohol 147 was successfully converted to amine 148 in good yields. However, compound 135 remained unreacted under these conditions (Scheme 3.37).
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Scheme 3.37 Direct alkylation of amine by alcohol

The proposed mechanism\textsuperscript{152} is shown in Scheme 3.38. The compound 146 is deprotonated by DIPEA and then undergoes a Michael addition by an alcohol to form intermediate 150. Thermolysis of this intermediate leads to the P-C bond cleavage and the release of MeCN, to afford alkoxyphosphonium iodide 151, which further decomposes to yield alkyl iodide 152. Finally, the active species 152 alkylates the amine to form the desired product. Based on this mechanism, the failure to provide target amine from compound 135 (Scheme 3.37) could be a result of the bulky C-4 side chain in alcohol 135 that blocks the Michael addition occurring, therefore stopping the rest of the steps to generate the desired amine.

Scheme 3.38 Proposed mechanism for the direct alkylation of amine by alcohol

Another approach to produce amine is an indirect way via aldehyde stage (Scheme 3.39). Alcohol 135 was oxidised to aldehyde 153 by activated manganese dioxide, followed by a reductive-amination and then deprotection to generate the C-2 amines 154 and 155. Primary amine 156 was further hydrogenated from compound 154. However, this route is only suitable for small-scale synthesis because at least 50 equivalents of MnO2 are required to drive the reaction complete in the oxidation step,
which is neither environmentally nor economically friendly.

\[ \text{Scheme 3.39} \quad (a) \text{MnO}_2, \text{DCM, r.t., 24 hr, 77%; (b) i. R}^1\text{NH}_2, \text{HOAc, THF, r.t., 2 hr; ii. NaBH(OAc)}_3, \text{THF, r.t., 12 hr, 50% for 154 and 80% for 155; (c) 5% TFA in DCM, r.t., 2 hr, quantitative; (d) Pd/C, 1,4-cyclohexadiene, EtOH, 80 °C, 4 hr, 38%}. \]

### 3.2.2.2 Structure-activity relationships

\[ \text{Y: -CONH-, -CO-, -CH}_2\text{-O-, -CH}_2\text{NH-}\]

\[ R^1, R^2: \text{alkyl or aromatic groups} \]

**Determination of suitable R**^1

A range of R^1 groups were added to the amino group of piperidine, aiming to investigate their effects on inhibition (Table 3.18). It is clear that a smaller R^1 gives better potency, which is consistent with the results in the C-2 ethyl ester series (Table 3.16).

**Table 3.18 Study of R**^1 **on 4-piperidinol side chain in the second generation of PfNMT inhibitors**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R^1</th>
<th>IC\text{$_{50}$} (µM)$^a$</th>
<th>Compound</th>
<th>R^1</th>
<th>IC\text{$_{50}$} (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>117</td>
<td>H</td>
<td>22.0</td>
<td>118</td>
<td>Me</td>
<td>$^\sim$133 (43.0%)$^b$</td>
</tr>
<tr>
<td>119</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NI (0%)$^b$</td>
</tr>
</tbody>
</table>

$^a$The IC\text{$_{50}$} values were averaged from two independent dose-response curves; the variation was generally <15%. $^b$The numbers in parentheses were the percentage inhibition values of a compound at 100 µM in triplicate; the variation was generally <10%. IC\text{$_{50}$} values are estimated from these to give a comparative indication of potency. NI: no inhibition.
Given the two different series (ethyl ester and phenyl ketone) ending up with a similar inhibition trend regarding the size of $R^1$, the facts further support that 4-piperidinol with no $N$-substituent is an optimal C-4 side chain in the second generation of PfNMT inhibitors (Scheme 3.40).

Scheme 3.40 Hydrogen was determined to be an optimal $R^1$ on piperidine in the second generation of PfNMT inhibitors

**Activity Evaluation of the C-2 side chain**

Inhibitory activities of amide series with various $R^2$ substitutions are listed in Table 3.19. None of compounds containing alkyl groups (121-125) were good inhibitors although it seemed to follow a trend that bigger $R^2$ leads to better inhibition. Compound 126 with phenyl was at least 4-fold more active than 124 and 125, indicating a possible $\pi$-$\pi$ interaction with the enzyme which was described earlier (Table 3.11). Branches on the aromatic rings were found to reduce the activity to a large extent (127, 129 vs. 126). Interestingly, insertion of a methylene group between amide and the aromatic ring improved the potency significantly (130 vs. 129), indicating the importance of such spacer. Compounds with different length of the alkyl spacer were thus prepared and their inhibitory activities were measured. The results showed that mono-methylene is an optimal length of the spacer (128 vs. 157 and 158). In addition, rigidification of the mono-methylene spacer failed to improve the potency (131 vs. 128). Among the compounds in the amide series, compound 130 displayed an IC$_{50}$ value of 2.3 µM, similar potency to the most active compound from the first generation of PfNMT inhibitors (IC$_{50}$: 1.4 µM).
### Table 3.19 Investigation of $R^2$ in the amide series in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td></td>
<td>~410 (19.6%)$^b$</td>
<td>122</td>
<td></td>
<td>~294 (25.4%)$^b$</td>
</tr>
<tr>
<td>123</td>
<td></td>
<td>~292 (25.5%)$^b$</td>
<td>124</td>
<td></td>
<td>~130 (43.5%)$^b$</td>
</tr>
<tr>
<td>125</td>
<td></td>
<td>~146 (40.7%)$^b$</td>
<td>126</td>
<td></td>
<td>28.8</td>
</tr>
<tr>
<td>128</td>
<td></td>
<td>13.0</td>
<td>157</td>
<td></td>
<td>37.2</td>
</tr>
<tr>
<td>158</td>
<td></td>
<td>~144 (40.9%)$^b$</td>
<td>127</td>
<td></td>
<td>~318 (23.9%)$^b$</td>
</tr>
<tr>
<td>129</td>
<td></td>
<td>&gt;100</td>
<td>130</td>
<td></td>
<td>2.3</td>
</tr>
<tr>
<td>131</td>
<td></td>
<td>21.0</td>
<td>132</td>
<td></td>
<td>~138 (42.1%)$^b$</td>
</tr>
</tbody>
</table>

$^a$The IC$_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%. $^b$The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC$_{50}$ values are estimated from these to give a comparative indication of potency.

Inhibitory activities of the ether compounds (141-145) with different $R^2$ groups are listed in Table 3.20. The results suggested that a big $R^2$, especially an aromatic group, is favoured by the enzyme (145 and 142 vs. 141, 143 and 144). Interestingly, little difference was observed between compounds with and without a mono-methylene spacer (142 vs. 145), which was in contrast to the previous findings from the amide series (Table 3.19).
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Table 3.20 Examination of $R^2$ in the ether series in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>141</td>
<td>H</td>
<td>$&gt;1000$ (3.9%)$^b$</td>
<td>143</td>
<td>$\uparrow$</td>
<td>$&gt;500$ (15.1%)$^b$</td>
</tr>
<tr>
<td>144</td>
<td>$\uparrow$</td>
<td>$\sim418$ (19.3%)$^b$</td>
<td>142</td>
<td>$\uparrow$</td>
<td>6.6</td>
</tr>
<tr>
<td>145</td>
<td>$\uparrow$</td>
<td>7.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$The IC$_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%. $^b$The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC$_{50}$ values are estimated from these to give a comparative indication of potency.

Amine compounds (154-156) were generally poor inhibitors against PfNMT (Table 3.21). However, the rule that bigger $R^2$ results in better enzyme inhibition was still followed.

Table 3.21 Exploration of $R^2$ in the amine series in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>$R^2$</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>156</td>
<td>H</td>
<td>NI (0%)$^b$</td>
<td>154</td>
<td>$\uparrow$</td>
<td>$\sim124$ (44.6%)$^b$</td>
</tr>
<tr>
<td>155</td>
<td>$\uparrow$</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$The IC$_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%. $^b$The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC$_{50}$ values are estimated from these to give a comparative indication of potency. NI: no inhibition.

To conclude, the trend that a bigger $R^2$, especially an aromatic ring, leads to better enzyme inhibition was observed in three investigated series (amide, ether and amine), therefore emerging as a clear SAR for the second generation of PfNMT inhibitors.
3.2.2.3 Summary

**Achievement**

4-Piperidinol was identified as an optimal rigid C-4 side chain, which made the molecule more drug-like and easier to synthesise while maintaining a similar potency to its trimethylene counterpart. Further exploration of the non-ester C-2 side chain resulted in the discovery of inhibitor 130 with an IC$_{50}$ value of 2.3 µM (Scheme 3.41), comparable with the most active inhibitor or compound 80 from the first generation of PfNMT inhibitors (IC$_{50}$: 1.4 µM).

![Scheme 3.41 Development of the initial second generation of PfNMT inhibitors](image)

**Problems to solve**

Four good inhibitors described in Scheme 3.41 were measured against both human NMTs (Table 3.22). However disappointedly, moderate selectivity over human NMTs, generally less than 25-fold, was obtained.
Table 3.22 Selectivity data of the good inhibitors from the initial second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y-R²</th>
<th>Enzyme IC₅₀ (µM)ᵃ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PfNMT</td>
<td>HsNMT1</td>
</tr>
<tr>
<td>117</td>
<td>-CO-</td>
<td>22.0</td>
<td>~78 (56%)ᵇ</td>
</tr>
<tr>
<td>142</td>
<td>-CH₂O-</td>
<td>6.6</td>
<td>~153 (39.6%)</td>
</tr>
<tr>
<td>145</td>
<td>-CH₂O-</td>
<td>7.1</td>
<td>~87 (53.6%)</td>
</tr>
<tr>
<td>130</td>
<td>-CONH-</td>
<td>2.3</td>
<td>~58 (63.2%)</td>
</tr>
</tbody>
</table>

ᵃThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇThe numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency. NI: no inhibition.

Compound 130 was the most promising compound out of the above second generation of PfNMT inhibitors, showing low single-digit µM of the IC₅₀ and moderate selectivity. From a synthetic perspective, much higher yields were achieved in the preparation of compound 130 than compound 80, the best inhibitor of the first generation. However, relatively high molecular weight of compound 130 (Mw: 414), toxicity concerns associated with naphthyl and scarcity of the commercially available naphthyl derivatives and their heterocyclic mimics significantly limited the further development based on this structure.

3.2.3 Breakthrough

At that stage, it seemed that the project was stagnant where conventional non-ester functionalities had been exhausted, but the IC₅₀ values of inhibitors were still in the µM range and only moderate selectivity over human NMTs were achieved. Therefore, revisiting the data obtained was necessary.
Table 3.23 compares the activity of compounds with three different functional groups Y. Ester 109 was far more potent than amide 121 and ether 144 when R² was fixed as ethyl (ethyl amine was not available at that time, so i-Pr amine was used instead). Combined with the rule that bigger R² leads to better enzyme inhibition, it was likely that ester coupled with a big R² would give high potency. Although ester might be hydrolysed by a range of esterases in the cell or body, it should be chemically stable under enzyme assay conditions, therefore worthwhile to be investigated.

### Table 3.23 Activity comparison among different series for the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y-R²</th>
<th>IC₅₀ (µM)a</th>
<th>Compound</th>
<th>Y-R²</th>
<th>IC₅₀ (µM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td>CONH</td>
<td>~410 (19.6)b</td>
<td>144</td>
<td>CH₂O</td>
<td>~418 (19.3%)b</td>
</tr>
<tr>
<td>109</td>
<td>COO</td>
<td>16.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. b The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency.

## 3.2.3.1 Synthesis of the C-2 ester

A range of methods were attempted to convert carboxylic acid 120 to ester. Deprotonating the acid using a strong base (NaH in this case) followed by S_N2 substitution with electrophilic R²X afforded the desired ester. However, the yields became poor when the size of R² increases (Scheme 3.42). Highly acidic conditions to make acyl chloride, a conventional precursor of ester, were found to complicate the reaction because of the vulnerability of Boc under such conditions (Scheme 3.42). Different coupling reagents were screened as well: PyBOP/DIPEA was only able to make methyl ester; DCC/DMAP (N,N'-dicyclohexylcarbodiimide/4-Dimethylaminopyridine) failed to provide any conversion; but good to excellent yields were obtained when
EDCI/HOBt (1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide/N-Hydroxybenzotriazole) was employed (Scheme 3.42). The mechanism of such conversion is similar to the one of PyBOP/DIPEA (Scheme 3.14) and compound 159 is the activated form of the carboxylic acid. Interestingly, intermediate 159 was found to be chemically stable and could be isolated under weakly acidic conditions. Considering that the actual form of EDCI used for synthesis was an HCl salt, DIPEA was thus added to basify the reaction mixture, ensuring the conversion from intermediate 159 to the desired ester.

Scheme 3.42 Trials of making the ester series

Esterification of carboxylic acid 120 by alcohols with various sizes of R² was conducted with the aid of the coupling reagents EDCI/HOBt, followed by the Boc-removal to produce compounds 160-164. Additionally, acid 165 was prepared from the deprotection of 120 directly (Scheme 3.43).

Scheme 3.43 (a) EDCI, HOBT, DIPEA, R²OH, CH₃CN, r.t., 12 hr, 70-90%; (b) 5% TFA in DCM, r.t., 2 hr, quantitative.

For full structural information of compounds 160-164 with different R² groups, please refer to the following SAR part, Section 3.2.3.2 or Appendix. Only the synthetic route is described here.
3.2.3.2 Activity evaluation

Discovery of a sub-μM inhibitor

Inhibitory activities of the ester series with different R² groups are listed in Table 3.24. As expected, the results suggest that big R² in the ester series is generally favoured by the enzyme which is consistent with the previous results (Section 3.2.2.2). More excitingly, compound 162 is the first compound to reach the sub-μM range, with an IC₅₀ value of 0.27 μM. Introduction of a mono-methylene between R² phenyl and the ester group dramatically improved the potency by 100-fold (162 vs. 161). It was thus worthwhile to investigate the most suitable length of such spacer and compounds 166 and 167 were hence prepared. The results reveal that the mono-methylene is optimal and longer spacer results in poorer activity (166, 167 vs. 162). Increasing the ring size reduced the potency by 5-fold (164 vs. 162), a contrast to the results from the amide series whereby 130 containing naphthyl was 6-fold more active than 128 with phenyl (Table 3.19), indicating the different environments surrounding R² between the ester and amide series. In addition, aromaticity of R² was determined to be critical and the removal of this led to a 30-fold reduction of activity (163 vs. 162), indicating the existence of a strong π-π interaction.
Table 3.24 Examination of R\textsuperscript{2} in the ester series in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>R\textsuperscript{2}</th>
<th>IC\textsubscript{50} (µM)\textsuperscript{a}</th>
<th>Compound</th>
<th>R\textsuperscript{2}</th>
<th>IC\textsubscript{50} (µM)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>165</td>
<td>H</td>
<td>~443 (18.4%)\textsuperscript{b}</td>
<td>160</td>
<td></td>
<td>~98 (50.6%)\textsuperscript{b}</td>
</tr>
<tr>
<td>109</td>
<td></td>
<td>16.5</td>
<td>161</td>
<td></td>
<td>24.4</td>
</tr>
<tr>
<td>162</td>
<td></td>
<td>0.27</td>
<td>163</td>
<td></td>
<td>9.7</td>
</tr>
<tr>
<td>166</td>
<td></td>
<td>4.1</td>
<td>167</td>
<td></td>
<td>36.2</td>
</tr>
<tr>
<td>164</td>
<td></td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}The IC\textsubscript{50} values were averaged from two independent dose-response curves; the variation was generally <15%. \textsuperscript{b} The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC\textsubscript{50} values are estimated from these to give a comparative indication of potency.

Investigation of phenyl substituents

Neither electronic nor hydrophobic effect of phenyl substitutions was found to improve enzyme inhibition in the amide series in the first generation of PfNMT inhibitors (Table 3.12). As mentioned above, the binding modes of R\textsuperscript{2} phenyl with the enzyme between the ester and amide series might be different. Therefore it was thought valuable to study the effects of phenyl substitutions in the ester series in the second generation of PfNMT inhibitors. The Topliss approach was again applied and the same initial set of substituents: methyl, chlorine and methoxy, were employed.

Interestingly, when compounds with methoxy on different phenyl positions were deprotected under acidic conditions, only compound with 3-methoxy could be made while the other two suffered from the decomposition (Scheme 3.44).
Scheme 3.44 Acid labile phenomena in some methylene esters

The decomposition was accompanied with a dramatic colour change after the addition of TFA, indicating the existence of the carbocation. Scheme 3.45 illustrates the fates of carbocations with methoxy added on the different phenyl positions. 2-OMe 168 and 4-OMe 170 can be stabilised by their resonance hybrids 169 and 171 respectively, responsible for the decomposition from their parent esters; in contrast, 3-OMe 172 lacks such stabilisation, therefore escaping a breakdown under acidic conditions.

Scheme 3.45 Resonance theory to explain the acid labile observation in some methylene esters

Seven derivatives 173-179 comprising methyl and chlorine at all three possible positions and methoxy at meta-position of phenyl were prepared and their inhibitory activities were measured (Table 3.25). Introduction of a para-substituent reduced the potency by at least 10-fold (173, 174 vs. 162), indicating that R² might sit in a shallow enzyme pocket. Meta and ortho-substitutions (175-179) failed to improve the inhibition although these two positions seemed to be more tolerated by the enzyme than the para-ones. These compounds were also tested against both human NMTs to determine the selectivity (Table 3.25). The most active compound (162) displayed excellent selectivity (greater than 400-fold over both human NMTs) and the investigated phenyl substitutions showed little effects on the inhibition against both human NMTs.
Table 3.5 Enzyme inhibition data of compounds with different phenyl R₃ substituents in the ester series in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₃</th>
<th>Enzyme IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PfNMT</td>
</tr>
<tr>
<td>162</td>
<td>H</td>
<td>0.27</td>
</tr>
<tr>
<td>173</td>
<td>4-Cl</td>
<td>5.6</td>
</tr>
<tr>
<td>174</td>
<td>4-Me</td>
<td>2.1</td>
</tr>
<tr>
<td>175</td>
<td>3-Cl</td>
<td>0.99</td>
</tr>
<tr>
<td>176</td>
<td>3-Me</td>
<td>0.62</td>
</tr>
<tr>
<td>177</td>
<td>3-OMe</td>
<td>0.68</td>
</tr>
<tr>
<td>178</td>
<td>2-Cl</td>
<td>0.98</td>
</tr>
<tr>
<td>179</td>
<td>2-Me</td>
<td>1.9</td>
</tr>
</tbody>
</table>

ᵃThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇThe numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency. NI: no inhibition.

Other interesting analogues

Given the fact that N-i-Pr piperidine 114 was slightly more active than piperidine 109 in the C-2 ethyl ester series (Table 3.16), N-i-Pr piperidine 180 was hence prepared via a reductive-amination from 162 and evaluated against PfNMT. Unfortunately, such i-propylation to the piperidine side chain failed to give the expected activity enhancement, but a 5-fold loss of enzyme potency observed instead (180 vs. 162, Figure 3.1).

Considering the similar activity between the trimethylene and 4-piperidinol side chains at the C-4 position of the benzofuran scaffold, compound 181 was synthesised via esterification and amination from compound 55. The resulting ester 181 showed an IC₅₀ value of 1.6 µM (Figure 3.1), equally potent with the best compound 80 (IC₅₀: 1.4 µM) in the first generation of PfNMT inhibitors, but it was still 6-fold less active than inhibitor 162. Additionally, compound 181 displayed moderate selectivity of 25-fold over both human NMTs, which was shared by the other first generation of PfNMT inhibitors (Table 3.14). Apart from simple synthesis and drug-like properties mentioned earlier (Table
3.17), 4-piperidinol possesses excellent selectivity, hence supporting the superiority of 4-piperidinol over the trimethylene side chain as an optimal C-4 side chain.

![Figure 3.1](image)

The reversed ester 182 was another interesting molecule to look at. The initial synthesis trial used Boc as a protecting group; however it failed at the Boc-removal stage when decomposition occurred under TFA conditions. The strategy then moved to utilise a Tr group instead and made the target compound via esterification and acid-catalysed deprotection (Scheme 3.46). However, poor yields were obtained due to the steric hindrance of Tr. Regarding the inhibitory activity, reversing the ester lost the potency by 35-fold (182 vs. 162, Figure 3.1), suggesting that the proper arrangement of methylene ester is essential.

![Scheme 3.46](image)

### 3.2.3.3 Summary

Re-examinations of previous compounds containing varied Y linkage groups at the C-2 position suggested that ester could form the basis for a promising series. Investigation of R² in the ester series led to the discovery of compound 162 with an IC₅₀ value of 0.27 µM, the first inhibitor to reach sub-µM range. In addition, the compound exhibited excellent selectivity over both human NMTs. Tunings of the electronic and hydrophobic effects of phenyl R³ substituents failed to improve the potency (Scheme
3.47. Preliminary attempts to design more active molecules based on the structure of \textit{162} were not successful. At this stage, aside from the role of \textit{Y} linkage group, other moieties of benzofuran inhibitors had been established. Therefore, the next step would focus on the investigation of \textit{Y} linkage group, aiming to understand its binding mode.

![Scheme 3.47 Development of the first sub-\(\mu\)M inhibitor](image)

### 3.2.4 Investigation of the role of \textit{Y} linkage group

#### 3.2.4.1 Huge activity difference between the amide and ester series

A 90-fold increase of inhibitory activity was observed in the ester series when \(R^2\) was changed from phenyl to benzyl; in contrast, only a 2-fold improvement was seen in the amide series (Table 3.26).

**Table 3.26 Comparison of the amide and ester series in the second generation of PfNMT inhibitors**

<table>
<thead>
<tr>
<th>(R^2)</th>
<th>Compound</th>
<th>IC(_{50}) ((\mu)M)(^a)</th>
<th>Compound</th>
<th>IC(_{50}) ((\mu)M)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="amide.png" alt="amide" /></td>
<td>126</td>
<td>28.8</td>
<td>161</td>
<td>24.4</td>
</tr>
<tr>
<td><img src="ester.png" alt="ester" /></td>
<td>128</td>
<td>13.0</td>
<td>162</td>
<td>0.27</td>
</tr>
</tbody>
</table>

\(^a\)The IC\(_{50}\) values were averaged from two independent dose-response curves; the variation was generally <15%.

The amide and ester groups are quite similar in terms of their propensities towards the dipole-dipole interaction and hydrogen bond. Given the fact that a partial double bond (38\%) formed between nitrogen and carbonyl carbon restricts the rotation...
of C-N bond\textsuperscript{[154]} while the C-O bond in the ester is more flexible (Figure 3.2), it is likely that the enhanced flexibility available to the ester analogues is beneficial within these inhibitors.

\[ \text{Figure 3.2 Flexibility theory to explain the activity difference between the amide and ester series in the second generation of PfNMT inhibitors} \]

Aside from the flexibility assumption, it was also worthwhile to examine whether Y involves other interactions, such as the dipole-dipole interaction and hydrogen bond; additionally, phenyl with no substitution was determined to be an optimal terminal group of the C-2 side chain; it was therefore easier to re-define Y as a linkage group to connect the benzofuran scaffold and phenyl (Figure 3.3). Compounds with various Y linkage groups based on the generic structure of \textbf{185} were thus proposed, synthesised and evaluated against PfNMT.

\[ \text{Figure 3.16 New definition of Y linkage group in the second generation of PfNMT inhibitors} \]

\section*{3.2.4.2 Synthesis}

\textit{Alkyl as the Y linkage group}

Compound \textbf{186} was designed to contain a flexible linker Y without any other possible interactions. Its intermediate alkenes \textbf{187} (mixture of \textit{cis} and \textit{trans} isomers), which were proposed to be prepared \textit{via} a Wittig reaction from aldehyde \textbf{153} (Scheme
were also interesting for biological tests, with the purpose to study the effects of the geometric isomerism of Y on enzyme inhibition.

Scheme 3.48 Synthetic analysis to make compounds containing alkyl as the Y linkage group

The synthetic route was straightforward. Aldehyde 186 underwent a Wittig reaction, followed by the Boc-removal to generate alkenes 188 (trans-isomer) and 189 (cis-isomer), which could only be separated by preparative HPLC. The latter was further hydrogenated to yield alkyl 186 in moderate yields (Scheme 3.49).

Scheme 3.49 (a) benzyltriphenyl phosphonium bromide, NaOH, THF/H₂O, r.t., 1 hr, 70% (including cis and trans); (b) 5% TFA in DCM, r.t., 2 hr, quantitative; (c) Pd/C, 1,4-cyclohexadiene, EtOH, 80 °C, 4 hr, 40%.

Carbonyl dimethylene formation

Compound 190 was proposed to mimic methylene ester (the series that showed the best inhibition so far). Its precursor or enone 191, synthesised in two steps involving a Mitsunobu reaction and aldol condensation (Scheme 3.50), was another interesting molecule for biological tests.

Scheme 3.50 Retro-synthetic analysis to make compounds containing carbonyl dimethylene moiety
To sum up the synthesis, \( N\text{-Boc}-4\text{-hydroxyl-piperidine} \) \( 103 \) was firstly added to methyl ketone \( 71 \) to form compound \( 192 \), which was then treated with benzaldehyde under basic conditions, followed by the Boc-removal to provide trans-enone \( 193 \) exclusively. Hydrogenation of compound \( 193 \) finally afforded the desired compound \( 190 \) (Scheme 3.51).

\[ \text{Scheme 3.51} \quad \text{(a) DIAD, PPh}_3, \ N\text{-Boc}-4\text{-OH piperidine (103), THF, r.t., 4 hr, 84%; (b) benzaldehyde, NaOH, EtOH/H}_2\text{O, 3 hr, 45%; (c) 5\% TFA in DCM, r.t., 2 hr, 90%; (d) Pd/C, 1,4-cyclohexadiene, EtOH, 80^\circ\text{C, 40}.} \]

\textit{Methylene sulfanyl derivatives}

In 2000, the Petrov group\[^{155, 156}\] reported a novel approach to synthesise 2-benzofuransulfanyl derivatives \( 196 \) from the precursor 1,2,3-thiadiazole \( 195 \), which was prepared in two steps involving a thermal dehydration and Hurd Mori reaction\[^{157, 158}\] from acetophenone \( 194 \) (Scheme 3.52). The route opens up a possibility to synthesise 4-hydroxy-2-benzofuransulfanyl derivatives, a desired inhibitor pattern described earlier (Section 3.1.3).

\[ \text{Scheme 3.52 Literature synthesis of 2-benzofuransulfanyl derivatives} \]

Carbazone \( 197 \) was prepared according to the literature method in moderate yields of 25\%\[^{156}\]. Thionyl chloride is usually used as both reagent and solvent for a normal Hurd Mori reaction. However, compound \( 197 \) was found to be insoluble in \( \text{SOCl}_2 \) at room temperature, under which conditions no desired conversion was observed; increasing the temperature helped dissolve the compound, but led to the formation of di-chloro compound \( 198 \) instead. Therefore, it was necessary to have another solvent to co-exist with \( \text{SOCl}_2 \) to dissolve the starting material. A range of solvents were thus
screened and DMF was found to give the desired product (Scheme 3.53).

\[ \text{DMF} \rightarrow \text{Product} \]

i) Not soluble in r.t., No reaction

ii) soluble at refluxing SOCl₂, 5% yield

\[ \text{Scheme 3.53 Optimisation of Mori-Hurd reaction conditions} \]

Base-catalysed alkylation of thiadiazole 199 was then conducted. Benzyl bromide was initially used. However, it was found to be too reactive and only O-benzylated products 201 and 202 were isolated. Therefore, benzyl chloride, a less reactive alkylating reagent, was attempted. It was able to provide the desired product 200, but in poor yields of 15% and three by-products 201-203 were isolated as well (Scheme 3.54a). Di-benzylated compound 203 could be removed by a careful control of the amount of benzyl chloride; however, O-benzylated products 201 and 202 were unavoidable even pre-refluxing compound 199 with the base for several hours before the addition of benzyl chloride. Possible explanation could be that the two adjacent hydroxyl groups stabilise the thiadiazole, resulting in only a partial breakdown by the base under thermal conditions to provide intermediate 204 (Scheme 3.54b).

\[ \text{Scheme 3.54 Investigation of base-catalyzed alkylation of 1,2,3-thiadiazole} \]
Compound 205 was prepared via a Mitsunobu reaction from compound 200. m-CPBA was reported to selectively oxidise the thioether to either sulfoxide or sulfone, depending on the equivalents of reagent used\textsuperscript{[159]}. However, when the same conditions were applied to compound 205, sulfone 207 was the exclusive product no matter how much m-CPBA involved in the reaction (Scheme 3.55). Given a chiral center in sulfoxide 206, the access to optically pure isomers might have proven to be difficult. Therefore, the sulfoxide 206 was not targeted at this stage.

Scheme 3.55 Oxidation of the benzofuransulfanyl compound

To conclude, 2,6-dihydroxy-acetophenone 1 was treated with ethyl carbazate under refluxing conditions to give ethylcarbozone 197, which then underwent a Hurd-Mori reaction to form 1,2,3-thiadiazole 199, followed by base-catalysed alkylation to yield sulfanyl 200. Incorporation of the piperidine side chain on compound 200 provided compound 205, which was either deprotected to obtain sulfanyl 208, or oxidised and then deprotected to afford sulfone 209, a truncated mimic of methylene ester (Scheme 3.56).
Scheme 3.56 (a) ethyl carbazate, EtOH/H$_2$O, 80 °C, 24 hr, 24%; (b) SOCl$_2$, DMF, -20 °C-r.t, 3 hr, 54%; (c) benzyl chloride, K$_2$CO$_3$, acetone, 60 °C, 6 hr, 24%; (d) DIAD, PPh$_3$, N-Boc-4-OH piperidine (103), THF, r.t., 4 hr, 50%; (e) 5% TFA in DCM, r.t., 2 hr, 90%; (f) m-CPBA, DCM, r.t., 1 hr, 50%.

**Bioisostere of methylene ester**

Aside from structural mimics, bioisosteres of the ester are another attractive area to investigate. In medicinal chemistry, bioisosteres are substituents or groups with similar physical or chemical properties which produce broadly similar biological properties to a chemical compound. Figure 3.4 lists a set of bioisosteres of ester reported by Cambridge Medchem Consulting$^{[160]}$. From a synthetic perspective, 1,2,4-oxadiazole is the most chemically tractable and has been successfully used in a number of biologically important molecules to mimic ester$^{[161-164]}$.

Figure 3.4 Structures of bioisosteres of ester

A one-pot reaction to make 1,2,4-oxadiazole was reported by Amarasinghe$^{[165]}$ in which ester 210 was formed in situ, followed by a dehydration under refluxing toluene conditions to provide the target compound (Scheme 3.57). However, no conversion occurred when the same conditions were applied to carboxylic acid 120.
Scheme 3.57 Proposed one-pot reaction to make 1,2,4-oxadiazole derivatives

The one-pot reaction was then separated into two individual steps. Ester 211 was readily prepared using an established EDCI/HOBt approach (Section 3.2.3.1), followed by the dehydration under thermal conditions in the presence of 4Å molecular sieves, to provide the target oxadiazole compounds 213 and 215 (Scheme 3.58).

Scheme 3.58 (a) R²(NH₂)C=N-OH, EDCI, HOBt, DIPEA, CH₃CN, r.t., 12 hr, 70%; (b) 4Å molecular sieve, dry toluene, 110 °C, 4 hr, 95%; (c) 5% TFA in DCM, r.t., 2 hr, 90%.

3.2.4.3 Structure-activity relationships

Rigid Y linkage groups

As for rigid Y linkage groups, all the compounds except cis-alkene 189 displayed similar inhibition with IC₅₀ values around 20 µM, indicating that the ester group itself is not special (Table 3.27). The geometric flip of alkenes from trans to cis-isomer resulted in a 3-fold drop of potency (188 vs. 189).
Table 3.27 Rigid Y linkage groups in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>Y</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>161</td>
<td>-COO-</td>
<td>24.4</td>
<td>126</td>
<td>-CONH-</td>
<td>28.8</td>
</tr>
<tr>
<td>188</td>
<td>H</td>
<td>26.1</td>
<td>189</td>
<td>H</td>
<td>72.0</td>
</tr>
<tr>
<td>117</td>
<td>-CO-</td>
<td>22.0</td>
<td>213</td>
<td>N</td>
<td>17.9</td>
</tr>
</tbody>
</table>

ᵃThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%.

Flexible Y linkage groups

Table 3.28 is used to compare the inhibitors with flexible Y linkage groups. Only a flexible ethyl of linker Y (186) was not able to ensure good inhibition. Changing methylene to the hetero atom, especially oxygen, led to a significant activity enhancement (142, 208 vs. 186), signaling the probable existence of a hydrogen bond associated with the hetero atom. Replacing carbonyl by methylene resulted in a 25-fold loss of activity (142, 145 vs. 162), indicating the necessity of the carbonyl group. The reversal of methylene ester reduced the potency dramatically (182 vs. 162) and the swap between the hetero atom and methylene was also found to affect enzyme inhibition (142 vs. 208), suggesting that the proper atom arrangement of Y is crucial.

Table 3.28 Flexible Y linkage groups in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>Y</th>
<th>IC₅₀ (µM)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>-COOCH₂⁻</td>
<td>0.27</td>
<td>186</td>
<td>-CH₂CH₂⁻</td>
<td>131.4</td>
</tr>
<tr>
<td>142</td>
<td>-CH₂O⁻</td>
<td>6.6</td>
<td>145</td>
<td>-CH₂OCH₂⁻</td>
<td>7.1</td>
</tr>
<tr>
<td>182</td>
<td>-CH₃OCO⁻</td>
<td>9.6</td>
<td>208</td>
<td>-SCH₂⁻ᵇ</td>
<td>38.2</td>
</tr>
</tbody>
</table>

ᵃThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇThe structure has no methyl on the C-3 position of the benzofuran scaffold.
Mimic of methylene ester as the Y linkage group

Compounds listed in Table 3.29 are designed to mimic methylene ester, to view the Y linkage group as a whole. The inhibitory activity plummeted by 50-fold when replacing the ester oxygen by methylene (190 vs. 162), suggesting the key role of this oxygen which could involve a hydrogen bond with the enzyme. Rigidification of the enone moiety gave a 3-fold improvement of activity (193 vs. 190), which appeared to contradict the previous findings that flexibility of Y was essential. It is possible that this rigid linker would direct the molecule into a proper enzyme pocket. Surprisingly, methylene sulfone 209 lost almost all the inhibitory activity. Shorter length of Y compared with methylene ester should not be a major contributor to such a dramatic loss of activity as even shorter one, C-2 phenyl ketone 171, displayed the inhibitory activity with an IC$_{50}$ value of 22 µM. Therefore, it is likely that methylene sulfone (209) would be incompatible with the good binding to the enzyme. Oxodiazole 215, a bioisostere of methylene ester, failed to reach the comparable level of inhibition with compound 162.

Table 3.29 Mimic of methylene ester as the Y linkage group in the second generation of PfNMT inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>Compound</th>
<th>Y</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>190</td>
<td>-COCH$_2$CH$_2$-</td>
<td>10.2</td>
<td>193</td>
<td>trans -COCH=CH-</td>
<td>3.5$^d$</td>
</tr>
<tr>
<td>209</td>
<td>-SO$_2$CH$_2$-</td>
<td>$\sim485$ (17.1%)$^{b,c}$</td>
<td>215</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>162</td>
<td>-COOCH$_2$-</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$The IC$_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%.  
$^b$ The number in parentheses was the percentage inhibition values of a compound at 100 µM in triplicate. The IC$_{50}$ value is estimated from these to give a comparative indication of potency; the variation was generally <10%.  
$^c$ The structure has no methyl on the C-3 position of the benzofuran scaffold.  
$^d$ The compound only showed moderate selectivity, with the IC$_{50}$s 43.9 µM and 39.5 µM against HsNMT1&2, respectively.
**Summary**

In conclusion, all three components of methylene ester were determined to be essential to maintain the activity: the carbonyl group might involve a dipole-dipole interaction or hydrogen bond; the ester oxygen could participate in a key hydrogen bond; the mono-methylene spacer would improve the flexibility of linker Y (Figure 3.5). None of other Y linkage groups synthesised so far exhibited comparable inhibition, indicating that methylene ester would be optimal to give excellent PfNMT inhibition.

![DDI or HBA, HBA, Flexibility](image)

**Figure 3.5** Optimal role of methylene ester to give excellent inhibition

*All three components of methylene ester were proven to be important to ensure excellent inhibition. The carbonyl group could be involved in dipole-dipole interaction or hydrogen bond with the enzyme; the ester oxygen would act as a key hydrogen bond acceptor; the methylene group is thought to give sufficient flexibility.*

### 3.2.5 Summary of the second generation of PfNMT inhibitors

4-Piperidinol was identified as an optimal C-4 side chain in the second generation of PfNMT inhibitors. Big R² groups, especially aromatic groups, were found to be favoured by the enzyme. A great effort was put into the study of Y linkage groups and methylene ester was determined to be optimal (Scheme 3.59). Compound 162 was the best inhibitor against PfNMT so far, with an IC₅₀ value of 0.27 µM and excellent selectivity over both human NMTs.

![Scheme 3.59](image)

**Scheme 3.59** Summary of development of the second generation of PfNMT inhibitors
3.3 Co-crystal structure

Excitingly, the structure of *P. vivax* NMT, a nonhydrolysable myr-CoA analogue [S-(2-oxo)pentadecyl-CoA] and compound 177 was successfully obtained in collaboration with Dr. J. Brannigan and Prof. A. Wilkinson in York University, which is to our knowledge the first co-crystal structure of an inhibitor bound to a *Plasmodium* NMT. The structural interpretations in this section are based on the initial unpublished information; this is still being refined and so the exact interatomic distances are still preliminary although the main features are well defined and are unlikely to change.

PvNMT shares 81% identity with PfNMT in primary sequence and only 2 out of 23 residues, which form the ligand binding site (residues within 5Å of the ligand in the enzyme) in PvNMT, are different between these two enzymes (Y212 and Y334 in PvNMT are both replaced by Phe in PfNMT, Figure 3.6). Therefore, the structural information of PvNMT would be greatly valuable to rationalise the experimental SARs for PfNMT inhibitors and give directions for future inhibitor design.

![Figure 3.6 Alignment of PvNMT and PfNMT](image)

*The sequences of various NMT proteins have been aligned with the program MultAlign (Version 5.4.1.Corpet 1988). PvNMT shares 81% identity with PfNMT in primary sequence; out of 23 residues forming the ligand binding pocket, only 2 residues are different between these two enzymes (Y212 and Y334 in PvNMT are both replaced by Phe in PfNMT). The colour codes are as follows: red, exact match; blue underline, residues forming the ligand binding site; green star, different residues between these two enzymes out of the residues forming the ligand binding pocket.*
3.3.1 Binding mode of compound 177 with PvNMT

The inhibitor 177 is found to bind at the peptide binding pocket and the key interactions are illustrated in Figure 3.7. The secondary amino group of piperidine at the C-4 position of the benzofuran scaffold picks up a hydrogen bond with the C-terminal carboxylate (Leu 410), a conserved interaction found in both CaNMT and TbNMT inhibitors. The benzofuran moiety is surrounded by hydrophobic residues and the ring is stacked parallel to Tyr211 to involve a π-π interaction. The observed interactions regarding methylene ester are as follows: the carbonyl oxygen is found to participate in a hydrogen bond with Tyr334 via a water bridge; the ester oxygen is associated with another hydrogen bond with Tyr211; the methylene directs the attached meta-methoxy phenyl group to a proper enzyme pocket, where the aromatic ring captures a weak π-π interaction with Phe105 (4.2 Å of distance) and the meta-methoxy oxygen forms a hydrogen bond with Ser319.

![Diagram of interactions](image)

**Figure 3.7 The schematic drawing of the interactions of inhibitor 177 with PvNMT**

The colour codes are as follows: oxygen atoms, red; nitrogen atoms, blue; carbon atoms of the key residues and the inhibitor are yellow and green respectively; residues not directly involved with the interactions are coloured orange; dashed magenta lines indicate hydrogen bond (HB) interactions and dashed brown lines represent π-π stackings; water molecules, red sphere.
3.3.2 Comparison of inhibition data between Pf and PvNMTs

Given the high identity of primary sequence between these two *Plasmodium* NMTs, it is expected that PfNMT inhibitors discussed earlier would give similar potency against PvNMT. Although there is a broad similarity in inhibition of the two NMTs, there are some differences (Table 3.30). Most significant is that the most potent PfNMT inhibitor, compound 162, is 10-fold less potent against PvNMT. As shown in Figure 3.7, R² phenyl involves a weak π-π stacking with Phe105 in PvNMT, giving a mere 3-fold improvement over its cyclohexyl counterpart (162 vs. 163). However, the same aromacity of R² is found to be responsible for a 35-fold activity enhancement in PfNMT. Such discrepancy could be explained as the different distances between R² and Phe105 in these two NMTs, whereby the distance in PfNMT is closer than the one in PvNMT, therefore giving a stronger π-π interaction.

Table 3.30 Comparison of inhibition data between Pf and PvNMTs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y</th>
<th>IC₅₀(PfNMT, µM)ᵃ</th>
<th>IC₅₀(PvNMT, µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>-COOCH₂⁻</td>
<td>0.27</td>
<td>2.9</td>
</tr>
<tr>
<td>128</td>
<td>-CONHCH₂⁻</td>
<td>19.2</td>
<td>15.7</td>
</tr>
<tr>
<td>193</td>
<td>(trans) -COCH=CH-</td>
<td>3.5</td>
<td>14.2</td>
</tr>
<tr>
<td>190</td>
<td>-COCH₂CH₂⁻</td>
<td>10.2</td>
<td>19.7</td>
</tr>
<tr>
<td>145</td>
<td>-CH₂OCH₂⁻</td>
<td>7.1</td>
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<td>166</td>
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</tr>
<tr>
<td>163</td>
<td></td>
<td>9.7</td>
<td>7.3</td>
</tr>
</tbody>
</table>

ᵃThe IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%.
3.3.3 Proposed origin of selectivity

Compound 177 displays excellent selectivity over human NMTs; while DDD85646, initially developed as a TbNMT inhibitor[124], is actually a universal NMT inhibitor, giving little selectivity (data not shown here). It was thus interesting to use the available structural information, with the purpose to rationalise the origin of selectivity.

At the start of this project, there was no information on the structure of any parasite NMT; now that this has become available for Leishmania NMTs[87, 124] and Plasmodium NMT (discussed above), it is apparent that the residues forming the peptide binding pocket are highly similar. Obtaining selective inhibitors is therefore welcome news, but would not have been obvious from the structural data. It is likely to be the different geometry of some key residues rather than the amino-acid differences, which is responsible for the selectivity. The X-ray structure of unligated and inhibitor-complexed PvNMT shows that the residue Y211 in PvNMT undergoes a twist, moving the aromatic ring and relocating the main chain to gain a stacking interaction with the benzofuran core of 177 (Figure 3.8a); presumably the associated energy cost is offset by the resulting π-π stacking with the scaffold and a hydrogen bond with the ester oxygen of the inhibitor (Figure 3.7). However, when modelling the same inhibitor into the structure of HsNMT1 with myr-CoA (PDB code: 3IU1), the equivalent tyrosine or Y296 in HsNMT1 is found to significantly clash with 177 (Figure 3.8b). Given the poor inhibition of 177 against HsNMT1, it is speculated that either the initial geometry of this residue is sufficiently different from that of PvNMT or the energy barrier required for the conformational change of Y296 in HsNMT1 is too high to be compensated by the resulting interactions, hence giving the selectivity. There are sequence differences between PvNMT and HsNMT1 in the region immediately “behind” that tyrosine (Figure 3.8a and 3.8b), which may be responsible for this difference in behaviour. In contrast to inhibitor 177, DDD85646 makes a minimal interaction with the corresponding tyrosine (Y217 in LmNMT and Y296 in HsNMT1) in both LmNMT (Figure 3.8c, PDB code: 2WSA) and HsNMT1 (Figure 3.8d, PDB code: 3IWE); this could be the reason that little selectivity is found for this inhibitor. It might be possible to validate this assumption by mutating this
particular tyrosine in PvNMT (Y211) and HsNMT1 (Y296); these studies are on-going and it will be interesting to see the effect of mutating this residue on the binding of 177 and DDD85646 by PvNMT and HsNMT1.

![Proposed origin of selectivity over human NMTs](image)

**Figure 3.8 Proposed origin of selectivity over human NMTs**

(a). Conformational change of Y212 in PvNMT after the binding of compound 177; (b). Modelling inhibitor 177 in HsNMT1; (c),(d). There is a minimal effect on the orientation of Y217 in LmNMT and Y296 in HsNMT1 upon the binding of DDD85646 in LmNMT and HsNMT1 respectively.

The colour codes are as follows: oxygen atoms, red; nitrogen atoms, blue; carbon atoms of residues and the inhibitor are yellow and green respectively; White carbons indicated the target Tyr residue before the insertion of the inhibitor, either compound 177 or DDD85646.
3.4 Evaluation of *P. falciparum* inhibition

Current antimalarial drug development aims at blocking the erythrocytic cycle. Preliminary work reported that small molecule PfNMT inhibitors, when incubating with *P. falciparum* infected erythrocytes, resulted in a reduction of parasitemia\(^{107, 89, 122}\). In this project, selected compounds from the library of PfNMT inhibitors were tested against parasites to study the correlation between enzyme inhibition and parasite growth killing ability, aiming to assess whether NMT is a target in *P. falciparum*. The assay used in the project was known as fluorescent-activated cell sorting (FACS) assay, which was conducted by Dr. D. Moss in NIMR, in conjunction with Dr. A. Holder.

3.4.1 Assay description

The erythrocytic cycle is a complex multi-step process, where the asexual reproduction occurs. For *P. falciparum* this typically takes 48 hours. Most parasites invade fresh red blood cells within 2 hours. Sharp morphological transitions for the ring-to-trophozoite and trophozoite-to-schizont stages occur at the 18- and 30-hour timepoints respectively\(^{166}\) (Figure 3.9A). Near the end of one cycle (44-48 hour), schizonts mature to merozoites, which then invade healthy erythrocytes to start a new infection after cell rupture (Figure 3.9B).
Figure 3.9 Life stages of P. falciparum throughout the intraerythrocytic cycle[166]

(A) Major morphological stages throughout the intraerythrocytic cycle are shown with the percentage representation of ring-, trophozoite- and schizont-stage parasites at every timepoint. The 2-hour invasion window is also indicated (gray area)[166]. (B) Detailed morphological stages throughout the intraerythrocytic cycle, where the merozoites grow first to a ring-shaped form and then to a larger trophozoite form. In the schizont stage, the parasites replicate rapidly to produce new merozoites which start a new infection after cell rupture.

FACS assay is a flow cytometric approach to analyse cell populations based on the light scattering properties and fluorescent characteristics, allowing rapid separation of cells. This method has been used to study growth inhibition of intraerythrocytic protozoan parasites that are hydrothidin (HE)-stained, a red fluorescent stain initially used to investigate the viability of prokaryotes[167]. HE is taken up by the living cells and metabolically converted to ethidium by NADPH oxidase (Figure 3.10A). Ethidium then intercalates into DNA (Figure 3.10B) and the resulting red fluorescence distinguishes infected erythrocytes with viable parasites from uninfected or infected erythrocytes.
containing dead parasites [168-170].

![Figure 3.10 Detection of living cells by hydroethidine](image)

(A) Mechanism of hydroethidine oxidation to fluorescent ethidium (coloured red). (B) The stain process of the DNA helix with ethidium

### 3.4.2 Selected compounds for parasite inhibition assay

25 selected compounds from the library of PfNMT inhibitors with diverse enzyme potency were tested against *P. falciparum* parasites. Compounds 3, the benzofuran scaffold, and WR99210, a highly potent antimalarial, were used as the negative and positive controls respectively (Figure 3.11).

Among the first generation of PfNMT inhibitors, the good inhibitors (15, 16, 56, 72, 80 and 181), showing IC\(_{50}\) values of single-digit µM range, were chosen to investigate their inhibition behaviours against parasites. In addition, compounds suffering from a total loss of enzyme inhibition by either relocation of the C-4 side chain (36) or incorporation of phenyl instead of t-Bu at the terminal end of the C-4 side chain (57, 73 and 81) were picked as well to find out whether these compounds would follow a similar trend in parasite inhibition (Figure 3.11).

Compounds selected from the second generation of PfNMT inhibitors were more comprehensive, showing a wide IC\(_{50}\) coverage, from 0.27 to 28.8 µM (Figure 3.11). In terms of the structures, these inhibitors only differ in the C-2 side chain, thus forming a good group to systematically study the influence of the C-2 side chain on the ability to kill the parasites.
3.4.3 Assay considerations

3.4.3.1 Determination of negative and positive controls

Establishment of good negative and positive controls is important in assay development. Compound 3, the bare benzofuran core, displayed no inhibitory effect against PfNMT at even 1 mM. As expected, no inhibition against parasites was observed at the highest concentration or 100 µM (Figure 3.12A), which is considered to the point at which any inhibition would come from the general toxicity of a test compound (personal communication, Dr. D. Moss, NIMR). Therefore, compound 3 was confirmed as
a negative control for the FACS assay. Highly active reference compound WR99210 was treated under the same conditions and the resulting EC₅₀ (0.24 nM, Figure 3.12B) was consistent with the literature value (0.15 nM)¹⁷¹, hence supporting the FACS assay as a reliable method to determine the inhibition against parasites.

Figure 3.12 Confirmation of negative and positive controls in *P. falciparum* inhibition assay

(A) Compound 3 displayed no inhibition against the parasites up to 100 µM, hence acting as the negative control in FACS assay. (B) WR99210, a highly active antimalarial, served as a positive control and showed an EC₅₀ value of 0.24 nM against *P. falciparum*, which was comparable with the literature data¹⁷¹.

3.4.3.2 Trifluoroacetic acid effect

All selected compounds from the library of PfNMT inhibitors were purified from preparative HPLC with the elution always containing 0.1% TFA. Therefore, they were actually TFA salts. It was reported that even low concentration of TFA (nM range) could alter the accuracy of biological studies¹⁷², prompting us to examine the effect of TFA on the growth of parasites. Encouragingly, as illustrated in Figure 3.13, TFA had no significant effect on the parasites, showing no inhibition or toxicity for cell growth over the concentrations up to 1 mM. Therefore, these TFA salts were used in the FACS assay without further manipulation and the resulting inhibitory activities should only come from the molecules under test.
Figure 3.13 The effects of TFA on the growth of parasites

Trifluoroacetic acid (TFA) demonstrated no inhibitory or toxic effects on P. falciparum infected erythrocytes at concentrations up to 1mM.

3.4.4 Parasite inhibition results

At that time, there had been no evidence to show in which stage(s) of the intraerythrocytic cycle N-myristoylation occurs (Figure 3.9). Given a highly metabolic activity in trophozoite stage\textsuperscript{166}, it was sensible that the inhibitors were added during this stage where myristoylation is expected to be happening.

3.4.4.1 Inhibition data of the first generation of PfNMT inhibitors

The inhibitory activities of compounds from the first generation of PfNMT inhibitors against parasites are summed up in Table 3.31. NMT inhibitors with the IC\textsubscript{50} values of low µM range were found to display the similar degree of parasite inhibition. However, compounds with little or no inhibition against the enzyme (36, 60, 73 and 81) unexpectedly exhibited good ability to kill parasites.
Table 3.31 Inhibition data of the compounds from the first generation of PfNMT inhibitors against *P. falciparum*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC$_{50}$ µM (PfNMT)</th>
<th>EC$_{50}$ µM (<em>P. falciparum</em>)</th>
<th>Comments on EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td><img src="image1" alt="Structure" /></td>
<td>9.0</td>
<td>(16.9 ± 2.7)$^a$</td>
<td>Prediction; good fit $^b$</td>
</tr>
<tr>
<td>16</td>
<td><img src="image2" alt="Structure" /></td>
<td>8.5</td>
<td>6.8 ± 0.7</td>
<td>Hill slope 4.4</td>
</tr>
<tr>
<td>36</td>
<td><img src="image3" alt="Structure" /></td>
<td>&gt;100</td>
<td>7.3 ± 0.9</td>
<td>Hill slope 3.1</td>
</tr>
<tr>
<td>59</td>
<td><img src="image4" alt="Structure" /></td>
<td>9.2</td>
<td>(1.2 ± 0.6)$^a$</td>
<td>Prediction; good fit</td>
</tr>
<tr>
<td>60</td>
<td><img src="image5" alt="Structure" /></td>
<td>&gt;100</td>
<td>(5.0)$^a$</td>
<td>Prediction; poor fit $^b$</td>
</tr>
<tr>
<td>72</td>
<td><img src="image6" alt="Structure" /></td>
<td>10.6</td>
<td>(5.5)$^a$</td>
<td>Prediction; poor fit</td>
</tr>
<tr>
<td>73</td>
<td><img src="image7" alt="Structure" /></td>
<td>&gt;100</td>
<td>(1.3 ± 0.5)$^a$</td>
<td>Prediction; poor fit</td>
</tr>
<tr>
<td>80</td>
<td><img src="image8" alt="Structure" /></td>
<td>1.4</td>
<td>(6.0)$^a$</td>
<td>Prediction; poor fit</td>
</tr>
<tr>
<td>81</td>
<td><img src="image9" alt="Structure" /></td>
<td>98.3</td>
<td>(5.5)$^a$</td>
<td>Prediction; poor fit</td>
</tr>
<tr>
<td>181</td>
<td><img src="image10" alt="Structure" /></td>
<td>1.5</td>
<td>(3.5)$^a$</td>
<td>Prediction; poor fit</td>
</tr>
</tbody>
</table>

$^a$ The number in parentheses was calculated from the two single points of inhibitory data, using GraFit (version 7.0.1). $^b$ good fit: $x < 0.3$; poor fit: $x > 0.5$, $x$ = standard deviation error / EC$_{50}$.

Discrepancy between the enzyme and cell-based results in the C-4 side chain

Compounds 15 and 16 showed similar enzyme inhibition. However, 16 containing the N-Bn moiety at the C-4 side chain was 3-fold more potent than its N-t-Bu counterpart 15 against the parasites. Such discrepancies were even more exaggerated whereby the N-Bn derivatives (60, 73, and 81) exhibiting no inhibition for enzyme, displayed comparable or even better activity than their N-t-Bu equivalents (59, 72, and 80).
in killing parasites (Table 3.31). In addition, relocation of the side chain from the C-4 to the C-5 position of the benzofuran core led to a total loss of enzyme activity, but maintained the anti-parasitic activity (16 vs. 36, Table 3.31). Given that the NMTs used in this project are slightly different from the wild-type ones whereby the former ones are His-tag attached at the N-terminus, it is not conclusive that the above activity discrepancy is a result of an off-target effect at the moment. The measurements of these compounds against the recombinant NMTs without His-tag are badly needed.

**Correlation between the enzyme and cell-based results**

When plotting the enzyme pIC$_{50}$ against the cell pEC$_{50}$ of five good NMT inhibitors from the first generation of the library (15, 59, 72, 80 and 181, Table 3.31), no linear correlation was observed although these anti-NMT compounds were also found to be anti-Plasmodial (Figure 3.14). The poor correlation could be rationalised as an off-target effect of this series.

![Poor correlation](image)

**Figure 3.14 Correlation between PfNMT and *P. falciparum* inhibition for the selected compounds from the first generation of PfNMT inhibitors**

*A poor correlation between enzyme and parasite inhibition was observed in the C-2 side chain (orange circle)*

### 3.4.4.2 Inhibition data of the second generation of PfNMT inhibitors

The inhibitory activities of the compounds from the second generation of PfNMT inhibitors against parasites are listed in Table 3.32. Test compounds showing a broad range of enzyme inhibition, with the IC$_{50}$ values from 0.27 to 28.8 µM, displayed a much
narrower EC_{50} range which was between 1.2 and 15.0 µM (Table 3.32).

Table 3.32 Inhibition data of the compounds from the second generation of PfNMT inhibitors against *P. falciparum*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC_{50} µM (PfNMT)</th>
<th>EC_{50} µM (P. falciparum)</th>
<th>Comments on EC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td><img src="image" alt="Structure" /></td>
<td>16.5</td>
<td>(15.0)^a</td>
<td>Prediction; poor fit^b</td>
</tr>
<tr>
<td>117</td>
<td><img src="image" alt="Structure" /></td>
<td>22.0</td>
<td>5.3 ± 0.6</td>
<td>Hill slope 3.2</td>
</tr>
<tr>
<td>124</td>
<td><img src="image" alt="Structure" /></td>
<td>100</td>
<td>(4.0 ± 0.7)^a</td>
<td>Prediction; good fit^b</td>
</tr>
<tr>
<td>126</td>
<td><img src="image" alt="Structure" /></td>
<td>28.8</td>
<td>(5.3 ± 1.1)^a</td>
<td>Prediction; good fit^b</td>
</tr>
<tr>
<td>128</td>
<td><img src="image" alt="Structure" /></td>
<td>13.0</td>
<td>7.8 ± 0.8</td>
<td>Hill slope 3.1^c;</td>
</tr>
<tr>
<td>130</td>
<td><img src="image" alt="Structure" /></td>
<td>2.3</td>
<td>2.4 ± 0.1</td>
<td>Steep Hill slope 12.4</td>
</tr>
<tr>
<td>145</td>
<td><img src="image" alt="Structure" /></td>
<td>7.1</td>
<td>4.5 ± 1.9</td>
<td>Hill slope 2.1</td>
</tr>
<tr>
<td>161</td>
<td><img src="image" alt="Structure" /></td>
<td>24.4</td>
<td>(10.0)^a</td>
<td>Prediction; poor fit^b</td>
</tr>
<tr>
<td>162</td>
<td><img src="image" alt="Structure" /></td>
<td>0.27</td>
<td>(1.2 ± 0.2)^a</td>
<td>Prediction; good fit^b</td>
</tr>
<tr>
<td>163</td>
<td><img src="image" alt="Structure" /></td>
<td>9.7</td>
<td>3.2 ± 0.8</td>
<td>Hill slope 2.5</td>
</tr>
<tr>
<td>173</td>
<td><img src="image" alt="Structure" /></td>
<td>5.6</td>
<td>(3.5)^a</td>
<td>Prediction; poor fit^b</td>
</tr>
<tr>
<td>174</td>
<td><img src="image" alt="Structure" /></td>
<td>2.1</td>
<td>(3.0)^a</td>
<td>Prediction; poor fit^b</td>
</tr>
<tr>
<td>190</td>
<td><img src="image" alt="Structure" /></td>
<td>10.2</td>
<td>4.0 ± 0.3</td>
<td>Hill slope 4.7</td>
</tr>
<tr>
<td>193</td>
<td><img src="image" alt="Structure" /></td>
<td>3.5</td>
<td>2.5 ± 0.1</td>
<td>Hill slope 3.6</td>
</tr>
<tr>
<td>215</td>
<td><img src="image" alt="Structure" /></td>
<td>2.0</td>
<td>2.1 ± 0.1</td>
<td>Hill slope 4.8</td>
</tr>
</tbody>
</table>

^a The number in parentheses was calculated from the two single points of inhibitory data, using GraFit (version 7.0.1). ^b good fit: x < 0.3; poor fit: x > 0.5, x = standard deviation error / EC_{50}. ^c 20% parasite inhibition even at the lowest concentration.
Figure 3.15 plots the enzyme pIC$_{50}$s against the cell pEC$_{50}$s of the test compounds (data listed in Table 3.32). In contrast to the previous set of inhibitors, a good correlation ($R^2 = 0.714$) was observed. This is highly suggestive that the anti-	extit{Plasmodial} effects are directly a result of NMT inhibition rather than an off-target effect. This is the first time to our knowledge that a series of compounds, covering a broad range of functional groups, such as ketone (117), amide (124, 126, 128 and 130), ether (145), ester (109, 161, 162, 163, 173, 174) and the mimics of methylene ester (190, 193, 215), have been found to show good correlation between PfNMT inhibition and anti-	extit{Plasmodial} ability. The results provide strong evidence from a chemical perspective to prove that NMT is a target in 	extit{P. falciparum}. In addition, the resulting good correlation indirectly suggests that methylene ester, currently the most promising Y linkage group, is able to survive under cell conditions.

![Good correlation](image)

**Figure 3.15** Correlation between PfNMT and 	extit{P. falciparum} inhibition for the selected compounds from the second generation of PfNMT inhibitors

A good correlation between the enzyme and parasite inhibition was observed in the C-2 side chain (red circle)

Considering that these inhibitors only differ at their C-2 side chains, it was hence worthwhile to study the influence of the C-4 side chain on parasite inhibition.
3.4.4.3 Investigation of the role of the C-4 side chain

Figure 3.16 lists the structures of further compounds selected for the cell-based assay, whereby these inhibitors differ in the C-4 side chain while keeping their C-2 side chain fixed.

The inhibitory activities of these six compounds against parasites are listed in Table 3.33. It appeared that inhibitors containing 4-piperidino without N-substitution generally displayed higher anti-parasitic activity than their N-substitution counterparts (109 vs. 112, 113 and 114; 162 vs. 180).

Table 3.33 Inhibition data of the further compounds from the second generation of PfNMT inhibitors against *P. falciparum*

<table>
<thead>
<tr>
<th>Compound</th>
<th>CLogP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pKa&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pIC&lt;sub&gt;50&lt;/sub&gt; (PfNMT)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; µM (P. falciparum)</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt; (P. falciparum)</th>
<th>Comments on EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>2.41</td>
<td>9.82</td>
<td>4.78</td>
<td>(15.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.89</td>
<td>Prediction; poor fit&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>112</td>
<td>2.80</td>
<td>8.66</td>
<td>4.30</td>
<td>16.6 ± 0.4</td>
<td>4.78</td>
<td>Hill slope 5.2</td>
</tr>
<tr>
<td>113</td>
<td>3.15</td>
<td>8.94</td>
<td>4.57</td>
<td>23.7 ± 1.4</td>
<td>4.62</td>
<td>Hill slope 3.4</td>
</tr>
<tr>
<td>114</td>
<td>3.57</td>
<td>9.22</td>
<td>5.10</td>
<td>(40.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.40</td>
<td>Prediction; poor fit&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>162</td>
<td>3.78</td>
<td>9.82</td>
<td>6.57</td>
<td>(1.2 ± 0.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.92</td>
<td>Prediction; good fit&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>180</td>
<td>4.94</td>
<td>9.22</td>
<td>5.85</td>
<td>4.7 ± 0.2</td>
<td>5.33</td>
<td>Hill slope 4.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> The values were calculated using the in-house compound database.  
<sup>b</sup> The number in parentheses was calculated from the two single points of inhibitory data, using GraFit (version 7.0.1).  
<sup>c</sup> good fit: x < 0.3; poor fit: x>0.5, x = standard deviation error / EC<sub>50</sub>. 

Aside from improving enzyme potency, tuning of physicochemical properties is also widely used to optimise cell inhibition<sup>[173-175]</sup>. N-substitution on the piperidine of the inhibitors resulted in a big change of either CLogP (calculated LogP) or pKa (Table
Combined with their enzyme inhibition data, these inhibitors formed a good group to identify the parameter(s) of the C-4 side chain to contribute to parasite inhibition. Among these three factors (CLogP, pKa and pIC$_{50}$), CLogP is the only one to show good correlation with cell inhibition (Figure 3.17) and lower hydrophobicity of R$_1$ leads to higher anti-parasitic activity, supporting that 4-piperidinol, possessing the lowest hydrophobicity among the series, is an optimised C-4 side chain of the inhibitors.

**Figure 3.17 Identification of factor(s) in the C-4 4-piperidinol series to affect parasite inhibition**

*Good correlation between CLogP and parasite inhibition was observed in the C-4 side chain (green circle)*

### 3.5 Summary

Starting from the hit compound 1, two generations of PfNMT inhibitors with different compositions at the C-4 position of the benzofuran scaffold were developed (Scheme 3.60). N-t-Bu trimethylene was identified as an optimal C-4 side chain for the first generation of inhibitors. Subsequent modification of the C-2 side chain led to the discovery of several promising inhibitors (72, 59, 80 and 181) showing good inhibition at low µM range against both the enzyme (PfNMT) and parasites (*P. falciparum*). However, moderate selectivity over human NMTs and poor correlation between enzyme potency and cell inhibition prevented these inhibitors from further optimisation. Rigidification of
the trimethylene side chain led to the discovery of 4-piperidinol as a favourable C-4 side chain for the second generation of PfNMT inhibitors. A great effort was then put into the investigation of Y linkage groups and methylene ester was determined to be a delicate combination to direct the attached R² phenyl group to pick up a strong π-π interaction, which was unequivocally supported by the crystal structure. More importantly, a tight correlation between enzyme potency and parasite inhibition was observed in the second generation of PfNMT, chemically validating NMT as a target in *P. falciparum*. 

**Scheme 3.60 Summary of development of PfNMT inhibitors**
4. Discovery of LdNMT inhibitors

The piggy-back strategy was again used here to identify LdNMT inhibitors, whereby the whole library of PfNMT inhibitors (discussed in Chapter 3) was screened against LdNMT to generate the initial SARs. Enzyme inhibition data were then combined with the parasite growth inhibition results, aiming to discover good hits or early lead candidates.

4.1 Structure-activity relationships

The full enzyme inhibition data are listed in Appendix. The key SARs observed for LdNMT, especially the ones different from the ones suggested in PfNMT, are presented in this chapter.

4.1.1 Identification of the C-4 side chain for LdNMT inhibitors

4-Piperidinol is a favourable C-4 side chain

Inhibitors with the trimethylene and 4-piperidinol side chains at the C-4 position of the benzofuran scaffold displayed comparable enzyme potency against PfNMT. However, as Table 4.1 suggests, across a range of representative C-2 side chains, compounds containing 4-piperidinol are generally more active than their trimethylene counterparts against LdNMT. Most significant is the pair of compounds 80 and 142, where a 22-fold activity difference was observed. These results reveal that 4-piperidinol is more favoured than trimethylene by LdNMT as the C-4 side chain.
Table 4.1 Identification of 4-piperidinol as a favourable C-4 side chain for LdNMT inhibitors

<table>
<thead>
<tr>
<th>-Y-R₂</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>IC₅₀ (µM)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>-COOEt</td>
<td>15</td>
<td>~167 (37.5%)ᵇ</td>
<td>109</td>
<td>22.9</td>
</tr>
<tr>
<td>-COPh</td>
<td>72</td>
<td>32.6</td>
<td>117</td>
<td>8.6</td>
</tr>
<tr>
<td>-CH₂OPh</td>
<td>80</td>
<td>56.0</td>
<td>142</td>
<td>2.6</td>
</tr>
<tr>
<td>-CONHPh</td>
<td>56</td>
<td>~132 (43.1%)ᵇ</td>
<td>126</td>
<td>94.8</td>
</tr>
<tr>
<td>-CONHBn</td>
<td>58</td>
<td>&gt;100</td>
<td>128</td>
<td>13.5</td>
</tr>
<tr>
<td>-COOBn</td>
<td>181</td>
<td>14.6</td>
<td>162</td>
<td>4.7</td>
</tr>
</tbody>
</table>

ᵃ The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency.

There is little room for R₁ substitution on C-4 4-piperidinol side chain

Table 4.2 lists the inhibitory activities of three series of compounds (ethyl ester, phenyl ketone and benzyl ester) with various N-substitutions on the C-4 4-piperidinol side chain. The results show that 4-piperidinol without N-substitution is most active among the series and large R₁ leads to a significant loss of activity. These findings are slightly different to the case with PfNMT where compound 114 was found to be 2-fold more active than compound 109 (Table 3.16).
Table 4.2 Investigation of R¹ substitutions on the C-4 4-piperidinol side chain

<table>
<thead>
<tr>
<th>Compound</th>
<th>R¹</th>
<th>IC₅₀ (µM)ᵃ</th>
<th>Compound</th>
<th>R¹</th>
<th>IC₅₀ (µM)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>H</td>
<td>22.9</td>
<td>112</td>
<td>Me</td>
<td>&gt;100</td>
</tr>
<tr>
<td>113</td>
<td>Et</td>
<td>~116</td>
<td>114</td>
<td>i-Pr</td>
<td>117</td>
</tr>
<tr>
<td>115</td>
<td>Bn</td>
<td>NI (0%)ᵇ</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. ᵇ The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency. NI: no inhibition.

4.1.2 Investigation of the C-2 side chain for LdNMT inhibitors

Big and aromatic R² is favoured

Inhibition data for compounds with different sizes of R² are presented in Table 4.3. The rule that bigger R² results in better potency is followed in LdNMT, which was also found for PfNMT. However, there are some differences between these two enzymes: compound 164, 6-fold less active than compound 162 against PfNMT (Table 3.24), was found to be 3-fold more potent than compound 162 for LdNMT; addition of a methylene spacer to the C-2 ester and amide was shown to improve the potency by similar extents (4-7 fold) against LdNMT (162 vs. 161; 128 vs. 126) while dramatic differences regarding the improvement factor between these two pairs were recorded for PfNMT (Table 3.26);
in contrast to a 35-fold activity difference for PfNMT between compounds 162 and 163, aromaticity of R² was only responsible for a 3-fold improvement for LdNMT.

Table 4.3 Examination of compounds with various sizes of R² against LdNMT

```
<table>
<thead>
<tr>
<th>Compound</th>
<th>R²</th>
<th>IC₅₀ (µM)</th>
<th>Compound</th>
<th>R²</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>~145</td>
<td>(40.8%)</td>
<td>109</td>
<td></td>
<td>22.9</td>
</tr>
<tr>
<td>161</td>
<td></td>
<td>19.8</td>
<td>162</td>
<td></td>
<td>4.7</td>
</tr>
<tr>
<td>163</td>
<td></td>
<td>16.5</td>
<td>164</td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>121</td>
<td></td>
<td>~216</td>
<td>123</td>
<td></td>
<td>~161</td>
</tr>
<tr>
<td>124</td>
<td></td>
<td>&gt;100</td>
<td>126</td>
<td></td>
<td>94.8</td>
</tr>
<tr>
<td>128</td>
<td></td>
<td>13.0</td>
<td>130</td>
<td></td>
<td>5.3</td>
</tr>
</tbody>
</table>
```

*The IC₅₀ values were averaged from two independent dose-response curves; the variation was generally <15%. The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%. IC₅₀ values are estimated from these to give a comparative indication of potency.

Comparison of the Y linkage groups

Inhibitory activities of molecules containing varied Y linkers are listed in Table 4.4. Among the rigid Y linkage groups (Table 4.4A), ketone 117 and enone 193 displayed the best inhibition, with the IC₅₀ values below 10 µM. The remaining compounds except cis-alkene 189 possessed the similar inhibition against LdNMT. For the flexible Y linkage
groups (Table 4.4B), methylene ester is not optimal in LdNMT: reversed methylene ester 182 even displayed higher potency than methylene ester 162; replacing the carbonyl by methylene even increased potency by 2-fold (145 vs. 162). The results suggest that inhibitors containing a methylene ether (142 and 145) are somewhat favoured by the enzyme; however, poor synthetic yields and chemical instability significantly limit their further optimisation. Surprisingly, compound 154, which shows little inhibition for PfNMT, was actually quite active against LdNMT with an IC$_{50}$ value of 9.0 µM.

Table 4.4 Investigation of effects of Y linkage groups on the inhibitory activity for LdNMT

(A) Rigid Y linker

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y</th>
<th>IC$_{50}$ (µM) $^a$</th>
<th>Compound</th>
<th>Y</th>
<th>IC$_{50}$ (µM) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>161</td>
<td>-COO-</td>
<td>19.8</td>
<td>126</td>
<td>-CONH-</td>
<td>28.8</td>
</tr>
<tr>
<td>188</td>
<td>-CH=CH- (trans)</td>
<td>20.8</td>
<td>189</td>
<td>-CH=CH- (cis)</td>
<td>68.8</td>
</tr>
<tr>
<td>193</td>
<td>-COCH=CH- (trans)</td>
<td>9.7$^b$</td>
<td>213</td>
<td></td>
<td>26.8</td>
</tr>
<tr>
<td>117</td>
<td>-CO-</td>
<td>8.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(B) Flexible Y linker

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y</th>
<th>IC$_{50}$ (µM) $^a$</th>
<th>Compound</th>
<th>Y</th>
<th>IC$_{50}$ (µM) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>-COOCH$_2$-</td>
<td>4.7</td>
<td>128</td>
<td>-CONHCH$_2$-</td>
<td>13.5</td>
</tr>
<tr>
<td>142</td>
<td>-CH$_2$O-</td>
<td>2.6</td>
<td>145</td>
<td>-CH$_2$OCH$_2$-</td>
<td>2.5</td>
</tr>
<tr>
<td>182</td>
<td>-CH$_2$OOC-</td>
<td>3.3</td>
<td>208</td>
<td>-SCH$_2$-</td>
<td>25.1$^c$</td>
</tr>
<tr>
<td>186</td>
<td>-CH$_2$CH$_2$-</td>
<td>30.9</td>
<td>154</td>
<td>-CH$_2$NH-</td>
<td>9.0</td>
</tr>
<tr>
<td>215</td>
<td></td>
<td>10.2</td>
<td>209</td>
<td>-SO$_2$CH$_2$-</td>
<td>&gt;100$^c$</td>
</tr>
</tbody>
</table>

$^a$The IC$_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%. $^b$The compound only has moderate selectivity, IC$_{50}$ for HsNMT1&2 are 43.9 µM and 39.5 µM, respectively. $^c$The actual structure has no methyl group at the C-3 position of the benzofuran scaffold.
Investigation of $R^3$ substitution

Table 4.5 shows the inhibition data of inhibitors with various phenyl substitutions $R^3$: *para*-substitutions (173 and 174) resulted in a 4-fold loss of activity; *ortho*-substitutions (178 and 179) were slightly less potent than the parent compound 162; *meta*-substitutions (175-177) were found to be promising since they displayed comparable or higher inhibition than compound 162. In general, introduction of phenyl substitutions appears to be more tolerated by LdNMT than PfNMT (Table 3.25). Moreover, addition of *meta*-OMe improved the inhibitory activity by 2-fold; this could imply that a hydrogen bond with the enzyme might be gained.

Table 4.5  Inhibitory activities of compounds with different phenyl $R^3$ substitutions against LdNMT

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R^3$</th>
<th>$IC_{50}$ ($\mu$M)$^a$</th>
<th>Compound</th>
<th>$R^3$</th>
<th>$IC_{50}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>H</td>
<td>4.7</td>
<td>173</td>
<td>4-Cl</td>
<td>16.4</td>
</tr>
<tr>
<td>174</td>
<td>4-Me</td>
<td>21.1</td>
<td>175</td>
<td>3-Cl</td>
<td>4.1</td>
</tr>
<tr>
<td>176</td>
<td>3-Me</td>
<td>2.8</td>
<td>177</td>
<td>3-OMe</td>
<td>2.2</td>
</tr>
<tr>
<td>178</td>
<td>2-Cl</td>
<td>7.9</td>
<td>179</td>
<td>2-Me</td>
<td>6.1</td>
</tr>
</tbody>
</table>

$^a$The $IC_{50}$ values were averaged from two independent dose-response curves; the variation was generally <15%.

4.1.3 Summary

The whole library of PfNMT inhibitors was screened against LdNMT. Surprisingly, the molecules with trimethylene were found to be generally poor LdNMT inhibitors. However, the inhibitors with 4-piperidinol were quite promising. In terms of the C-4 piperidine side chain, hydrogen as $R^1$ was shown to be superior to other substitutions (Figure 4.1). With regard to the C-2 side chain, aromaticity of $R^2$ and the addition of
meta-OMe to the R² phenyl contributed to the improved enzyme potency (Figure 4.1). However, the role of Y linkage group remained ambiguous and there were a number of candidate Y linkage groups based on the enzyme inhibition results (Table 4.4). With the purpose to select the early lead candidates for LdNMT inhibitors, it would be greatly helpful at that stage to have these molecules covering a variety of Y linkage groups tested in the cell-based assay.

![Summary of SARs for LdNMT inhibitors](image)

**Figure 4.1 Summary of SARs for LdNMT inhibitors**

### 4.2 Evaluation of *L. donovani* inhibition

In collaboration with Prof. D. Smith and Dr. D. Pappe in York University, inhibitors containing various Y linkage groups were tested against *L. Donovani* amastigotes, aiming to select promising candidates for further optimisation. In addition, it was also interesting to know whether anti-LdNMT molecules would kill the parasites in cells.

#### 4.2.1 Assay description

The development of new antileishmanial drugs has been impeded by the lack of a simple and reliable drug evaluation system because the parasites exist in two forms (which differs from the stages in malaria parasites), known as extracellular flagellated promastigotes and intracellular amastigotes within mammalian macrophages. These two different forms are exposed to extreme environmental changes such as pH and temperature\(^{176}\). Many studies still measure the antileishmanial activity using promastigotes, the form of parasites found in the sandfly vector, because it is easy to...
grow in culture. However, since the development of cultured amastigotes, the actual causative form to infect human body\cite{177}, much attention has been turned to establishing a rapid assay using amastigotes for the discovery of antileishmanial agents\cite{178, 179}. In this project, a fluorescent assay using Alamar blue (see below) was used to determine the inhibition against *L. donovani* amastigotes.

Alamar Blue, an oxidation-reduction indicator, is reduced in the living cell, hence changing the colour from blue to red (Figure 4.2)\cite{180}. The assay was initially developed to target promastigotes\cite{181} and later axenic amastigotes\cite{179}, providing a simple, rapid and reproducible method for screening antileishmanial agents.

![Figure 4.2 Mechanism of the oxidation-reduction indicator Alamar Blue](image)

*The oxidative form, as the name suggested, is coloured as blue while the reduced form is red.*

### 4.2.2 Selection strategy

There are two types of amastigotes, known as the axenic and *ex-vivo* amastigotes. Given the sources available to our collaborators in York University, *ex-vivo* amastigotes, purified from the spleen of a 6-week-infected donor hamster, were used in this project. Compounds were tested against both *ex-vivo* amastigotes and macrophages to obtain the extracellular inhibitory activity and toxicity respectively, aiming to identify the promising hits with good activity and sufficient inhibition window, which determines the ratio between the macrophage toxicity and anti-leishmanial activity (Figure 4.3). The resulting hits would be further confirmed by an intracellular amastigote assay which is currently laborious, time consuming and difficult to scale up into a system for HTS\cite{182}. 


Figure 4.3 Strategy to select promising LdNMT inhibitors using the cell-based assay

A funnel representing the selection of hits or early lead candidates for LdNMT inhibitors is based on the results of inhibition against ex-vivo amastigotes and toxicity for macrophages.

4.2.3 Compounds for *L. donovani* inhibition test

As mentioned earlier, the role of Y linkage group in the inhibitors remained ambiguous while the SARs for the other parts of the inhibitors were relatively clear (Figure 4.1). Therefore, ten compounds which only differ in the Y linkage group were selected for analysis (Figure 4.4). Compound 3, the negative control used in *P. falciparum* inhibition assay, was also viewed as a negative control here while amphotericin B, currently the most effective first-line antileishmanial drug\(^ {48}\), was used as a positive control.

![Figure 4.4 Structures of compounds differing only in the Y linkage group selected for anti-leishmanial activity assays](image-url)
4.2.4  *L. donovani* inhibition results

4.2.4.1  Negative and positive controls

As expected, compound 3, the negative control in *P. falciparum*, had no considerable inhibition against *L. donovani* amastigotes at the highest concentration of 75 µM (Figure 4.5A). Therefore, compound 3 was confirmed as a negative control for the assay. Amphotericin B, a highly active reference compound, was treated under the same conditions; and a good dose-response curve was obtained (Figure 4.5B), confirming the feasibility of the assay.

![Figure 4.5 Confirmation of negative and positive controls in the *L. donovani* amastigote assay](image)

(A) Compound 3 displayed little inhibition against amastigotes at even 75 µM, hence acting as a negative control in the assay. (B) Amphotericin B, currently the most effective first-line antileishmanial, served as a positive control, showed an EC$_{50}$ value of 0.03 µM.

4.2.4.2  Study of ex-vivo amastigote inhibition

Both inhibitory activities against ex-vivo *L. donovani* amastigotes and toxicity for macrophages of test compounds are summarised in Table 4.6. With respect to parasite inhibition, compounds with enzyme IC$_{50}$ at low µM range (2.6 to 19.8 µM) generally showed a similar range of EC$_{50}$ (1.3 to 25.0 µM), indicating that anti-LdNMT could be also anti-leishmanial. Some EC$_{50}$ values were observed to be lower than their corresponding IC$_{50}$ values (compounds 161, 162, 190, 193 and 215). Such unexpected discrepancy can be rationalised by considering that IC$_{50}$ values derived from the enzyme assay are actually apparent enzyme potency, which is found to change significantly with different
concentrations of assay components and varied peptide substrates (data not shown here). In terms of the macrophage toxicity, a viability threshold was found to exist since only two forms of macrophage, the live (intact cell) and the dead (lysed cell), were able to be detected during the experiments.

Table 4.6 *Ex-vivo L. donovani* amastigote inhibition and macrophage toxicity data for the selected compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC_{50} μM (LdNMT)</th>
<th>EC_{50} μM (L. donovani)^a</th>
<th>Comments on EC_{50}</th>
<th>Toxicity μM^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>128</td>
<td><img src="image1" alt="Structure" /></td>
<td>13.5</td>
<td>(15.0)^c</td>
<td>Prediction; poor fit</td>
<td>Between 8.3 and 25</td>
</tr>
<tr>
<td>142</td>
<td><img src="image2" alt="Structure" /></td>
<td>2.6</td>
<td>(1.9 ± 0.1)^c</td>
<td>Prediction; good fit</td>
<td>Between 8.3 and 25</td>
</tr>
<tr>
<td>145</td>
<td><img src="image3" alt="Structure" /></td>
<td>2.6</td>
<td>(11.0 ± 2.0)^c</td>
<td>Prediction; good fit</td>
<td>Between 8.3 and 25</td>
</tr>
<tr>
<td>154</td>
<td><img src="image4" alt="Structure" /></td>
<td>9.0</td>
<td>(25.0)^c</td>
<td>Prediction; poor fit</td>
<td>Between 8.3 and 25</td>
</tr>
<tr>
<td>161</td>
<td><img src="image5" alt="Structure" /></td>
<td>19.8</td>
<td>4.9 ± 1.1</td>
<td>Hill slope 0.9</td>
<td>Between 25 and 75</td>
</tr>
<tr>
<td>162</td>
<td><img src="image6" alt="Structure" /></td>
<td>4.7</td>
<td>1.4 ± 0.3</td>
<td>Hill slope 1.1</td>
<td>~8.3^e</td>
</tr>
<tr>
<td>182</td>
<td><img src="image7" alt="Structure" /></td>
<td>2.6</td>
<td>(15.0)^c</td>
<td>Prediction; poor fit</td>
<td>Between 25 and 75</td>
</tr>
<tr>
<td>190</td>
<td><img src="image8" alt="Structure" /></td>
<td>18.2</td>
<td>(5.5)^c</td>
<td>Prediction; poor fit</td>
<td>Between 8.3 and 25</td>
</tr>
<tr>
<td>193</td>
<td><img src="image9" alt="Structure" /></td>
<td>9.7</td>
<td>1.3 ± 0.02</td>
<td>Hill slope 4.4</td>
<td>Between 8.3 and 25</td>
</tr>
<tr>
<td>215</td>
<td><img src="image10" alt="Structure" /></td>
<td>10.2</td>
<td>1.5 ± 0.04</td>
<td>Hill slope 4.2</td>
<td>Between 8.3 and 25</td>
</tr>
</tbody>
</table>

^a *Ex-vivo L. donovani* amastigotes; ^b bone marrow derived macrophage (BMDM); ^c The numbers in parentheses were calculated from the two single points of inhibitory data, using GraFit (version 7.0.1); ^d good fit: x < 0.3; poor fit: x>0.5, x = standard deviation error / EC_{50}; ^e macrophage started to round up.
The enzyme pIC$_{50}$s are plotted against the cell pEC$_{50}$s in Figure 4.6. However, a poor correlation was observed which was in contrast to the good correlation seen in *P. falciparum* (Figure 3.18). There are several possibilities for that. Firstly, high metabolic activity and/or slightly acidic assay environment (pH 6.5, tested in the cultures throughout the incubation period) in the *ex-vivo* amastigotes might result in the partial decomposition of compounds 145 and 182 under culture conditions, perhaps responsible for lower-than-expected cell inhibition of these two compounds. But it remains mysterious that compound 142, another acid-labile molecule, exhibited strong cell inhibition. Secondly, the ideal temperature to culture amastigotes is 37 °C[176]. However, *ex vivo* amastigotes, the amastigotes without macrophages, were found to only survive at 26 °C throughout the incubation period (personal communication, Dr. D. Pappe, York University), under which conditions amastigotes gradually differentiate into promastigotes[176]. Therefore, actual parasite inhibition under assay conditions could be the one against both *ex-vivo* amastigotes and promastigotes. Considering that the different susceptibilities of these two forms of parasites to antileishmanial reference drugs have been reported[183], it is possible that the test inhibitors in this project would display different extents of inhibition against amastigotes and promastigotes, thus accounting for the observed poor correlation between enzyme potency and cell inhibition. Thirdly, low cell absorption could contribute to the poor cell killing ability of compound 154, whereby the two secondary amino groups might prevent the molecules from being effectively absorbed into cells. Fourthly, there has been no confirmative evidence to date to show that NMT is critical for the viability of amastigotes although the enzyme has been reported to be essential in *L.donovani* promastigotes[87]. Lastly, some of these inhibitors might be off target in *L. donovani* amastigotes. Under this circumstance, the on-target studies of these molecules are badly required.
Figure 4.6 Correlation between LdNMT potency and *L. donovani* ex-vivo amastigote inhibition for inhibitors differing in the Y linkage group

The dots with red circles indicate the compounds which are used to give the possible explanations to the poor correlation between enzyme potency and anti-leishmanial activity.

### 4.2.4.3 Selection of the early lead candidates

Figure 4.7 lists the structures of the four compounds which were selected based on their high anti-leishmanial activity and sufficient inhibition window (at least 5-fold), which is the ratio between the macrophage toxicity and anti-leishmanial activity. The evaluation of these four molecules in terms of the synthetic ease, stability and selectivity concerns was as follows.

![Diagram showing structures of compounds](image)

**Figure 4.7** Structures of compounds with good antileishmanial activity and sufficient inhibition window

*Inhibitors containing the ether and enone moiety*

Low synthetic yields of 2% and acid lability significantly limited the further development of ether 142. Using carbonyl vinyl as the Y linkage group overcame the synthesis and stability issues. However, the resulting enone 193 suffered from poor selectivity over human NMTs (IC$_{50}$: 43.9 and 39.5 µM against HsNMT1 and HsNMT2
respectively), raising potential toxicity concerns. In addition, the Michael acceptor moiety is always thought to be responsible for a range of false positives in the biochemical assays\cite{184,185}, therefore discarding enone 193 as an early lead candidate.

Inhibitors with the ester functional group

Given the excellent selectivity over human NMTs, ester 162, the lead candidate of PfNMT inhibitors, was also found to be a promising LdNMT inhibitors. Therefore, it was worthwhile to investigate a range of esters and the compounds listed in Figure 4.8 were further evaluated in cell-based assays.

![Figure 4.8 Structures of the ester series for L. donovani ex-vivo amastigote inhibition assays](image)

Table 4.7 presents the parasite inhibition and macrophage toxicity data of the ester series. Generally, higher enzyme potency leads to stronger cell inhibition except for compound 177, which gave the lower-than-expected antileishmanial activity. In addition, compounds 163 and 164 displayed macrophage toxicity at lower concentrations than other esters, indicating a toxicity concern of cyclohexyl or naphthalene as the R² group.
Table 4.7 *Ex-vivo* *L. donovani* amastigote inhibition and macrophage toxicity data for esters

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; µM (LdNMT)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; µM (&lt;i&gt;L. donovani&lt;/i&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Comments on EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Toxicity µM&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td><img src="image1" alt="Structure" /></td>
<td>23.0</td>
<td>(4.6 ± 0.5)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Prediction; good fit&lt;sup&gt;d&lt;/sup&gt;</td>
<td>~25&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>161</td>
<td><img src="image2" alt="Structure" /></td>
<td>19.8</td>
<td>4.9 ± 1.1</td>
<td>Hill slope 0.9</td>
<td>Between 25 and 75</td>
</tr>
<tr>
<td>162</td>
<td><img src="image3" alt="Structure" /></td>
<td>4.7</td>
<td>1.4 ± 0.3</td>
<td>Hill slope 1.1</td>
<td>~8.3&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>163</td>
<td><img src="image4" alt="Structure" /></td>
<td>15.6</td>
<td>3.6 ± 0.7</td>
<td>Hill slope 5.1</td>
<td>Between 2.8 and 8.3</td>
</tr>
<tr>
<td>164</td>
<td><img src="image5" alt="Structure" /></td>
<td>1.9</td>
<td>1.5 ± 0.2</td>
<td>Hill slope 1.5</td>
<td>Between 2.8 and 8.3</td>
</tr>
<tr>
<td>173</td>
<td><img src="image6" alt="Structure" /></td>
<td>16.4</td>
<td>(4.0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Prediction; poor fit&lt;sup&gt;d&lt;/sup&gt;</td>
<td>~8.3&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>177</td>
<td><img src="image7" alt="Structure" /></td>
<td>2.2</td>
<td>7.4 ± 2.9</td>
<td>Hill slope 1.2</td>
<td>Between 8.3 and 25</td>
</tr>
</tbody>
</table>

<sup>a</sup> *Ex-vivo* *L. donovani* amastigotes; <sup>b</sup> bone marrow derived macrophage (BMDM); <sup>c</sup> The numbers in parentheses were calculated from the two single points of inhibitory data, using GraFit (version 7.0.1); <sup>d</sup> good fit: x < 0.3; poor fit: x>0.5, x = standard deviation error / EC<sub>50</sub>; <sup>e</sup> macrophage started to round up.

Figure 4.9 demonstrates the correlation between enzyme potency and parasite inhibition of the ester series. Interestingly, it shows a tight correlation if compound 177 is excluded from the series. The discrepancy of 177 might be a result of demethylases in *L. donovani* amastigotes, which remove methyl from the methoxy group. Or it could be that the ester in this particular compound is a better substrate for cellular esterases than the other ester compounds. Given that the only one series is included in these data, it is not fully conclusive that the antileishmanial effects are directly a result of the NMT inhibition rather than an off-target effect. However, the linear response is at least indicative of this and also suggests that these esters are able to survive under culture conditions. Therefore, ester 162 was selected as an early lead candidate of LdNMT inhibitors at this stage.
Inhibitors containing an oxadiazole moiety

As mentioned earlier, oxadiazole is a bioisostere of ester. Given the excellent selectivity over human NMTs and leadlike properties (removing the stability concerns of the ester group), compound 215 was determined to be a second early lead candidate of LdNMT inhibitors. However, incorporation of oxadiazole was found to decrease the solubility by at least 5-fold (data not shown here), which should be bore in mind for future optimisation.

4.3 Summary

The whole library of PfNMT inhibitors was screened against LdNMT. The molecules with the trimethylene side chain generally showed poor inhibitory activity against LdNMT while the inhibitors with 4-piperidinol were found to be a promising class for further investigation. Subsequent SARs suggested that hydrogen and phenyl were the optimal $R^1$ and $R^2$ groups respectively, leaving the $Y$ linkage group to be selected. With the aid of the amastigote growth inhibition and macrophage toxicity assays, combined with synthesis and selectivity considerations, ester 162 and oxadiazole 215 were determined to be the early lead candidates for LdNMT inhibitors.
Scheme 4.1 Identification of the early lead candidates for LdNMT inhibitors
5. Final Summary

This PhD project has the goal to identify, design and synthesise inhibitors against NMTs in \emph{P. falciparum} and \emph{L. donovani}, the causative agents of malaria and visceral leishmaniasis respectively, aiming to validate NMT as a target for anti-parasitic drugs.

5.1 \emph{P. falciparum} NMT inhibitors

5.1.1 Achievements

A piggy-back strategy of screening a 25-component library of reported NMT inhibitors was used to identify a hit or compound 1, based on a benzofuran core, which showed promising enzyme potency and scope for synthetic diversity. Subsequent effort focused on the modification of the C-2 and C-4 side chains of the scaffold, creating a library of 150 analogues. The potency against PfNMT and selectivity over human NMTs were measured in an optimised scintillation proximity assay, leading to the discovery of compound 162, a lead candidate for PfNMT inhibitors. The experimental SARs were then rationalised by the structural analysis of inhibitor 177 in PvNMT, which is to our knowledge the first co-crystal structure of an inhibitor bound to a \emph{Plasmodium} NMT. More importantly, a tight correlation between enzyme potency and parasite inhibition was observed, providing strong evidence from a chemical perspective to validate NMT as a target in \emph{P. falciparum}.

![Structures of key PfNMT inhibitors at each important stage of the thesis](image)

Figure 5.1 Structures of key PfNMT inhibitors at each important stage of the thesis
The current situation of evaluating NMT as a target in *P. falciparum* is summarised in Table 5.1. Compared with the situation when the project started in 2007, several improvements have been achieved:

- A lead candidate for PfNMT inhibitors with excellent enzyme potency and selectivity over human homologues was identified.
- Cell inhibition results suggested that the anti-*Plasmodial* effects are directly a result of the NMT inhibition, chemically validating NMT as a target in *P. falciparum*.
- The first co-crystal structure of an inhibitor bound to a *Plasmodium* NMT was obtained, which helps to rationalise the experimental SARs and gives the direction for future inhibitor design.

**Table 5.1 Traffic light system**\(^{[126]}\) to evaluate PfNMT as an anti-malarial target in early 2011

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target validation</td>
<td><strong>No genetic validation</strong> but chemical evidence is achieved; downstream proteins associated with biological pathways of parasites</td>
</tr>
<tr>
<td>Assay feasibility</td>
<td><strong>Well developed enzyme</strong>(^{[89]}) and cell-based assays(^{[127]})</td>
</tr>
<tr>
<td>Toxicity concern</td>
<td>Existence of human homologues; <strong>selective inhibition is obtained</strong></td>
</tr>
<tr>
<td>Resistance potential</td>
<td>Single gene copy and the potential to avoid resistance</td>
</tr>
<tr>
<td>Structural information</td>
<td><strong>Co-crystal structure of a ligand in <em>P. vivax</em> NMT is available</strong></td>
</tr>
</tbody>
</table>

Red represents that no information has been obtained; Amber indicates that only little information has been achieved and more progress is required; Green means that information or concept has been well understood and established.

Underlined text demonstrates the improvement compared with the situation of PfNMT as an antimalarial target when the project started in 2007.

### 5.1.2 Future work

As shown in Figure 5.2a, the function of Y linkage group is to direct the attached \(R^2\) group into a pocket. Interestingly, the residues (highlighted in red, Figure 5.2b) forming this pocket are highly conserved among the four parasite NMTs listed in Figure 5.2b, indicating a similar shape of the pocket. Two “hot residues”, F105 and S319 in *PvNMT*, were identified to involve a \(\pi-\pi\) interaction and hydrogen bond with aromatic \(R^2\)
respectively (Figure 3.7). Therefore, to maximise these two interactions is key to further improve enzyme potency. Clearly, this needs to be done without affecting selectivity over human NMTs, though it seems that the origin of this specificity for these inhibitors lies elsewhere (Section 3.3.3)

Figure 5.2 The enzyme pocket containing the $R^2$ group of the inhibitor in PvNMT

(a) Close-up view of the PvNMT enzyme pocket containing $R^2$ of inhibitor 177; the 3-methoxyphenyl ester sits in the middle of this pocket, involving a $\pi-\pi$ interaction with F105 and a hydrogen bond with S319.

(b) Comparison of the residues forming the observed (Pv and Lm NMTs) or expected (Pf and Ld NMTs) ligand binding pocket. The red-coloured residues, which form the pocket containing the $R^2$ group, are highly conserved among the four parasite NMTs.

The colour codes are as follows: oxygen atoms, red; nitrogen atoms, blue; carbon atoms of residues and the inhibitor are yellow and green, respectively; residues forming the pocket to accommodate the $R^2$ group are presented as a surface model; other residues not directly involved with the interactions are displayed as a line model; water molecules, red stars.

Compared with compound 177 in PvNMT, there is a missing hydrogen bond with S319 from the phenyl $R^2$ of compound 162, currently the lead candidate of PfNMT inhibitors. Therefore, the short-term goal is to investigate the diverse phenyl substitutions or heterocycles with hydrogen bond donor properties to pick up the
missing interaction with PfNMT (216, Figure 5.3). However, acid lability described in Scheme 3.45 will possibly limit the full study of this ester series. Under this circumstance, it is also desirable to identify other good Y linkage groups to replace methylene ester (217, Figure 5.3).

Figure 5.3 Future design for PfNMT inhibitors

Aside from enhancing enzyme potency, the long-term goal should be to improve cell inhibition, aiming to identify the determinant factors other than enzyme IC$_{50}$, such as CLogP described in Figure 3.17, and understand the structural influence on the cellular uptake behaviour.

5.2 *L. donovani* NMT inhibitors

5.2.1 Achievements

A second piggy-back approach was applied to identify good hits or early lead candidates for LdNMT inhibitors whereby the 150-component library of PfNMT inhibitors was screened against LdNMT. The resulting SARs determined 4-piperidinol to be an optimal C-4 side chain. In addition, phenyl at the C-2 side chain was found to be important as well. *L. donovani* ex-vivo amastigote inhibition and macrophage toxicity assays were further conducted to select the promising Y linkage groups, resulting in the identification of compounds 162 and 215 with good anti-parasitic activity and sufficient inhibition window (at least greater than 5-fold), which determines the ratio between macrophage toxicity and its corresponding EC$_{50}$ value (Figure 5.4).
Table 5.2 lists the current situation of evaluating NMT as an anti-leishmanial target. Compared with the situation when LdNMT project started in 2009, several improvements have been achieved:

- Two early lead candidates for LdNMT inhibitors with good enzyme potency and selectivity over human homologues were achieved.
- LdNMT inhibitors were also found to kill parasites in the ex-vivo amastigote inhibition assays although there was no conclusive clue from the chemical aspect to show that NMT is a target in *L. donovani*.

Table 5.2 Traffic light system[126] to evaluate LdNMT as an anti-leishmania target in early 2011

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target validation</td>
<td>weak genetic validation[87]; <em>LdNMT inhibitors show cell killing ability</em>; weak functional knowledge of downstream proteins</td>
</tr>
<tr>
<td>Assay feasibility</td>
<td>Well developed enzyme assay; <em>Ex-vivo</em> and <em>intracellular</em> amastigotes is available</td>
</tr>
<tr>
<td>Toxicity concern</td>
<td>Existence of human homologues; Selective inhibition is achieved</td>
</tr>
<tr>
<td>Resistance potential</td>
<td>Single gene copy and the potential to avoid resistance</td>
</tr>
<tr>
<td>Structural information</td>
<td>Structure of LdNMT with myr-CoA analogue[87] (PDB code: 2WUU)</td>
</tr>
</tbody>
</table>

*Amber* indicates that only little information has been achieved and more progress is required; *Green* means that information or concept has been well understood and established.

Underlined text demonstrates the improvement compared with the situation of PfNMT as an antimalarial target when the project started in 2007.
5.2.2 Future work

Given the conserved pocket containing the $R^2$ group in LdNMT (described in Figure 5.2), the strategy for future PfNMT inhibitor design can also be applied, to discover more active LdNMT inhibitors (217, Figure 5.5). Given that there is no preference of the Y linkage group in terms of enzyme potency, it is likely that either an optimal Y has not been identified or the benzofuran scaffold is not favoured by the enzyme. Scaffold hopping studies are on-going in our group in an attempt to address this (218, Figure 5.5). In addition, obtaining a co-crystal structure of an inhibitor bound to LdNMT will be enormously helpful in the near future.

![Chemical structures](image)

**Figure 5.5 Future design for LdNMT inhibitors**

Understanding the cell behaviour of inhibitors in parasites is even more challenging than just improving enzyme potency, since a poor correlation between enzyme potency and the cell killing ability has been observed (Figure 4.6). There are various explanations to such discrepancies, and a sub-group of the inhibitor variations does show an excellent correlation (ester series, Figure 4.9). However, from a chemistry point of view, to synthesise inhibitors with good stability, including chemical and biological stability, will be at the top of the agenda. More compounds with good stability and diverse functional groups will thus be badly needed to support the target validation.
Chapter 6 – Experimental section

6 Experimental Section

6.1 Chemistry

6.1.1 General Methods

All solvents and reagents were purchased from commercial sources and used without further purification.

The compounds were spotted on silica TLC plates (Merck, Si60, F254), visualized under UV-light at 254 nm or iodine over silica.

Purification of the final products was performed on a Gilson semi-preparative Reverse Phase-HPLC system (Anachem Ltd., Luton, UK) equipped with a HICHROM C18 Column (250 × 21.2 mm), #306 pumps and a Gilson 155 UV/Vis detector. UV detection was at 220 nm. The mobile phase consisted of water plus 0.1% TFA (solvent A) and methanol plus 0.1% TFA (solvent B) with a gradient of 30% B for 2 min changing to 98% B over 30 min, maintaining for 2 min, and then down to 2% B over 0.5 min at a flow rate of 12 mL/min.

The purities of the reported compounds for biological assay tests were evaluated by analytical LC-MS which were carried out on a Waters 2767 system equipped with a photodiode array and a mass spectrometer using an X-Bridge™ C18 column (5 μM, 4.6 x 100 mM). The mobile phase consisted of water plus 0.1% formic acid (solvent A) and methanol plus 0.1 formic acid (solvent B) with a gradient starting from 2% to 98% B over the first 10 min, maintaining for 2 min, and down to 2% B over 1 min followed by the maintenance period for another 4 min at 2% B at a flow rate of 1.2 mL/min.

Considering that the actual forms of these compounds are TFA and water adducts, determination of the accurate chemical formulae might have proven to be difficult. Therefore, only Mass, NMR and LC-MS data, which are used to confirm the structures and purities of test compounds, are presented in this thesis.
One-dimensional $^1$H- and $^{13}$C-NMR spectra as well as two-dimensional NMR spectra were recorded on Bruker AV at 400, 500 MHz or 100, 125 MHz respectively. Chemical shifts were reported in ppm.

Mass spectra were obtained from the Mass Spectrometry Service of Department of Chemistry, Imperial College London.

6.1.2 Synthesis and characterisation of compounds

![Chemical structure](image)

Ethyl 3-methyl-4-(3-(pyridin-3-ylmethylamino)propoxy)benzofuran-2-carboxylate (1)$^{[82]}$

Compound 2 (8 mg, 0.023 mmol) was stirred with 3-(aminomethyl)pyridine (9.6 µL, 0.092 mmol) in DMF (1 mL) at 80 °C for 4 hours. The reaction mixture was diluted with EtOAc (10 mL), followed by partitioning with water (10 mL). The aqueous layer was further extracted by EtOAc (2 x 5 mL). The combined organic layers were sequentially washed by saturated NH$_4$Cl, water and brine (each 10 mL), dried over anhydrous sodium sulphate, evaporated under pressure to dryness. The residue was purified by semi-preparative reverse phase HPLC to give the title compound as a white solid (5.9 mg, 53% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.82 (s, 1H), 8.74 (s, 1H), 8.20 (d, J=8.0 Hz, 1H), 7.70 (dd, J=8.0, 5.2 Hz, 1H), 7.39 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.4 Hz, 1H), 6.79 (d, J=8.0 Hz, 1H), 4.44 (s, 2H), 4.41 (q, J=7.2 Hz, 2H), 4.28 (t, J=5.6 Hz, 2H), 3.41 (t, J=7.6 Hz, 2H), 2.70 (s, 3H), 2.38-2.31 (m, 2H), 1.43 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.48, 155.71, 155.04, 148.96, 148.69, 140.07, 139.52, 128.70, 125.74, 124.88, 118.21, 104.63, 104.00, 64.65, 60.69, 47.96, 45.02, 25.78, 13.23, 10.38. Calculated exact mass for the protonated molecule (C$_{21}$H$_{25}$N$_2$O$_4$): 369.1814; measured accurate mass (ESI): 369.1806. LC-MS purity: 100%, $t_R = 11.74$ min.
Chapter 6 – Experimental section

Ethyl 4-(3-bromopropoxy)-3-methylbenzofuran-2-carboxylate (2)[82]

Compound 3 (100 mg, 0.45 mmol), potassium carbonate (326 mg, 2.36 mmol) were suspended in DMF (2 mL). The mixture was stirred at room temperature for 30 minutes and 1,3-dibromopropane (240 µL, 2.36 mmol) was then added. The resulting mixture was allowed to stir at room temperature for 4 hours. The reaction mixture was added with 10 mL water and extracted with EtOAc 3×15 mL. The organic layer was then dried over anhydrous sodium sulphate, concentrated and purified by column chromatography on silica gel, eluting with hexane:ethyl acetate, 95:5 to give the title compound as a white solid (95 mg, 62% yield). 1H-NMR (CDCl$_3$, 400 MHz): δ 7.35 (t, J=8.0 Hz, 1H), 7.17 (d, J=8.0 Hz, 1H), 6.68 (d, J=8.0 Hz, 1H), 4.47 (q, J=7.2 Hz, 2H), 4.26 (t, J=5.6 Hz, 2H), 3.68 (t, J=6.4 Hz, 2H), 2.76 (s, 3H), 2.47-2.41 (m, 2H), 1.46 (t, J=7.2 Hz, 3H).

Ethyl 4-hydroxy-3-methylbenzofuran-2-carboxylate (3)[135]

To a sodium ethoxide solution, prepared by dissolving sodium (225 mg, 9.8 mmol) in 20 mL anhydrous ethanol (purchased from Aldrich, with 3 Å molecular sieve over it) under a nitrogen atmosphere and cooled down to around -18 °C, compound 4 (1.55 g, 6.5 mmol) was added with stirring over a period of 5 min. The solution was stirred for 18 hours while the temperature was slowly warmed up to room temperature. The mixture was quenched by 20 mL water and extracted by 20 mL EtOAc immediately. The aqueous layer was further extracted by 2 × 15 mL EtOAc. The above organic layer was combined and dried over anhydrous sodium sulphate, concentrated and purified by column chromatography on silica gel, eluting with hexane:ethyl acetate, 86:14, 80:20 to give the title compound as an light-yellow solid (670 mg, 46% yield). 1H-NMR (CD$_3$COCD$_3$, 400 MHz): δ 9.05 (s, 1H, OH), 7.25 (t, J=8.0 Hz, 1H), 6.99 (d, J=8.0 Hz, 1H), 6.69 (d, J=8.0 Hz, 1H), 4.36 (q, J=7.2 Hz, 2H), 2.73 (s, 3H), 1.37 (t, J=7.2 Hz, 3H).
A solution of 2,6-dihydroxyacetophenone (5 g, 32.9 mmol) in 60 mL of acetone was added potassium carbonate (11.3 g, 82 mmol) and ethyl bromoacetate (3.82 mL, 34.5 mmol). The reaction mixture was stirred at 60 °C for 2 hours. The solid was filtered off and the filtrate was concentrated. The residue was purified by column chromatography on silica gel, eluting with hexane:ethyl acetate, 78:22 to obtain the title compound as a light-yellow solid (6.95 g, 89% yield). 

\[
\text{H-NMR (CDCl}_3, 400 MHz) : \delta 13.26 (s, 1H), 7.34 (t, J=8.0 \text{ Hz}, 1H), 6.64 (d, J=8.0 \text{ Hz}, 1H), 6.26 (d, J=8.0 \text{ Hz}, 1H), 4.72 (s, 2H), 4.31 (q, J=7.8 \text{ Hz}, 2H), 2.82 (s, 3H), 1.33 (t, J=7.8 \text{ Hz}, 3H).
\]

The aqueous layer after the extraction with EtOAc in the preparation of compound 3, was acidified using 2 mL concentrated HCl (20 mmol) and then extracted with 20 mL EtOAc. The aqueous layer was further extracted with 2 × 15 mL EtOAc. The combined organic layers were dried over anhydrous sodium sulphate, concentrated and purified by column chromatography on silica gel, eluting with hexane:ethyl acetate, 86:14, to give the title compound as a white solid (276 mg, 29% yield). 

\[
\text{H-NMR (CD}_2\text{COCD}_3, 400 MHz) : \delta 8.89 (s, 1H, OH), 7.42 (s, 1H), 7.06 (t, J=8.0 \text{ Hz}, 1H), 6.94 (d, J=8.0 \text{ Hz}, 1H), 6.64 (d, J=8.0 \text{ Hz}, 1H), 2.38 (s, 3H).
\]

Prepared from compound 3 (100 mg, 0.45 mmol), dibromoethane (0.28 mL, 2.4 mmol) and potassium carbonate (0.69 g, 5.0 mmol) in DMF (2 mL) according to the preparation of compound 2, the title compound was obtained as a white solid (55 mg, 37% yield). 

\[
\text{H-NMR (CDCl}_3, 400 MHz) : \delta 7.34 (t, J=8.0 \text{ Hz}, 1H), 7.18 (d, J=8.0 \text{ Hz}, 1H), 6.61 (d, J=8.0 \text{ Hz}, 1H), 4.47 (q, J=7.2 \text{ Hz}, 2H), 4.44 (t, J=6.0 \text{ Hz}, 2H), 3.76 (t, J=6.0 \text{ Hz}, 2H), 2.81 (s, 3H), 1.46 (t, J= 7.2 \text{ Hz}, 3H).
\]
Prepared from compound 3 (100 mg, 0.45 mmol), dibromobutane (0.28 mL, 2.4 mmol) and potassium carbonate (0.69 g, 5.0 mmol) in DMF (2 mL) according to the preparation of compound 2, the title compound was obtained as a white solid (89 mg, 56% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 7.33 (t, J=8.0 Hz, 1H), 7.15 (d, J=8.0 Hz, 1H), 6.63 (d, J=8.0 Hz, 1H), 4.46 (q, J=7.2 Hz, 2H), 4.14 (t, J=6.0 Hz, 2H), 3.54 (t, J=6.4 Hz, 2H), 2.77 (s, 3H), 2.19-2.04 (m, 4H), 1.46 (t, J=7.2 Hz, 3H).

Prepared from compound 11 (13.1 mg, 0.04 mmol) and t-butylamine (16.8 µL, 0.16 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (9.5 mg, 55% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.43 (t, J=8.0 Hz, 1H), 7.19 (d, J=8.0 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.43 (t, J=4.4 Hz, 2H), 4.41 (q, J=7.2 Hz, 3H), 3.59 (t, J=4.4 Hz, 2H), 2.79 (s, 3H), 1.48 (s, 9H), 1.42 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.45, 155.75, 154.49, 139.74, 128.67, 125.75, 118.45, 105.35, 104.37, 64.13, 60.74, 57.30, 41.21, 24.33, 13.21, 10.35. Calculated exact mass for the protonated molecule (C$_{18}$H$_{26}$NO$_4$): 320.1862; measured accurate mass (ESI): 320.1849. LC-MS purity: 94%, $t_R$ = 11.67 min.

Prepared from compound 11 (10 mg, 0.031 mmol) and 3-(aminomethyl)-pyridine (12.6 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (2.7 mg, 19% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.79 (d, J=2.0 Hz, 1H), 8.72 (dd, J=5.0, 1.6 Hz, 1H), 8.15 (dt, J=8.0, 1.6 Hz, 1H), 7.67 (dd, J=8.0, 5.0 Hz, 1H), 7.43 (t,
J=8.4 Hz, 1H), 7.20 (d, J=8.0 Hz, 1H), 6.87 (d, J=8.0 Hz, 1H), 4.51 (s, 2H), 4.51 (t, J=4.8 Hz, 2H), 4.41 (q, J=7.2 Hz, 2H), 3.69 (t, J=4.8 Hz, 2H), 2.76 (s, 3H), 1.42 (t, J=7.2 Hz, 3H). 13C-NMR (CD3OD, 125 MHz): δ 161.81, 157.18, 155.72, 151.66, 151.49, 141.17, 139.98, 130.03, 129.12, 127.07, 125.74, 120.00, 106.91, 105.98, 65.24, 62.15, 50.04, 47.94, 14.63, 11.71. Calculated exact mass for the protonated molecule (C20H23N2O4): 355.1658; measured accurate mass (ESI): 355.1645. LC-MS purity: 100%, tR = 11.34 min.

 Preparation of compound 15

Prepared from compound 2 (5 mg, 0.015 mmol) and tert-butylamine (6.2 µL, 0.06 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (2.9 mg, 45% yield). 1H-NMR (CD3OD, 400 MHz): δ 7.41 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.0 Hz, 1H), 6.81 (d, J=8.0 Hz, 1H), 4.41 (q, J=7.0 Hz, 2H), 4.29 (t, J=6.0 Hz, 2H), 3.29 (t, J=6.0 Hz, 2H), 2.76 (s, 3H), 2.32-2.25 (m, 2H), 1.44 (s, 9H), 1.40 (t, J=7.0 Hz, 3H). 13C-NMR (CD3OD, 100 MHz): δ 160.60, 155.75, 155.23, 139.70, 128.87, 128.58, 150.75, 104.75, 104.15, 64.77, 60.83, 56.98, 38.81, 26.58, 24.58, 13.37, 10.52. Calculated exact mass for the protonated molecule (C19H28NO4): 334.2018; measured accurate mass (ESI): 334.2003. LC-MS purity: 100%, tR = 12.28 min.

 Preparation of compound 16

Prepared from compound 2 (10 mg, 0.029 mmol) and benzylamine (12.7 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as an off-white solid (6.0 mg, 43% yield). 1H-NMR (CD3OD, 400 MHz): δ 7.54-7.48 (m, 5H), 7.40 (t, J=8.0 Hz, 1H), 7.14 (d, J=8.0 Hz, 1H), 6.79 (d, J=8.0 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 4.30 (s, 2H), 4.27 (t, J=5.6 Hz, 2H), 3.33 (t, J=6.0 Hz, 2H), 2.66 (s, 3H), 2.35-2.28 (m, 2H), 1.44 (t, J=7.2 Hz, 3H). 13C-NMR (CD3OD, 100 MHz): δ 160.47, 155.70, 155.23, 139.70, 128.87, 128.58, 150.75, 104.75, 104.15, 64.77, 60.83, 56.98, 38.81, 26.58, 24.58, 13.37, 10.52. Calculated exact mass for the protonated molecule (C19H28NO4): 334.2018; measured accurate mass (ESI): 334.2003. LC-MS purity: 100%, tR = 12.28 min.
Calculated exact mass for the protonated molecule (C_{22}H_{26}NO_4): 368.1862; measured accurate mass (ESI): 368.1876. LC-MS purity: 100%; t_R = 12.41 min.

**Ethyl 3-methyl-4-(3-(pyridin-2-ylmethy lamino)propoxy)benzofuran-2-carboxylate (17)**

Prepared from compound 2 (10 mg, 0.029 mmol) and 2-(aminomethyl)-pyridine (12.0 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (7.2 mg, 52% yield). 1H-NMR (CD_3OD, 400 MHz): δ 8.60 (d, J=4.8 Hz, 1H), 7.89 (dt, J=8.0, 1.6 Hz, 1H), 7.49 (d, J=8.0 Hz, 1H), 7.41 (d, J=8.0 Hz, 1H), 7.41 (t, J=8.0 Hz, 1H), 7.14 (d, J=8.0 Hz, 1H), 6.81 (d, J=8.0 Hz, 1H), 4.47 (s, 2H), 4.42 (q, J=7.2 Hz, 2H), 4.30 (t, J=6.0 Hz, 2H), 3.41 (t, J=7.6 Hz, 2H), 2.70 (s, 3H), 2.41-2.34 (m, 2H), 1.43 (t, J=7.2 Hz, 3H). 13C-NMR (CD_3OD, 100 MHz): δ 160.37, 155.70, 155.06, 151.04, 149.23, 139.49, 137.43, 128.71, 125.80, 125.80, 123.65, 122.69, 118.18, 104.63, 104.02, 64.87, 60.68, 50.44, 44.98, 25.73, 13.22, 10.37. Calculated exact mass for the protonated molecule (C_{21}H_{25}N_2O_4): 369.1814; measured accurate mass (ESI): 369.1807. LC-MS purity: 100%; t_R = 12.13 min.

**Ethyl 3-methyl-4-(3-(piperidin-1-yl)propoxy)benzofuran-2-carboxylate (18)**

Prepared from compound 2 (10 mg, 0.029 mmol) and piperidine (11.5 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as an off-white solid (12.0 mg, 90% yield). 1H-NMR (CD_3OD, 400 MHz): δ 7.40 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.0 Hz, 1H), 6.79 (d, J=8.0 Hz,1H), 4.26 (q, J=7.2 Hz, 2H), 4.26 (t, J=6.0 Hz, 2H), 3.64 (d, J=12.4 Hz, 2H), 1.43 (t, J=7.2 Hz, 3H). 13C-NMR (CD_3OD, 100 MHz): δ 160.49, 155.70, 155.05, 139.52, 128.71, 125.80, 118.22, 104.62, 103.97, 64.81, 60.69, 54.40, 53.10, 23.77, 22.95, 21.24, 13.22, 10.41. Calculated exact mass for the protonated molecule (C_{20}H_{28}NO_4): 364.2018; measured accurate mass (ESI): 364.2009. LC-MS purity: 100%, t_R = 11.88 min.
Ethyl 3-methyl-4-(3-(methylamino)propoxy)benzofuran-2-carboxylate (19)

Prepared from compound 2 (10 mg, 0.029 mmol) and methylamine (33% wt solution in ethanol, 11.3 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (7.5 mg, 64% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.40 (t, $J=8.0$ Hz, 1H), 7.14 (d, $J=8.0$ Hz, 1H), 6.80 (d, $J=8.0$ Hz, 1H), 4.41 (q, $J=7.2$ Hz, 2H), 4.27 (t, $J=5.6$ Hz, 2H), 3.29 (t, $J=7.6$ Hz, 2H), 2.80 (s, 3H), 2.76 (s, 3H), 2.33-2.26 (m, 2H), 1.43 (t, $J=7.2$ Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 160.50, 155.70, 155.09, 139.57, 128.70, 125.66, 118.24, 104.62, 104.01, 64.72, 60.69, 46.53, 32.36, 25.70, 13.22, 10.37. Calculated exact mass for the protonated molecule (C$_{16}$H$_{22}$NO$_4$): 292.1549; measured accurate mass (ESI): 292.1545. LC-MS purity: 96%, $t_R = 11.73$ min.

Ethyl 3-methyl-4-(3-(phenylamino)propoxy)benzofuran-2-carboxylate (20)

Prepared from compound 2 (10 mg, 0.029 mmol) and aniline (10.9 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a pale brown solid (9.5 mg, 70% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 7.34-7.29 (m, 3H), 7.15 (d, $J=8.0$ Hz, 1H), 7.08 (d, $J=8.0$ Hz, 2H), 7.07 (t, $J=8.0$ Hz, 1H), 6.58 (d, $J=8.0$ Hz, 1H), 4.46 (q, $J=7.2$ Hz, 2H), 4.16 (t, $J=6.0$ Hz, 2H), 3.50 (t, $J=7.6$ Hz, 2H), 2.73 (s, 3H), 2.31-2.24 (m, 2H), 2.20 (s, NH), 1.46 (t, $J=7.2$ Hz, 3H). $^{13}$C-NMR (CDCl$_3$, 100 MHz): $\delta$ 160.46, 155.66, 155.18, 142.12, 139.71, 129.81, 128.55, 126.37, 123.37, 118.65, 117.64, 105.35, 103.77, 65.26, 61.03, 45.54, 27.38, 14.43, 11.38. Calculated exact mass for the protonated molecule (C$_{21}$H$_{24}$NO$_4$): 354.1705; measured accurate mass (ESI): 354.1716. LC-MS purity: 100%, $t_R = 22.94$ min (optimal gradient: from 2% to 98% B over the first 20 min, maintaining for 2 min, and down to 2% B over 1 min followed by the maintenance period for another 4 min at 2% B at a flow rate of 1.2 mL/min).
Ethyl 3-methyl-4-(3-(pyridin-4-ylmethylamino)propoxy)benzofuran-2-carboxylate (21)

Prepared from compound 2 (10 mg, 0.029 mmol) and 4-(aminomethyl)-pyridine (12.1 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a yellow solid (4.8 mg, 34% yield). $^1$H-NMR (CD$_3$OD, 500 MHz): δ 8.72 (s, 2H), 7.67 (d, J=6.0 Hz, 2H), 7.38 (t, J=7.5 Hz, 1H), 7.11 (d, J=7.5 Hz, 1H), 6.78 (d, J= 7.5 Hz, 1H), 4.42 (s, 2H), 4.39 (q, J=7.2 Hz, 2H), 4.27 (t, J=6.0 Hz, 2H), 3.39 (t, J=7.5 Hz, 2H), 2.69 (s, 3H), 2.36-2.30 (m, 2H), 1.40 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 161.88, 157.14, 156.43, 149.88, 144.44, 140.98, 130.11, 127.11, 126.30, 119.65, 106.10, 105.43, 66.05, 62.11, 50.87, 46.74, 27.16, 14.64, 11.79. Calculated exact mass for the protonated molecule (C$_{21}$H$_{25}$N$_2$O$_4$): 369.1814; measured accurate mass (ESI): 369.1814. LC-MS purity: 100%, $t_r$ = 11.65 min.

Ethyl 3-methyl-4-(3-(naphthalen-1-ylmethyl-amino)-propoxy)-benzofuran-2-carboxylate (22)

Prepared from compound 2 (10 mg, 0.029 mmol) and 1-(aminomethyl)-naphthalin (17.0 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (3.7 mg, 24% yield). $^1$H-NMR (CD$_3$OD, 500 MHz): δ 8.16 (d, J=8.0 Hz, 1H), 8.00 (d, J=8.0 Hz, 1H), 7.97 (d, J=8.0 Hz, 1H), 7.69 (d, J=7.5 Hz, 1H), 7.66-7.62 (m, 1H), 7.60-7.54 (m, 1H), 7.36 (t, J=8.0 Hz, 1H), 7.10 (d, J=8.0 Hz, 1H), 6.75 (d, J=8.0 Hz, 1H), 4.80 (s, 2H), 4.40 (q, J=7.0 Hz, 2H), 4.25 (t, J=6.0 Hz, 2H), 3.42 (t, J=7.5 Hz, 2H), 2.56 (s, 3H), 2.35-2.29 (m, 2H), 1.41 (t, J=7.0 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 161.88, 157.12, 156.39, 140.92, 135.52, 132.71, 131.78, 130.38, 130.24, 130.09, 128.55, 128.34, 127.69, 127.11, 126.52, 123.69, 119.63, 106.07, 105.41, 66.22, 62.11, 48.91, 46.52, 27.11, 14.67, 11.69. Calculated exact mass for the protonated molecule (C$_{26}$H$_{28}$NO$_4$): 418.2018; measured accurate mass (ESI): 418.2036. LC-MS purity: 100%, $t_r$ = 19.14 min (gradient: from 2% to 98% B over the first 20 min, maintaining for 2 min, and down to 2% B over 1 min followed by another 4 min at 2% B; flow rate: 1.2 mL/min).
Ethyl 3-methyl-4-[3-(propan-2-ylamino)propoxy]-1-benzofuran-2-carboxylate \([23]^{[82]}\)

Prepared from compound \(2\) (10 mg, 0.029 mmol) and isopropylamine (10.2 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound \(1\), the title compound was obtained as a white solid (9.7 mg, 77% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): δ 7.40 (t, \(J=8.0\) Hz, 1H), 7.14 (d, \(J=8.0\) Hz, 1H), 6.80 (d, \(J=8.0\) Hz, 1H), 4.41 (q, \(J=7.2\) Hz, 2H), 4.27 (t, \(J=6.0\) Hz, 2H), 3.50-3.43 (m, 1H), 3.30 (d, \(J=8.0\) Hz, 2H), 2.76 (s, 3H), 2.32-2.25 (m, 2H), 1.43 (t, \(J=7.2\) Hz, 3H), 1.39 (d, \(J=6.8\) Hz, 6H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): δ 160.55, 155.77, 155.09, 139.53, 128.87, 125.79, 118.28, 104.61, 104.04, 64.66, 60.76, 50.66, 42.19, 26.20, 17.89, 13.30, 10.45. Calculated exact mass for the protonated molecule (C\(_{18}\)H\(_{26}\)NO\(_4\)): 320.1862; measured accurate mass (ESI): 320.1858. LC-MS purity: 100%, \(t_R = 12.66\) min.

Ethyl 4-(3-(cyclohexylamino)propoxy)-3-methylbenzofuran-2-carboxylate (24)

Prepared from compound \(2\) (10 mg, 0.029 mmol) and cyclohexylamine (13.7 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound \(1\), the title compound was obtained as a white solid (10.9 mg, 79% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): δ 7.40 (t, \(J=8.0\) Hz, 1H), 7.13 (d, \(J=8.0\) Hz, 1H), 6.80 (d, \(J=8.0\) Hz, 1H), 4.41 (q, \(J=7.2\) Hz, 2H), 4.27 (t, \(J=6.0\) Hz, 2H), 3.32 (d, \(J=8.0\) Hz, 2H), 3.21-3.10 (m, 1H), 2.75 (s, 3H), 2.32-2.25 (m, 2H), 2.20-2.11 (brd, 2H), 1.98-1.87 (brd, 2H), 1.80-1.70 (m, 2H), 1.46-1.19 (m, 5H), 1.43 (t, \(J=7.2\) Hz, 3H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): δ 160.48, 155.72, 155.10, 139.54, 128.71, 125.72, 118.23, 104.60, 103.99, 64.70, 60.69, 57.11, 41.77, 28.99, 26.00, 24.67, 24.03, 13.22, 10.38. Calculated exact mass for the protonated molecule (C\(_{21}\)H\(_{30}\)NO\(_4\)): 360.2175; measured accurate mass (ESI): 360.2186. LC-MS purity: 100%, \(t_R = 12.71\) min.
Ethyl 3-methyl-4-(3-(2-methylbenzylamino)propoxy)benzofuran-2-carboxylate (25)

Prepared from compound 2 (10 mg, 0.029 mmol) and 2-methylbenzylamine (14.9 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (7.6 mg, 53% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.46 (d, J=7.6 Hz, 1H), 7.41 (t, J=8.0 Hz, 1H), 7.37-7.29 (m, 3H), 7.14 (d, J=8.0 Hz, 1H), 6.80 (d, J=8.0 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 4.35 (s, 2H), 4.29 (t, J=6.0 Hz, 2H), 3.41 (t, J=8.0 Hz, 2H), 2.70 (s, 3H), 2.47 (s, 3H), 2.38-2.31 (m, 2H), 1.44 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 160.47, 155.72, 155.05, 139.52, 137.56, 137.40, 130.88, 129.83, 129.52, 128.71, 126.48, 125.75, 118.23, 104.63, 104.00, 64.76, 60.69, 48.71, 44.97, 25.69, 17.80, 13.23, 10.39. Calculated exact mass for the protonated molecule (C$_{23}$H$_{28}$NO$_4$): 382.2018; measured accurate mass (ESI): 382.2007. LC-MS purity: 100%, $t_R$ = 12.52 min.

Ethyl 3-methyl-4-(3-(4-methylbenzylamino)propoxy)benzofuran-2-carboxylate (26)

Prepared from compound 2 (10 mg, 0.029 mmol) and 4-methylbenzylamine (15.2 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (8.2 mg, 57% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.40 (t, J=8.0 Hz, 1H), 7.39 (d, J=8.0 Hz, 2H), 7.29 (d, J=8.0 Hz, 2H), 7.13 (d, J=8.0 Hz, 1H), 6.78 (d, J=8.0 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 4.25 (s, 2H), 4.25 (t, J=5.6 Hz, 2H), 3.30 (t, J=8.0 Hz, 2H), 2.61 (s, 3H), 2.39 (s, 3H), 2.33-2.26 (m, 2H), 1.44 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 160.46, 155.69, 154.99, 139.71, 139.47, 129.55, 128.70, 127.85, 125.76, 118.19, 104.60, 103.94, 64.61, 60.68, 50.68, 44.14, 25.64, 19.84, 13.25, 10.32. Calculated exact mass for the protonated molecule (C$_{23}$H$_{28}$NO$_4$): 382.2018; measured accurate mass (ESI): 382.2023. LC-MS purity: 100%, $t_R$ = 12.68 min.
Ethyl 4-(3-(2-fluorobenzylamino)propoxy)-3-methylbenzofuran-2-carboxylate (27)

Prepared from compound 2 (10 mg, 0.029 mmol) and 2-fluorobenzylamine (13.7 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (8.2 mg, 57% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 7.62-7.51 (m, 2H), 7.40 (t, J=8.0 Hz, 1H), 7.36-7.24 (m, 2H), 7.14 (d, J=8.0 Hz, 1H), 6.79 (d, J=8.0 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 4.40 (s, 2H), 4.27 (t, J=5.6 Hz, 2H), 3.37 (t, J=8.0 Hz, 2H), 2.68 (s, 3H), 2.38-2.28 (m, 2H), 1.44 (t, J=7.2 Hz, 3H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 160.47, 160.18, 155.70, 155.02, 139.51, 132.07, 131.96, 128.70, 125.73, 124.90, 118.22, 115.6, 104.63, 103.99, 64.67, 60.69, 44.74, 44.29, 25.63, 13.23, 10.36. Calculated exact mass for the protonated molecule (C\(_{22}\)H\(_{25}\)NO\(_4\)F): 386.1768; measured accurate mass (ESI): 386.1771. LC-MS purity: 100%, \(t_R = 12.34\) min.

Ethyl 4-(3-(4-fluorobenzylamino)propoxy)-3-methylbenzofuran-2-carboxylate (28)

Prepared from compound 2 (10 mg, 0.029 mmol) and 4-fluorobenzylamine (13.7 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (8.6 mg, 59% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 7.57 (dd, J=8.8, 5.2 Hz, 2H), 7.40 (t, J=8.0 Hz, 1H), 7.23 (t, J=8.8 Hz, 2H), 7.14 (d, J=8.0 Hz, 1H), 6.79 (d, J=8.0 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 4.30 (s, 2H), 4.27 (t, J=5.6 Hz, 2H), 3.33 (t, J=8.0 Hz, 2H), 2.66 (s, 3H), 2.36-2.26 (m, 2H), 1.44 (t, J=7.2 Hz, 3H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 162.26, 160.47, 155.71, 155.01, 139.52, 131.97, 128.70, 127.16, 125.69, 118.21, 115.78, 104.62, 103.98, 64.66, 60.70, 50.16, 44.45, 25.69, 13.22, 10.36. Calculated exact mass for the protonated molecule (C\(_{22}\)H\(_{25}\)NO\(_4\)F): 386.1768; measured accurate mass (ESI): 386.1766. LC-MS purity: 100%, \(t_R = 12.33\) min.
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Ethyl 3-methyl-4-(3-piperazin-1-yl)propoxybenzofuran-2-carboxylate(29)

Prepared from compound 2 (10 mg, 0.029 mmol) and piperazine (10 mg, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (4.9 mg, 37% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.39 (t, J=8.4 Hz, 1H), 7.12 (d, J=8.4 Hz, 1H), 6.78 (d, J=8.0 Hz, 1H), 4.41 (q, J=7.2 Hz, 2H), 4.25 (t, J=6.0 Hz, 2H), 3.45 (t, J=5.2 Hz, 4H), 3.23 (brd, 4H), 3.13 (t, J=7.6 Hz, 2H), 2.76 (s, 3H), 2.29-2.22 (m, 2H), 1.42 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 161.94, 157.15, 156.76, 140.88, 130.12, 127.32, 119.66, 105.81, 105.36, 66.57, 62.08, 55.80, 50.48, 43.55, 26.26, 14.63, 11.79. Calculated exact mass for the protonated molecule (C$_{19}$H$_{27}$N$_2$O$_4$): 347.1971; measured accurate mass (ESI): 347.1956. LC-MS purity: 100%, $t_R$ = 11.39 min.

Ethyl 4-(4-(tert-butylationo)butoxy)-3-methylbenzofuran-2-carboxylate(30)[82]

Prepared from compound 12 (10.7 mg, 0.03 mmol) and tert-butyamine (12.6 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (6.8 mg, 49% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.38 (t, J=8.0 Hz, 1H), 7.10 (d, J=8.0 Hz, 1H), 6.78 (d, J=8.0 Hz, 1H), 4.40 (q, J=7.2 Hz, 2H), 4.21 (t, J=6.0 Hz, 2H), 3.10 (t, J=4.0 Hz, 2H), 2.76 (s, 3H), 2.06-1.90 (m, 4H), 1.42 (t, J=7.2 Hz, 3H), 1.40 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.52, 155.73, 155.49, 139.42, 128.75, 125.94, 118.22, 104.27, 103.94, 67.08, 60.65, 56.54, 41.00, 26.05, 24.41, 23.62, 13.23, 10.38. Calculated exact mass for the protonated molecule (C$_{20}$H$_{30}$NO$_4$): 348.2175; measured accurate mass (ESI): 348.2165. LC-MS purity: 100%, $t_R$ = 12.45 min.
Ethyl 3-methyl-4-(4-(pyridin-3-ylmethylamino)butoxy)benzofuran-2-carboxylate (31)

Prepared from compound 12 (26 mg, 0.07 mmol) and 3-(aminomethyl)-pyridine (29.5 µL, 0.29 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (13.6 mg, 39% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.86 (s, 1H), 8.78 (d, J=4.8 Hz, 1H), 8.33 (d, J=8.0 Hz, 1H), 7.80 (dd, J=8.0, 4.8 Hz, 1H), 7.36 (t, J=8.0 Hz, 1H), 7.09 (d, J=8.0 Hz, 1H), 6.75 (d, J=8.0 Hz, 1H), 4.43 (s, 2H), 4.39 (q, J=7.2 Hz, 2H), 4.18 (t, J=5.2 Hz, 2H), 3.27 (t, J=7.6 Hz, 2H), 2.72 (s, 3H), 2.07-1.97 (m, 4H), 1.41 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.55, 155.71, 155.47, 147.34, 146.90, 142.24, 139.38, 129.65, 128.72, 125.99, 125.53, 118.21, 104.24, 103.89, 67.00, 60.66, 47.56, 47.41, 25.91, 23.00, 13.21, 10.39. Calculated exact mass for the protonated molecule (C$_{22}$H$_{27}$N$_2$O$_4$): 383.1971; measured accurate mass (ESI): 383.1956. LC-MS purity: 100%, $t_R$ = 12.07 min.

Ethyl 4-(3-aminopropoxy)-3-methylbenzofuran-2-carboxylate (32)$^{[82]}$

10% Palladium on carbon (6 mg, 0.005 mmol) and 1,4-cyclohexadiene (50 µL, 0.54 mmol) were added to a solution of compound 16 (10 mg, 0.027 mmol) in ethanol (1 mL). The resulting mixture was stirred at 80 °C for 4 hours. After that, the reaction mixture was filtered off and the filtration was concentrated in vacuo, which was purified by semi-preparative reverse phase HPLC to give the title compound as a white solid (7.1 mg, 67% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.39 (t, J=8.4 Hz, 1H), 7.13 (d, J=8.4 Hz, 1H), 6.79 (d, J=8.0 Hz, 1H), 4.41 (q, J=7.2 Hz, 2H), 4.26 (t, J=6.0 Hz, 2H), 3.23 (t, J=7.6 Hz, 2H), 2.75 (s, 3H), 2.30-2.23 (m, 2H), 1.42 (s, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.50, 155.70, 155.15, 139.50, 128.71, 125.78, 118.23, 104.57, 103.99, 64.70, 60.69, 36.90, 27.00, 13.22, 10.33. Calculated exact mass for the protonated molecule (C$_{15}$H$_{20}$NO$_4$): 278.1392; measured accurate mass (ESI): 278.1400. LC-MS purity: 97%, $t_R$ = 11.48 min.
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Ethyl 3-methyl-4-(3-phenoxypropoxy)benzofuran-2-carboxylate (33)

Prepared from compound 3 (10 mg, 0.045 mmol), benzyl 3-bromopropyl ether (16 µL, 0.091 mmol) and potassium carbonate (39 mg, 0.27 mmol) in DMF (1 mL), according to the preparation of compound 2, the title compound was obtained as colourless oil (3.0 mg, 19% yield). ¹H-NMR (CD₃OD, 400 MHz): δ 7.36 (t, J=8.0 Hz, 1H), 7.31-7.29 (m, 2H), 7.25-7.18 (m, 2H), 7.07 (d, J=8.0 Hz, 1H), 6.73 (d, J=8.0 Hz, 1H), 4.54 (s, 2H), 4.40 (q, J=7.2 Hz, 2H), 4.20 (t, J=6.0 Hz, 2H), 3.72 (t, J=6.0 Hz, 2H), 2.58 (s, 3H), 2.18-2.12 (m, 2H), 1.42 (t, J=7.2 Hz, 3H).

¹³C-NMR (CD₃OD, 100 MHz): δ 160.57, 155.71, 139.24, 138.30, 128.69, 127.91, 127.41, 127.19, 126.23, 118.25, 103.98, 103.78, 72.49, 65.89, 64.65, 60.59, 29.22, 13.24, 10.23. Calculated exact mass for the protonated molecule (C₂₂H₂₅O₅): 369.1702; measured accurate mass (ESI): 369.1714. LC-MS purity: 100%, tᵣ = 23.99 min (gradient: from 2% to 98% B over the first 20 min, maintaining for 2 min, and down to 2% B over 1 min, followed by 4 min at 2% B; flow rate: 1.2 mL/min).

N-benzyl-3-(2-(ethoxycarbonyl)-3-methylbenzofuran-4-yloxy)-N,N-dimethylpropan-1-aminium bromide (34)

A solution of compound 2 (10 mg, 0.029 mmol) and N,N-dimethyl benzylamine (4.4 µL, 0.029 mmol) in acetonitrile (1 mL) was stirred at 85 °C for 16 hours. The reaction mixture was then cooled and a white solid precipitated. The solid was washed by ether and dried under pressure, to give the title compound as a white solid (12.4 mg, 90% yield). ¹H-NMR (CD₃OD, 400 MHz): δ 7.63-7.51 (m, 5H), 7.40 (d, J=8.4 Hz, 1H), 7.13 (d, J=8.4 Hz, 1H), 6.80 (d, J=8.0 Hz, 1H), 4.67 (s, 2H), 4.40 (q, J=7.2 Hz, 2H), 4.30 (t, J=5.6 Hz, 2H), 3.65-3.61 (m, 2H), 3.17 (s, 6H), 2.60 (s, 3H), 2.57-2.46 (m, 2H), 1.42 (t, J=7.2 Hz, 3H). ¹³C-NMR (CD₃OD, 400 MHz): δ 160.46, 155.69, 154.92, 139.51, 132.79, 130.63, 128.73, 127.39, 125.74, 118.21, 104.69, 104.07, 68.20, 64.76, 61.74, 60.70, 49.18, 22.55, 13.24, 10.46. Calculated exact mass for the protonated molecule (C₂₄H₃₀NO₄): 396.2175; measured accurate mass (ESI): 396.2169. LC-MS purity: 100%, tᵣ = 11.98 min.
Ethyl 5-(3-(tert-butylamino)propoxy)-3-methylbenzofuran-2-carboxylate (35)

Prepared from compound 42 (10 mg, 0.029 mmol) and tert-butylamine (12.3 µL, 0.12 mmol) in DMF (1 mL), according to the preparation for compound 1, the title compound was obtained as a white solid (7.0 mg, 54% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.46 (d, J=9.2 Hz, 1H), 7.21 (d, J=1.8 Hz, 1H), 7.14 (dd, J=9.2, 1.8 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 4.21 (t, J=6.0 Hz, 2H), 3.25 (t, J=7.6 Hz, 2H), 2.57 (s, 3H), 2.24-2.17 (m, 2H), 1.43 (t, J=7.2 Hz, 3H), 1.42 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.46, 155.23, 149.60, 141.50, 129.37, 125.28, 117.72, 112.25, 103.07, 65.22, 60.79, 56.72, 38.87, 26.52, 24.39, 13.23, 8.07. Calculated exact mass for the protonated molecule (C$_{19}$H$_{28}$NO$_4$): 334.2018; measured accurate mass (ESI): 334.2024. LC-MS purity: 100%, $t_R = 11.81$ min.

Ethyl 5-(3-(benzylamino)propoxy)-3-methylbenzofuran-2-carboxylate (36)

Prepared from compound 42 (10 mg, 0.029 mmol) and benzylamine (12.8 µL, 0.12 mmol) in DMF (1 mL), according to the preparation for compound 1, the title compound was obtained as a white solid (5.6 mg, 40% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.54-7.48 (m, 5H), 7.46 (d, J=9.2 Hz, 1H), 7.19 (d, J=1.8 Hz, 1H), 7.11 (dd, J=8.8, 1.8 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 4.28 (s, 2H), 4.20 (t, J=5.6 Hz, 2H), 3.33 (m, 2H), 2.57 (s, 3H), 2.29-2.22 (m, 2H), 1.43 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.45, 155.18, 149.60, 141.51, 131.16, 129.52, 128.96, 125.26, 117.68, 112.24, 104.96, 103.10, 65.42, 60.78, 51.07, 44.97, 25.77, 13.22, 8.06. Calculated exact mass for the protonated molecule (C$_{22}$H$_{26}$NO$_4$): 368.1862; measured accurate mass (ESI): 368.1862. LC-MS purity: 100%, $t_R = 12.22$ min.

1-[5-(Benzyloxy)-2-hydroxyphenyl]ethan-1-one (38)

A solution of 2,5-dihydroxyacetophenone (2 g, 13.1 mmol) in 30 mL of acetone was added potassium carbonate (5.4 g, 39.4 mmol) and benzyl bromide (1.64 mL, 13.8 mmol). The reaction mixture was stirred at 60 °C for 3 hrs. The solid was
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filtered off and the filtrate was concentrated in vacuo, to give the title compound without further purification, as yellow oil (3.2 g, quantitative yield). $^1\text{H-NMR (CDCl}_3, 400 \text{ MHz)}: \delta 11.88 (s, 1\text{H}), 7.48-7.35 (m, 5\text{H}), 7.28 (d, J=3.2 \text{ Hz, 1\text{H}}), 7.21 (dd, J=8.8, 3.2 \text{ Hz, 1\text{H}}), 6.95 (d, J=8.8 \text{ Hz, 1\text{H}}), 5.07 (s, 2\text{H}), 2.61 (s, 3\text{H})$.

$\text{BnO-} \begin{array}{c} \text{COCH}_3 \\ \text{CH}_2\text{COOEt} \end{array} \quad \text{Ethyl 2-[2-acetyl-4-(benzyl)phenox]-acetate (39)}$

A solution of compound 38 (3.2 g, 13.1 mmol) in 5 mL of DMF was added potassium carbonate (9.0 g, 65.5 mmol) and ethyl bromoacetate (2.9 mL, 26.2 mmol). The reaction mixture was stirred at 80 °C for 4 hours. The solid was filtered off and the filtrate was concentrated. The residue was purified by column chromatography on silica gel, eluting with hexane:ethyl acetate, 91:9, 84:16, to obtain the title compound as a off-white solid (2.37 g, 55% yield). $^1\text{H-NMR (CDCl}_3, 400 \text{ MHz)}: \delta 7.48-7.32 (m, 6\text{H}), 7.08 (dd, J=8.8, 3.2 \text{ Hz, 1\text{H}}), 6.81 (d, J=8.8 \text{ Hz, 1\text{H}}), 5.06 (s, 2\text{H}), 4.69 (s, 2\text{H}), 4.29 (q, J=7.2 \text{ Hz, 2\text{H}}), 2.74 (s, 3\text{H}), 1.32 (t, J=7.2 \text{ Hz, 3\text{H}})$.

$\text{BnO-} \begin{array}{c} \text{COOEt} \\ \text{O} \end{array} \quad \text{Ethyl 5-(benzyl)oxy-3-methyl-1-benzofuran-2-carboxylate (40)}$

Prepared from compound 39 (1.25 g, 3.9 mmol) and sodium (90 mg, 3.9 mmol) in ethanol (10 mL) according to the preparation of compound 3, the title compound was obtained as a pale-yellow solid (0.53 g, 44 % yield). $^1\text{H-NMR (CDCl}_3, 400 \text{ MHz)}: \delta 7.50-7.35 (m, 6\text{H}), 7.17 (dd, J=8.8, 2.4 \text{ Hz, 1\text{H}}), 7.12 (d, J=2.4 \text{ Hz, 1\text{H}}), 5.14 (s, 2\text{H}), 4.47 (q, J=7.2 \text{ Hz, 2\text{H}}), 2.58 (s, 3\text{H}), 1.47 (t, J=7.2 \text{ Hz, 3\text{H}})$.

$\text{HO} \begin{array}{c} \text{COOEt} \\ \text{O} \end{array} \quad \text{Ethyl 5-hydroxy-3-methyl-1-benzofuran-2-carboxylate (41)}$

1,4-Cyclohexadiene (0.33 mL) and 10% palladium on carbon (20 mg) was added to a solution of compound 40 (110 mg, 0.36 mmol) in ethanol (5 mL). The resulting mixture was stirred at 80 °C for 4 hours. After that, the reaction mixture was filtered off and the filtration was concentrated in vacuo, to give the title compound without further purification as an off-white solid (78 mg, quantitative yield). $^1\text{H-NMR (CD}_2\text{COCD}_3, 400 \text{ MHz)}: \delta 8.40 (brs, 1\text{H}), 7.41 (d, J=8.8 \text{ Hz, 1\text{H}}), 7.08 (dd, J=8.8, 2.4 \text{ Hz, 1\text{H}})$,
7.05 (d, J=2.4 Hz, 1H), 4.39 (q, J=7.2 Hz, 2H), 2.52 (s, 3H), 1.40 (t, J=7.2 Hz 3H).

**Ethyl 5-(3-bromopropoxy)-3-methyl-1-benzofuran-2-carboxylate (42)**

Prepared from compound 41 (78 mg, 0.36 mmol), dibromopropane (112 µL, 1.1 mmol) and potassium carbonate (276 mg, 2.2 mmol) in DMF (1 mL) according to the preparation of compound 2, the title compound was obtained as colourless oil (58 mg, 48% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 7.46 (d, J=8.8 Hz, 1H), 7.08 (dd, J=8.8, 2.4 Hz, 1H), 7.05 (d, J=2.4 Hz, 1H), 4.47 (q, J=7.2 Hz, 2H), 4.18 (t, J=6.0 Hz, 2H), 3.66 (t, J=6.4 Hz, 2H), 2.58 (s, 3H), 2.41-2.34 (m, 2H), 1.46 (t, J=7.2 Hz, 3H).

**Ethyl 8-hydroxyimidazo[1,2-a]pyridine-2-carboxylate (45)**

A suspension of 2-aminopyridin-3-ol (600 mg, 5.45 mmol) in anhydrous THF (12 mL) was treated with ethyl bromopyruvate (681 µL, 5.45 mmol) at room temperature and then heated at reflux temperature for 40 hours. The resulting mixture was evaporated in vacuo to give the residue which was dissolved in DCM (30 mL), followed by partitioning with 5% NaHCO$_3$ solution. The aqueous phase was further extracted with DCM (2 x 20 mL). The combined organic layers were dried over anhydrous sodium sulphate and evaporated in vacuo. The residue was purified by column chromatography over silica gel, eluting with hexane:ethyl acetate 30:70, 0:100 to give the title compound as a yellow solid (180 mg, 15% yield). $^1$H-NMR (CD$_3$SOCD$_3$, 400 MHz): $\delta$ 10.63 (s, 1H, -OH), 8.53 (s, 1H), 8.06 (d, J=7.0 Hz, 1H), 6.81 (t, J=7.0 Hz, 1H), 6.55 (d, J=7.0 Hz, 1H), 4.31 (q, J=7.2 Hz, 2H), 1.32 (t, J=7.2 Hz, 1H).

**Ethyl 8-(3-bromopropoxy)imidazo[1,2-a]pyridine-2-carboxylate (47)**

Prepared from compound 45 (93 mg, 0.45 mmol), 1, 3-dibromopropane (91 µL, 0.90 mmol) and potassium carbonate (370 mg, 2.7 mmol) in DMF (2 mL) according to the preparation of compound 2, the title compound was obtained as an off-white solid (36 mg, 24% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 8.20 (s,
Ethyl 8-(3-({tert-butylamino}propoxy)imidazo[1,2-a]pyridine-2-carboxylate (48)

Prepared from compound 47 (10 mg, 0.03 mmol) and tert-butylamine (13 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as yellow oil (11.6 mg, 90% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 8.57 (s, 1H), 8.23 (d, J=7.2 Hz, 1H), 7.12 (t, J=7.2 Hz, 1H), 7.03 (d, J=7.2 Hz, 1H), 4.47 (q, J=7.2 Hz, 2H), 4.41 (t, J=5.6 Hz, 2H), 3.37 (t, J=7.2 Hz, 2H), 2.36-2.29 (m, 2H), 1.44 (s, 9H), 1.44 (t, J=7.2 Hz, 3H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 161.12, 146.72, 138.61, 132.49, 120.43, 118.89, 115.30, 106.27, 66.78, 61.22, 56.85, 39.24, 26.01, 24.39, 13.18. Calculated exact mass for the protonated molecule (C\(_{17}\)H\(_{26}\)N\(_3\)O\(_3\)):

320.1974; measured accurate mass (ESI): 320.1990. LC-MS purity: 100%, \(t_\text{R}\) = 9.87 min.

Ethyl 8-((3-((benzylamino)propoxy)imidazo[1,2-a]pyridine-2-carboxylate (49)

Prepared from compound 47 (10 mg, 0.03 mmol) and benzylamine (13 µL, 0.12 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as pale yellow oil (8.2 mg, 59% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 8.50 (s, 1H), 8.17 (d, J=7.2 Hz, 1H), 7.54-7.51 (m, 2H), 7.46-7.44 (m, 3H), 7.01 (t, J=7.2 Hz, 1H), 6.91 (d, J=7.2 Hz, 1H), 4.46 (q, J=7.2 Hz, 2H), 4.37 (t, J=5.6 Hz, 2H), 4.34 (s, 2H), 3.44 (t, J=7.0 Hz, 2H), 2.38-2.32 (m, 2H), 1.44 (t, J=7.2 Hz, 3H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 161.97, 147.07, 139.40, 133.49, 131.15, 129.60, 129.23, 128.85, 120.50, 118.70, 114.67, 106.16, 66.76, 61.06, 51.21, 44.75, 25.44, 13.23. Calculated exact mass for the protonated molecule (C\(_{20}\)H\(_{24}\)N\(_3\)O\(_3\)):

354.1818; measured accurate mass (ESI): 354.1817. LC-MS purity: 97%, \(t_\text{R}\) = 10.78 min.
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4-(3-Bromopropoxy)-3-methylbenzofuran (50)

Prepared from compound 7 (75 mg, 0.51 mmol), 1,3-dibromopropane (0.27 mL, 2.6 mmol) and potassium carbonate (0.69 g, 5.0 mmol) in DMF (2 mL) according to the preparation of compound 2, the title compound was obtained as colourless oil (35 mg, 30% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 7.29 (d, J=1.2 Hz, 1H), 7.19 (t, J=8.0 Hz, 1H), 7.09 (d, J=8.0 Hz, 1H), 6.65 (d, J=8.0 Hz, 1H), 4.24 (t, J=6.0 Hz, 2H), 3.68 (t, J=6.4 Hz, 2H), 2.45-2.40 (m, 2H), 2.38 (d, J=1.2 Hz, 3H).

N-tert-butyl-3-(3-methylbenzofuran-4-yloxy)propan-1-amine (51)

Prepared from compound 50 (10 mg, 0.037 mmol) and t-butylamine (15.8 µL, 0.15 mmol) in DMF (1 mL), according to the preparation for compound 1, the title compound was obtained as a white solid (7.6 mg, 55% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.38 (d, J=0.8 Hz, 1H), 7.18 (t, J=8.0 Hz, 1H), 7.05 (d, J=8.0 Hz, 1H), 6.72 (d, J=8.0 Hz, 1H), 4.24 (t, J=6.0 Hz, 2H), 3.27 (t, J=8.0 Hz, 2H), 2.28-2.21 (m, 2H), 1.42 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.95, 157.71, 144.16, 128.65, 121.89, 119.11, 108.40, 107.31, 60.63, 42.62, 30.48, 28.34, 12.89. Calculated exact mass for the protonated molecule (C$_{16}$H$_{24}$NO$_2$): 262.1807; measured accurate mass (ESI): 262.1797. LC-MS purity: 90%, $t_R$ = 11.93 min.

{3-((3-Methyl-1-benzofuran-4-yl)oxy)propyl}(pyridin-3-ylmethyl)amine (52)

Prepared from compound 50 (10 mg, 0.037 mmol) and 3-(aminomethyl)-pyridine (15.3 µL, 0.15 mmol) in DMF (1 mL), according to the preparation for compound 1, the title compound was obtained as pale yellow oil (5.6 mg, 37% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.81 (d, J=1.2 Hz, 1H), 8.74 (dd, J=4.8, 1.2 Hz, 1H), 8.20 (dt, J=8.0, 1.2 Hz, 1H), 7.71 (dd, J=8.0, 4.8 Hz, 1H), 7.38 (d, J=1.2 Hz, 1H), 7.18 (t, J=8.0 Hz, 1H), 7.05 (d, J=8.0 Hz, 1H), 6.71 (d, J=8.0 Hz, 1H), 4.43 (s, 2H), 4.24 (t, J=5.6 Hz, 2H), 3.40 (t, J=7.6 Hz, 2H), 2.35-2.28 (m, 2H), 2.33 (d, J=1.2 Hz, 3H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 157.00, 153.17, 148.68, 148.41, 140.43, 140.18, 128.76, 124.96, 124.68,
117.95, 115.52, 104.50, 103.41, 64.35, 46.94, 45.11, 25.89, 8.93. Calculated exact mass for the protonated molecule \((C_{18}H_{21}N_{2}O_{2})\): 297.1603; measured accurate mass (ESI): 297.1589. LC-MS purity: 100%, \(t_R = 11.36\) min.

\(\text{N-benzyl-3-(3-methylbenzofuran-4-yloxy) propan-1-amine (53)}\)

Prepared from compound 50 (10 mg, 0.037 mmol) and benzylamine (16.4 µL, 0.15 mmol) in DMF (1 mL), according to the preparation for compound 1, the title compound was obtained as a white solid (6.6 mg, 44% yield). \(^1\)H-NMR (CD$_3$OD, 400 MHz): 6 7.54-7.48 (m, 5H), 7.37 (d, \(J=1.2\) Hz, 1H), 7.18 (t, \(J=8.0\) Hz, 1H), 7.05 (d, \(J=8.0\) Hz, 1H), 6.71 (d, \(J=8.0\) Hz, 1H), 4.29 (s, 2H), 4.23 (t, \(J=5.6\) Hz, 2H), 3.32 (t, \(J=6.0\) Hz, 2H), 2.32-2.26 (m, 2H), 2.28 (d, \(J=1.2\) Hz, 3H). \(^{13}\)C-NMR (CD$_3$OD, 100 MHz): 6 156.96, 153.62, 140.15, 131.00, 129.57, 129.39, 128.98, 124.67, 117.94, 115.23, 104.46, 103.36, 64.60, 51.00, 44.62, 25.81, 8.93. Calculated exact mass for the protonated molecule \((C_{19}H_{22}NO_2)\): 296.1650; measured accurate mass (ESI): 296.1643. LC-MS purity: 100%, \(t_R = 12.21\) min.

\(\text{4-(3-bromopropoxy)-3-methyl-1-benzofuran-2-carboxylic acid (55)}^{[80]}\)

LiOH·H$_2$O (1.49 g, 35.4 mmol) was added to a solution of compound 2 (2 g, 5.9 mmol) in THF/MeOH (40 mL, 1:1). The reaction mixture was stirred for 24 hours at room temperature. The volatile components were then evaporated in vacuo. The residue was dissolved in H$_2$O (30 mL), followed by the addition of 3 mL concentrated HCl while stirring. Precipitation occurred in the resulting solution and title compound was collected as a white solid (1.66 g, 90% yield). \(^1\)H-NMR (CD$_3$OD, 400 MHz): 6 7.35 (t, \(J=8.0\) Hz, 1H), 7.17 (d, \(J=8.0\) Hz, 1H), 6.68 (d, \(J=8.0\) Hz, 1H), 4.26 (t, \(J=5.6\) Hz, 2H), 3.68 (t, \(J=6.4\) Hz, 2H), 2.76 (s, 3H), 2.47-2.41 (m, 2H).
3-Methyl-4-(3-(4-methylbenzylamino)propoxy)-N-phenylbenzo-furan-2-carboxamide (60)

Prepared from a solution of compound 55 (15 mg, 0.05 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (9.2 µL, 0.055 mmol), PyBOP (28 mg, 0.055 mmol) and aniline (5 µL, 0.055 mmol), the brominated precursor was obtained. After that, amination with 4-methyl
benzylamine (25.3 µL, 0.2 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (5.0 mg, 19% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.72 (d, J=8.4 Hz, 2H), 7.42-7.37 (m, 5H), 7.29 (d, J=8.4 Hz, 2H), 7.21 (d, J=8.4 Hz, 1H), 7.18 (t, J=8.0 Hz, 1H), 6.80 (d, J=8.0 Hz, 1H), 4.26 (t, J=5.6 Hz, 2H), 4.24 (s, 2H), 3.30 (t, J=8.0 Hz, 2H), 2.68 (s, 3H), 2.38 (s, 3H), 2.33-2.26 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.44, 156.28, 142.74, 141.20, 139.13, 130.99, 129.84, 129.56, 129.21, 125.75, 124.94, 122.40, 120.19, 106.07, 105.60, 66.06, 52.12, 45.62, 27.09, 21.24, 11.54. Calculated exact mass for the protonated molecule (C$_{27}$H$_{29}$N$_2$O$_3$): 429.2178; measured accurate mass (ESI): 429.2169. LC-MS purity: 100%, $t_R$ = 12.73 min.

\[ \text{N-benzyl-4-(3-(tert-butylamino)propanoxy)-3-methylbenzofuran-2-carboxamide (61)} \]

Prepared from a solution of compound 55 (30 mg, 0.1 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (18.4 µL, 0.11 mmol), PyBOP (56 mg, 0.11 mmol) and benzylamine (12.0 µL, 0.11 mmol), the brominated precursor was obtained. After that, the amination with t-butyamine (21 µL, 0.2 mmol) in DMF (1 mL), was carried out according to preparation of compound 59, to afford the title compound as a white solid (17.3 mg, 44% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.39-7.32 (m, 5H), 7.26 (t, J=7.2 Hz, 1H), 7.13 (d, J=8.4 Hz, 1H), 6.78 (d, J=8.0 Hz, 1H), 4.58 (s, 2H), 4.26 (t, J=6.0 Hz, 2H), 3.27 (t, J=8.0 Hz, 2H), 2.77 (s, 3H), 2.29-2.22 (m, 2H), 1.42 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 162.33, 156.31, 156.27, 142.85, 140.17, 129.54, 129.35, 128.50, 128.20, 123.81, 120.06, 105.93, 105.48, 65.94, 58.12, 43.53, 40.04, 27.85, 25.81, 11.45. Calculated exact mass for the protonated molecule (C$_{24}$H$_{31}$N$_2$O$_3$): 395.2335; measured accurate mass (ESI): 395.2319. LC-MS purity: 100%, $t_R$ = 11.98 min.
4-(3-(t-Butylamino)propoxy)-N-cyclohexyl-3-methylbenzofuran-2-carboxamide (62)

Prepared from a solution of compound 55 (15 mg, 0.05 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (9.2 µL, 0.055 mmol), PyBOP (28 mg, 0.055 mmol) and cyclohexylamine (6.3 µL, 0.055 mmol), the brominated precursor was obtained. After that, the amination with tert-butylamine (21 µL, 0.2 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (6.9 mg, 28% overall yield). 1H-NMR (CD$_3$OD, 400 MHz): δ 7.37 (t, J=8.0 Hz, 1H), 7.16 (d, J=8.4 Hz, 1H), 6.81 (d, J=8.0 Hz, 1H), 4.28 (t, J=6.0 Hz, 2H), 3.95-3.84 (m, 1H), 3.29 (t, J=8.0 Hz, 2H), 2.77 (s, 3H), 2.30-2.23 (m, 2H), 2.00-1.90 (m, 2H), 1.90-1.80 (m, 2H), 1.76-1.67 (m, 1H), 1.48-1.38 (m, 4H), 1.43 (s, 9H), 1.35-1.22 (m, 1H). 13C-NMR (CD$_3$OD, 100 MHz): δ 161.48, 156.28, 156.18, 142.99, 129.21, 123.39, 120.10, 105.95, 105.47, 65.92, 58.15, 49.71, 40.06, 33.76, 27.88, 26.56, 26.44, 25.81, 11.44. Calculated exact mass for the protonated molecule (C$_{23}$H$_{35}$N$_2$O$_3$): 387.2648; measured accurate mass (ESI): 387.2659. LC-MS purity: 100%, t$_R$ = 12.50 min.

N-t-butyl-4-(3-(t-Butylamino)propoxy)-3-methylbenzofuran-2-carboxamide (63)

Prepared from a solution of compound 55 (10 mg, 0.03 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (6.1 µL, 0.037 mmol), PyBOP (19 mg, 0.037 mmol), the brominated precursor was obtained. After that, the amination with t-butyl amine (3.4 µL, 0.037 mmol) in DMF (1 mL) was carried out according to the method of compound 59, to afford the title compound as a white solid (5 mg, 35% overall yield). 1H-NMR (CD$_3$OD, 400 MHz): δ 7.37 (t, J=8.4 Hz, 1H), 7.16 (d, J=8.4 Hz, 1H), 6.81 (d, J=8.0 Hz, 1H), 4.28 (t, J=6.0 Hz, 2H), 3.29 (t, J=8.0 Hz, 2H), 2.75 (s, 3H), 2.31-2.20 (m, 2H), 1.50 (s, 9H), 1.43 (s, 9H). 13C-NMR (CD$_3$OD, 100 MHz): δ 161.83, 156.23, 155.95, 143.36, 129.12, 122.84, 120.11, 105.98, 105.48, 65.90, 58.18, 52.64, 40.07, 29.10, 27.90, 25.81, 11.42. Calculated exact mass for the protonated molecule (C$_{21}$H$_{33}$N$_2$O$_3$): 361.2491; measured accurate mass (ESI): 361.2493. LC-MS purity:
100%, \( t_R = 12.15 \) min.

\[
(4\text{-benzylpiperazin-1-yl})(4\text{-}(t\text{-butylamino)propoxy})\text{-3-methylbenzofuran-2-yl \ methanone (64)}
\]

Prepared from a solution of compound 55 (10 mg, 0.03 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (6.1 \( \mu \)L, 0.037 mmol), PyBOP (19 mg, 0.037 mmol) and 1-benzyl piperazine (6.4 \( \mu \)L, 0.037 mmol), the brominated precursor was obtained. After that, the amination with tert-butylamine (13.6 \( \mu \)L, 0.13 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (2.7 mg, 16%overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \( \delta \) 7.59-7.51 (m, 5H), 7.40 (t, \( J=8.4 \) Hz, 1H), 7.15 (d, \( J=8.4 \) Hz, 1H), 6.84 (d, \( J=8.0 \) Hz, 1H), 4.43 (s, 2H), 4.29 (t, \( J=6.0 \) Hz, 2H), 3.44 (brd, 4H), 3.33 (brd, 4H), 3.28 (t, \( J=8.0 \) Hz, 2H), 2.64 (s, 3H), 2.30-2.24 (m, 2H), 1.43 (s, 9H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \( \delta \) 162.29, 156.37, 156.16, 142.64, 132.37, 131.48, 130.51, 130.05, 129.51, 125.03, 119.40, 105.96, 105.73, 66.04, 61.78, 58.18, 52.69, 40.03, 27.88, 25.81, 11.57. Calculated exact mass for the protonated molecule (C\(_{28}\)H\(_{38}\)N\(_3\)O\(_3\)): 464.2913; measured accurate mass (ESI): 464.2921. LC-MS purity: 100%, \( t_R = 9.52 \) min.

4-(3-(Benzylamino)propoxy)-N-cyclohexyl-3-methylbenzofuran-2-carboxamide (65)

Prepared from a solution of compound 55 (15 mg, 0.05 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (9.2 \( \mu \)L, 0.055 mmol), PyBOP (28 mg, 0.055 mmol) and cyclohexylamine (6.3 \( \mu \)L, 0.055 mmol), the brominated precursor was obtained. After that, the amination with benzyamine (22 \( \mu \)L, 0.2 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (9.4 mg, 35% overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \( \delta \) 7.58-7.48 (m, 5H), 7.36 (t, \( J=8.4 \) Hz, 1H), 7.15 (d, \( J=8.4 \) Hz, 1H), 6.78 (d, \( J=8.0 \) Hz, 1H), 4.30 (s, 2H), 4.26 (t, \( J=6.0 \) Hz, 2H), 3.94-3.84 (m, 1H), 3.29 (t, \( J=8.0 \) Hz, 2H), 2.66 (s, 3H), 2.34-2.27 (m, 2H), 2.00-1.90 (m, 2H), 1.90-1.81 (m, 2H), 1.77-1.66 (m, 1H), 1.49-1.38 (m,
2-Benzoyl-3-methyl-1-benzofuran-4-ol (70)

A mixture of 2, 6-dihydroxy acetophenone (50 mg, 0.33 mmol), 2-bromo-1-phenylethan-1-one (69 mg, 0.34 mmol) and potassium carbonate (136 mg, 1.0 mmol) in acetonitrile (3 mL) was stirred at reflux temperature for 24 hours. The reaction mixture was filtered through a celite pad and the filtrate was concentrated in vacuo. The residue was purified by column chromatography over silica gel, eluting with hexane:ethyl acetate, 91:9, 84:16, to afford the title compound as a light yellow solid (34 mg, 41% yield). \(^1\)H-NMR (CD\(_3\)COCD\(_3\), 400 MHz): \(\delta\) 9.31 (brs, 1H), 8.07 (d, \(J=7.2\) Hz, 2H), 7.68 (t, \(J=7.2\) Hz, 1H), 7.61 (t, \(J=7.2\) Hz, 2H), 7.33 (t, \(J=8.0\) Hz, 1H), 7.06 (d, \(J=8.0\) Hz, 1H), 6.75 (d, \(J=8.0\) Hz, 1H), 2.81 (s, 3H).

1-(4-Hydroxy-3-methyl-1-benzofuran-2-yl)ethan-1-one (71)

Prepared from 2, 6-dihydroxy acetophone (2 g, 13.1 mmol), 1-chloropropan-2-one (1.05 mL, 13.1 mmol) and potassium carbonate (5.4 g, 39.4 mmol) in acetonitrile (30 mL) according to the preparation of compound 70, the title compound was obtained as an off-white solid (420 mg, 17% yield). \(^1\)H-NMR (CD\(_3\)COCD\(_3\), 400 MHz): \(\delta\) 9.27 (brs, 1H), 7.32 (t, \(J=8.0\) Hz, 1H), 7.05 (d, \(J=8.0\) Hz, 1H), 6.72 (d, \(J=8.0\) Hz, 1H), 2.76 (s, 3H), 2.53 (s, 3H).

(4-((3-(t-Butylamino)propoxy)-3-methylbenzofuran-2-yl)-(phenyl)-methanone (72)

Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL) according to the preparation of compound 2, the brominated precursor
was obtained. After that, the amination with t-butylamine (14 µL, 0.12 mmol) in DMF (1 mL) was carried out according to the preparation of compound 1, to afford the title compound as a white solid (5.3 mg, 24% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.01 (d, J=7.2 Hz, 2H), 7.66 (t, J=7.2 Hz, 1H), 7.56 (t, J=7.2 Hz, 2H), 7.46 (t, J=8.4 Hz, 1H), 7.18 (d, J=8.4 Hz, 1H), 6.84 (d, J=8.0 Hz, 1H), 4.32 (t, J=6.0 Hz, 2H), 3.30 (t, J=8.0 Hz, 2H), 2.82 (s, 3H), 2.32-2.26 (m, 2H), 1.43 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 185.98, 155.68, 155.47, 137.86, 132.40, 129.39, 129.18, 128.04, 127.27, 118.49, 104.84, 104.15, 64.69, 56.78, 38.65, 26.46, 24.41, 10.97. Calculated exact mass for the protonated molecule (C$_{23}$H$_{28}$NO$_3$): 366.2069; measured accurate mass (ESI): 366.2076. LC-MS purity: 100%, $t_R = 12.70$ min.

Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with benzylamine (14 µL, 0.12 mmol) in DMF (1 mL) was carried out according to the method of compound 72, to afford the title compound as an off-white solid (5.9 mg, 25% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.01 (d, J=7.2 Hz, 2H), 7.66 (t, J=7.2 Hz, 1H), 7.58-7.43 (m, 8H), 7.17 (d, J=8.4 Hz, 1H), 6.82 (d, J=8.0 Hz, 1H), 4.30 (s, 2H), 4.29 (t, J=6.0 Hz, 2H), 3.36-3.33 (m, 2H), 2.70 (s, 3H), 2.36-2.29 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 185.98, 155.68, 155.42, 146.98, 137.90, 132.40, 130.98, 129.56, 129.44, 129.38, 129.16, 129.00, 128.05, 127.37, 118.47, 104.82, 104.15, 64.79, 51.01, 44.50, 25.70, 10.99. Calculated exact mass for the protonated molecule (C$_{26}$H$_{26}$NO$_3$): 400.1913; measured accurate mass (ESI): 400.1922. LC-MS purity: 100%, $t_R = 12.90$ min.
Prepared from a solution of compound 71 (18 mg, 0.09 mmol), 1,3-dibromopropane (18.8 µL, 0.18 mmol) and potassium carbonate (75 mg, 0.54 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with t-butylamine (25 µL, 0.24 mmol) in DMF (1 mL) was carried out according to the preparation of compound 72, to afford the title compound as a white solid (8.7 mg, 32% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.44 (t, J=8.4 Hz, 1H), 7.16 (d, J=8.4 Hz, 1H), 6.81 (d, J=8.0 Hz, 1H), 4.28 (t, J=6.0 Hz, 2H), 3.28 (t, J=8.0 Hz, 2H), 2.77 (s, 3H), 2.57 (s, 3H), 2.31-2.24 (m, 2H), 1.42 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 191.40, 155.55, 155.39, 146.94, 129.29, 124.64, 118.61, 104.76, 104.00, 64.63, 56.74, 47.42, 38.62, 26.42, 24.40, 10.42. Calculated exact mass for the protonated molecule (C$_{18}$H$_{26}$NO$_3$): 304.1913; measured accurate mass (ESI): 304.1922. LC-MS purity: 100%, $t_R = 11.40$ min.

Prepared from a solution of compound 71 (18 mg, 0.09 mmol), 1,3-dibromopropane (18.8 µL, 0.18 mmol) and potassium carbonate (75 mg, 0.54 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with benzylamine (28 µL, 0.24 mmol) in DMF (1 mL) was carried out according to the preparation of compound 72, to afford the title compound as a white solid (14.3 mg, 47% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.55-7.46 (m, 5H), 7.43 (t, J=8.4 Hz, 1H), 7.14 (d, J=8.0 Hz, 1H), 6.78 (d, J=8.0 Hz, 1H), 4.30 (s, 2H), 4.26 (t, J=6.0 Hz, 2H), 3.35-3.31 (m, 2H), 2.66 (s, 3H), 2.57 (s, 3H), 2.37-2.26 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 192.77, 156.89, 156.77, 148.28, 132.41, 130.98, 130.79, 130.66, 130.37, 126.17, 119.99, 106.14, 105.40, 66.15, 52.28, 45.87, 27.82, 27.08, 11.83. Calculated exact mass for the protonated molecule (C$_{21}$H$_{24}$NO$_3$): 338.1756; measured accurate mass (ESI): 338.1749. LC-MS purity: 100%, $t_R = 11.81$ min.
[4-(3-bromopropoxy)-3-methyl-1-benzofuran-2-yl]methanol (78)

Prepared from compound 79 (550 mg, 3.1 mmol), 1,3-dibromopropane (0.63 mL, 6.2 mmol) and potassium carbonate (2.56 g, 18.6 mmol) in DMF (2 mL) according to the preparation of compound 2, the title compound was obtained as colourless oil (0.42 g, 46% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 7.22 (t, J=8.0 Hz, 1H), 7.11 (d, J=8.0 Hz, 1H), 6.66 (d, J=8.0 Hz, 1H), 5.12 (s, 2H), 4.24 (t, J=6.0 Hz, 2H), 3.67 (t, J=6.4 Hz, 2H), 2.45 (s, 3H), 2.44-2.39 (m, 2H).

OH

1-(4-Hydroxy-3-methyl-1-benzofuran-2-yl)ethan-1-one (79)

LiAlH$_4$ (0.26 g, 6.8 mmol) was added slowly to a solution of compound 3 (1g, 4.5 mmol) in anhydrous THF (20 mL) at 0 °C. The resulting mixture was gradually warmed up to room temperature for 2 hours. 100 µL of water was then added to the reaction mixture, followed by the filtration through a celite pad. The filtrate was concentrated in vacuo, to give the title compound as a light-yellow solid (0.55g, 68% yield) without further purification. $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 7.24 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.0 Hz, 1H), 6.64 (d, J=8.0 Hz, 1H), 5.15 (s, 2H), 2.45 (s, 3H).

N-t-butyl-3-(3-methyl-2-(phenoxy)methyl)benzofuran-4-yloxy)-propan-1-amine (80)

To a stirred solution of the compound of compound 78 (29 mg, 0.1 mmol), phenol (24 mg, 0.25 mmol) and triphenylphosphine (65 mg, 0.25 mmol) in anhydrous THF (2 mL) was added DIAD (47 µL, 0.25 mmol) at room temperature. The resulting mixture was stirred at the same temperature for 4 hours, and then concentrated in vacuo. The residue was purified by column chromatography over silica gel, eluting with hexane:ethyl acetate, 5:1 to afford the brominated precursor. After that, the amination with t-butylamine (11.5 µL, 0.11 mmol) in DMF (1 mL) was carried out according to the preparation of compound 1, to afford the title compound as a white solid (2.4 mg, 7% overall yield). $^1$H-NMR (CD$_2$OD, 400 MHz): $\delta$ 7.30 (t, J=8.0 Hz, 2H), 7.23 (t, J=8.4 Hz, 1H), 7.08 (d, J=8.4 Hz, 1H), 7.03 (d, J=8.0 Hz, 2H), 6.98 (t, J=7.6 Hz, 1H), 6.75 (d, J=8.0 Hz, 1H), 5.14 (s, 2H), 4.26 (t, J=6.0 Hz, 2H), 3.27 (t, J=8.0 Hz, 2H), 2.45 (s, 3H), 2.45 (s, 3H).
2.28-2.21 (m, 2H), 1.42 (s, 9H). 13C-NMR (CD3OD, 100 MHz): δ 158.52, 155.82, 153.81, 147.07, 129.11, 125.35, 120.97, 118.45, 114.78, 114.44, 104.32, 103.61, 64.28, 60.48, 56.72, 38.67, 26.52, 24.40, 8.97. Calculated exact mass for the protonated molecule (C23H30NO3): 368.2226; measured accurate mass (ESI): 368.2227. LC-MS purity: 90%, tR = 12.87 min.

3-(3-methyl-2-(phenoxy)methyl)benzofuran-4-yloxy)-N-(4-methyl-benzyl)propan-1-amine (81)

Prepared from compound 78 (50 mg, 0.17 mmol), phenol (40 mg, 0.42 mmol), triphenyl phosphine (110 mg, 0.42 mmol) and DIAD (80 µL, 0.42 mmol) in anhydrous THF (2 mL), the brominated precursor was obtained. After that, the amination with 4-methyl benzylamine (23 µL, 0.18 mmol) in DMF (1 mL) was carried out according to the preparation of compound 80, to afford the title compound as a white solid (8.0 mg, 11% overall yield). 1H-NMR (CD3OD, 400 MHz): δ 7.38 (d, J=8.0 Hz, 2H), 7.33-7.27 (m, 4H), 7.21 (t, J=8.0 Hz, 1H), 7.08-7.02 (m, 3H), 6.98 (t, J=7.6 Hz, 1H), 6.72 (d, J=8.0 Hz, 1H), 5.12 (s, 2H), 4.23 (s, 2H), 4.20 (t, J=6.8 Hz, 2H), 3.28 (t, J=8.0 Hz, 1H), 2.36 (s, 3H), 2.31 (s, 3H), 2.29-2.22 (m, 2H). 13C-NMR (CD3OD, 100 MHz): δ 158.55, 155.80, 153.77, 146.98, 139.71, 129.53, 129.13, 127.86, 125.32, 120.98, 114.77, 114.53, 104.29, 103.62, 64.41, 60.50, 50.73, 44.32, 25.75, 19.81, 19.18, 8.91. Calculated exact mass for the protonated molecule (C27H30NO3): 416.2226; measured accurate mass (ESI): 416.2232. LC-MS purity: decompose under weakly acidic or LC-MS conditions.

N-t-butyl-3-(2-((4-chlorophenoxy)methyl)-3-methylbenzo-furan-4-yloxy)propan-1-amine (82)

Prepared from compound 78 (50 mg, 0.17 mmol), 4-chlorophenol (41 µL, 0.42 mmol), triphenyl phosphine (110 mg, 0.42 mmol) and DIAD (80 µL, 0.42 mmol) in anhydrous THF (2 mL), the brominated precursor was obtained. After that, the amination with t-butylamine (13.8 µL, 0.13 mmol) in DMF (1 mL) was carried out according to the preparation of compound 80, to afford the title compound
as a white solid (4.3 mg, 5% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.29 (d, J=8.8 Hz, 2H), 7.24 (t, J=8.0 Hz, 1H), 7.08 (d, J=8.4 Hz, 1H), 7.03 (d, J=8.8 Hz, 2H), 6.76 (d, J=8.0 Hz, 1H), 5.15 (s, 2H), 4.26 (t, J=6.0 Hz, 2H), 3.28 (t, J=8.0 Hz, 2H), 2.45 (s, 3H), 2.29-2.21 (m, 2H), 1.42 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 158.69, 157.26, 155.26, 148.12, 130.38, 127.28, 126.88, 119.82, 117.82, 116.14, 105.73, 105.10, 65.74, 62.31, 58.17, 40.10, 27.94, 25.83, 10.37. Calculated exact mass for the protonated molecule (C$_{23}$H$_{29}$NO$_3$Cl): 402.1836; measured accurate mass (ESI): 402.1831. LC-MS purity: 96%, $t_R$ = 13.34 min.

N-t-butyl-3-{2-[(3-chlorophenoxy)methyl]-3-methylbenzofuran-4-yl}oxopropan-1-amine (83)

Prepared from compound (50 mg, 0.17 mmol), 3-chlorophenol (44 µL, 0.42 mmol), triphenyl phosphine (110 mg, 0.42 mmol) and DIAD (80 µL, 0.42 mmol) in anhydrous THF (2 mL), the brominated precursor was obtained. After that, the amination with t-butylamine (11.5 µL, 0.11 mmol) in DMF (1 mL) was carried out according to the preparation of compound 80, to afford the title compound as a white solid (1.2 mg, 2% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.26 (t, J=8.4 Hz, 1H), 7.20 (d, J=8.4 Hz, 1H), 7.07-7.05 (m, 2H), 6.98-6.96 (m, 1H), 6.96-6.94 (m, 1H), 6.73 (d, J=8.0 Hz, 1H), 5.14 (s, 2H), 4.23 (t, J=6.0 Hz, 2H), 3.25 (t, J=8.0 Hz, 2H), 2.44 (s, 3H), 2.26-2.18 (m, 2H), 1.39 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.76, 157.27, 155.25, 147.96, 135.91, 131.61, 126.93, 122.38, 119.78, 116.78, 116.50, 116.24, 114.78, 105.75, 105.07, 65.70, 62.11, 58.15, 40.09, 27.95, 25.82, 10.41. Calculated exact mass for the protonated molecule (C$_{23}$H$_{29}$NO$_3$Cl): 402.1836; measured accurate mass (ESI): 402.1842. LC-MS purity: 94%, $t_R$ = 13.40 min.

t-Butyl[3-{(3-methyl-2-[[naphthalen-1-yl]oxy)methyl]-1-benzofuran-4-yl}oxy]propylamine (84)

Prepared from compound 78 (50 mg, 0.17 mmol), 1-naphthol (60.5 mg, 0.42 mmol), triphenyl phosphine (110 mg, 0.42 mmol) and DIAD (80 µL, 0.42 mmol) in anhydrous THF (2 mL), the brominated
precursor was obtained. After that, the amination with t-butylamine (29 µL, 0.27 mmol) in DMF (1 mL) was carried out according to the preparation of compound 80, to afford the title compound as an off-white solid (11.2 mg, 12% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.18 (d, J=8.0 Hz, 1H), 7.81 (d, J=8.0 Hz, 1H), 7.49-7.39 (m, 4H), 7.25 (t, J=8.0 Hz, 1H), 7.11 (d, J=8.0 Hz, 1H), 6.76 (d, J=8.0 Hz, 1H), 5.34 (s, 2H), 4.25 (t, J=6.0 Hz, 2H), 3.25 (t, J=8.0 Hz, 2H), 2.48 (s, 3H), 2.28-2.21 (m, 2H), 1.40 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 157.29, 155.52, 155.26, 148.43, 148.43, 138.51, 128.51, 127.39, 127.39, 127.20, 126.90, 126.80, 126.21, 122.87, 121.94, 119.93, 116.11, 107.11, 105.76, 105.08, 65.73, 62.44, 58.09, 40.07, 27.90, 25.80, 10.45. Calculated exact mass for the protonated molecule (C$_{27}$H$_{32}$NO$_3$): 418.2382; measured accurate mass (ESI): 418.2368. LC-MS purity: 95%, $t_R = 13.64$ min.

t-Butyl[3-((3-methyl-2-[[naphtalen-2-yl]oxy]methyl]-1-benzo-furan-4-yl)oxy]propylamine (85)

Prepared from compound 78 (50 mg, 0.17 mmol), 2-naphthol (60.5 mg, 0.42 mmol), triphenylphosphine (110 mg, 0.42 mmol) and DIAD (80 µL, 0.42 mmol) in anhydrous THF (2 mL), the brominated precursor was obtained. After that, the amination with t-butylamine (29 µL, 0.27 mmol) in DMF (1 mL) was carried out according to the preparation of compound 80, to afford the title compound as a white solid (16.8 mg, 19% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.79-7.76 (m, 3H), 7.46-7.41 (m, 2H), 7.34 (t, J=8.0 Hz, 1H), 7.26-7.17 (m, 2H), 7.09 (d, J=8.0 Hz, 1H), 6.74 (d, J=8.0 Hz, 1H), 5.25 (s, 2H), 4.23 (t, J=6.0 Hz, 2H), 3.24 (t, J=8.0 Hz, 2H), 2.48 (s, 3H), 2.27-2.20 (m, 2H), 1.40 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 157.77, 157.26, 155.26, 148.28, 148.28, 138.51, 136.02, 130.49, 127.39, 127.20, 126.80, 126.83, 124.89, 119.93, 116.09, 108.60, 105.70, 105.07, 65.72, 61.91, 58.08, 40.05, 27.88, 25.80, 10.47. Calculated exact mass for the protonated molecule (C$_{27}$H$_{32}$NO$_3$): 418.2382; measured accurate mass (ESI): 418.2368. LC-MS purity: 95%, $t_R = 13.52$ min.
Prepared from a solution of compound 78 (10 mg, 0.034 mmol) and benzylamine (14.2 µL, 0.13 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (6.6 mg, 44% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 7.38-7.23 (m, 5H), 7.16 (t, J=8.4 Hz, 1H), 7.00 (d, J=8.4 Hz, 1H), 6.68 (d, J=8.0 Hz, 1H), 4.62 (s, 2H), 4.14 (t, J=6.0 Hz, 2H), 3.81 (s, 2H), 2.86 (t, J=7.6 Hz, 2H), 2.30 (s, 3H), 2.12-2.05 (m, 2H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 159.55, 158.26, 154.22, 142.83, 132.12, 132.05, 130.81, 128.78, 122.58, 116.40, 107.66, 107.31, 69.82, 57.79, 56.96, 49.47, 32.66, 12.71. Calculated exact mass for the protonated molecule (C\(_{20}\)H\(_{24}\)NO\(_3\)): 326.1756; measured accurate mass (ESI): 326.1767. LC-MS purity: 100%, \(t_R = 10.71\) min.

Prepared from a solution of compound 78 (10 mg, 0.034 mmol) and \(t\)-butylamine (14 µL, 0.13 mmol) in DMF (1 mL), according to the preparation of compound 1, the title compound was obtained as a white solid (5.0 mg, 36% yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 7.19 (t, J=8.4 Hz, 1H), 7.05 (d, J=8.4 Hz, 1H), 6.73 (d, J=8.0 Hz, 1H), 4.64 (s, 2H), 4.25 (t, J=5.6 Hz, 2H), 3.30-3.26 (m, 2H), 2.43 (s, 3H), 2.28-2.21 (m, 2H), 1.42 (s, 9H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 155.66, 153.77, 150.68, 124.90, 118.66, 112.13, 104.26, 103.48, 64.24, 56.71, 53.83, 38.68, 26.55, 24.40, 8.88. Calculated exact mass for the protonated molecule (C\(_{17}\)H\(_{26}\)NO\(_3\)): 292.1913; measured accurate mass (ESI): 292.1919. LC-MS purity: 95%, \(t_R = 9.97\) min.

Prepared from a solution of compound 86 (20 mg, 0.06 mmol) and benzenethiol (9.4 µL, 0.09 mmol) in anhydrous DCM (1 mL) was added TFA (0.2 mL).
mixture was stirred at room temperature for 2.5 hours. After that, the reaction mixture was concentrated in vacuo and the residue was purified by semi-preparative reverse phase HPLC to give the title compound as a white solid (5.3 mg, 17% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 9.63 (brs, 1H, NH), 7.41-7.23 (m, 10H), 7.11 (t, J=8.0 Hz, 1H), 7.04 (d, J=8.0 Hz, 1H), 6.48 (d, J=8.0 Hz, 1H), 4.13 (s, 2H), 3.99 (s, 2H), 3.97 (t, J=5.6 Hz, 2H), 3.09 (brs, 2H), 2.01-1.99 (m, 2H), 1.96 (s, 3H). $^{13}$C-NMR (CDCl$_3$, 100 MHz): δ 155.47, 153.16, 147.53, 135.14, 131.80, 129.62, 128.90, 127.26, 124.76, 118.87, 112.61, 104.99, 103.60, 64.58, 51.45, 44.57, 30.72, 25.79, 9.61. Calculated exact mass for the protonated molecule (C$_{26}$H$_{28}$NO$_2$S): 418.1841; measured accurate mass (ESI): 418.1837. LC-MS purity: 95%, $t_r = 13.42$ min.

Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with 3-(aminomethyl)-pyridine (13 µL, 0.12 mmol) in DMF (1 mL) was carried out according to the method of compound 72, to afford the title compound as yellow oil (4.5 mg, 19% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.78 (s, 1H), 8.72 (d, J=4.4 Hz, 1H), 8.14 (d, J=8.0 Hz, 1H), 8.01 (d, J=7.2 Hz, 2H), 7.66 (t, J=7.6 Hz, 2H), 7.56 (t, J=7.6 Hz, 2H), 7.46 (t, J=8.0 Hz, 1H), 7.18 (d, J=8.0 Hz, 1H), 6.84 (d, J=8.0 Hz, 1H), 4.43 (s, 2H), 4.31 (t, J=5.6 Hz, 2H), 3.42 (t, J=8.0 Hz, 2H), 2.75 (s, 3H), 2.39-2.32 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 185.95, 155.68, 155.41, 149.39, 149.20, 146.99, 139.46, 137.88, 132.41, 129.39, 129.71, 128.32, 128.05, 127.34, 124.66, 118.48, 104.86, 104.16, 64.74, 48.15, 45.01, 25.79, 11.01. Calculated exact mass for the protonated molecule (C$_{25}$H$_{25}$N$_2$O$_4$): 401.1865; measured accurate mass (ESI): 401.1866. LC-MS purity: 100%, $t_r = 12.33$ min.
Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with 4-methyl benzylamine (14 µL, 0.11 mmol) in DMF (1 mL) was carried out according to the preparation of compound 72, to afford the title compound as an off-white solid (5.3 mg, 22% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 8.01 (d, J=7.2 Hz, 2H), 7.66 (t, J=7.2 Hz, 1H), 7.56 (t, J=7.2 Hz, 2H), 7.45 (t, J=8.0 Hz, 1H), 7.39 (d, J=8.0 Hz, 2H), 7.29 (d, J=8.0 Hz, 2H), 7.17 (d, J=8.0 Hz, 1H), 6.82 (d, J=8.0 Hz, 1H), 4.28 (t, J=5.6 Hz, 2H), 4.25 (s, 2H), 3.30 (t, J=8.0 Hz, 2H), 2.66 (s, 3H), 2.38 (s, 3H), 2.34-2.27 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 185.96, 155.67, 155.38, 146.96, 142.89, 139.78, 137.89, 132.40, 129.58, 129.53, 129.37, 129.16, 128.05, 127.80, 127.36, 118.46, 104.81, 104.13, 64.72, 50.70, 44.16, 25.66, 19.83, 10.94. Calculated exact mass for the protonated molecule (C$_{27}$H$_{28}$NO$_3$): 414.2069; measured accurate mass (ESI): 414.2083. LC-MS purity: 100%, $t_r$ = 13.21 min.

Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with 4-fluoro-benzylamine (15 µL, 0.12 mmol) in DMF (1 mL) was carried out according to the preparation of compound 72, to afford the title compound as an off-white solid (5.9 mg, 24% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 8.01 (d, J=7.6 Hz, 2H), 7.66 (t, J=7.6 Hz, 1H), 7.58-7.54 (m, 4H), 7.46 (t, J=8.0 Hz, 1H), 7.22 (t, J=7.6 Hz, 2H), 7.17 (d, J=8.0 Hz, 1H), 6.83 (d, J=8.0 Hz, 1H), 4.30 (s, 2H), 4.30 (t, J=5.6 Hz, 2H), 3.34 (t, J=8.0 Hz, 2H), 2.73 (s, 3H), 2.36-2.29 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 185.95, 159.43, 155.73, 155.40, 147.05, 137.88, 132.39, 131.88, 129.38, 129.16, 128.05, 127.25, 118.44,
115.79, 104.84, 104.16, 64.77, 50.18, 44.50, 25.71, 10.98. Calculated exact mass for the protonated molecule (C_{26}H_{25}NO_{3}F): 418.1818; measured accurate mass (ESI): 418.1825. LC-MS purity: 100%, t_R = 12.88 min.

(3-Methyl-4-(3-(methylamino)propoxy)benzofuran-2-yl)(phenyl)methanone(94)

Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with methylamine (33% w/w in ethanol, 14 µL, 0.11 mmol) in DMF (1 mL) was carried out according to the method of compound 72, to afford the title compound as yellow oil (2.0 mg, 11% overall yield). \(^1\)H-NMR (CD_3OD, 400 MHz): δ 8.02 (d, J=7.2 Hz, 2H), 7.67 (t, J=7.2 Hz, 1H), 7.56 (t, J=7.2 Hz, 2H), 7.47 (t, J=8.4 Hz, 1H), 7.19 (d, J=8.4 Hz, 1H), 6.86 (d, J=8.0 Hz, 1H), 4.31 (t, J=6.0 Hz, 2H), 3.31 (t, J=8.0 Hz, 2H), 2.83 (s, 3H), 2.80 (s, 3H), 2.35-2.28 (m, 2H). \(^{13}\)C-NMR (CD_3OD, 100 MHz): δ 186.02, 155.68, 155.46, 147.00, 137.86, 132.42, 129.40, 129.17, 128.05, 127.42, 118.50, 104.86, 104.18, 64.81, 46.54, 32.37, 25.72, 11.00. Calculated exact mass for the protonated molecule (C_{20}H_{22}NO_{3}): 324.1600; measured accurate mass (ESI): 324.1602. LC-MS purity: 100%, t_R = 12.35 min.

(4-(3-(Isopropylamino)propoxy)-3-methylbenzofuran-2-yl)-(phenyl)-methanone (95)

Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with isopropylamine (9.2 µL, 0.11 mmol) in DMF (1 mL) was carried out according to the preparation of compound 72, to afford the title compound as a white solid (4.1 mg, 20 % overall yield). \(^1\)H-NMR (CD_3OD, 400 MHz): δ 8.02 (d, J=7.2 Hz, 2H), 7.67 (t, J=7.2 Hz, 1H), 7.57 (t, J=7.2 Hz, 2H), 7.47 (t, J=8.4 Hz, 1H), 7.19 (d, J=8.4 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.31 (t, J=6.0 Hz, 2H), 3.53-3.42 (m, 1H), 3.33-3.30 (m, 2H), 2.83 (s, 3H), 2.34-2.27 (m, 2H), 1.40 (d, J=6.4 Hz, 6H). \(^{13}\)C-NMR (CD_3OD, 100 MHz): δ 187.42,
157.14, 156.88, 148.41, 139.27, 133.81, 130.81, 130.57, 129.45, 128.76, 119.90, 106.24,
105.58, 66.18, 52.01, 43.54, 27.45, 19.23, 12.41. Calculated exact mass for the protonated molecule (C\textsubscript{22}H\textsubscript{26}NO\textsubscript{3}): 352.1913; measured accurate mass (ESI): 352.1927.
LC-MS purity: 98%, t\textsubscript{R} = 12.59 min.

\[
\text{(4-(3-(Cyclopropylamino)propoxy)-3-methylbenzofuran-2-yl)-(phenyl)-methanone (96)}
\]
Prepared from a solution of compound 70 (15 mg, 0.06 mmol), 1,3-dibromopropane (12.5 µL, 0.12 mmol) and potassium carbonate (50 mg, 0.36 mmol) in DMF (1 mL), the brominated precursor was obtained. After that, the amination with cyclo-propylamine (8.0 µL, 0.11 mmol) in DMF (1 mL) was carried out according to the preparation of compound 72, to afford the title compound as yellow oil (2.0 mg, 16% yield). \(^1\)H-NMR (CD\textsubscript{3}OD, 400 MHz): \(\delta\) 8.02 (d, J=7.2 Hz, 2H), 7.66 (t, J=7.2 Hz, 1H), 7.56 (t, J=7.2 Hz, 1H), 7.47 (t, J=8.4 Hz, 1H), 7.19 (d, J=8.4 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.31 (t, J=6.0 Hz, 2H), 3.43 (t, J=8.0 Hz, 2H), 2.91-2.85 (m, 1H), 2.83 (s, 3H), 2.38-2.28 (m, 2H), 1.02-0.96 (m, 2H), 0.94-0.89 (m, 2H). \(^{13}\)C-NMR (CD\textsubscript{3}OD, 100 MHz): \(\delta\) 187.45, 157.10, 156.87, 139.27, 138.74, 133.83, 130.82, 130.58, 129.46, 128.76, 119.90, 106.27, 105.59, 66.17, 46.91, 31.31, 27.11, 12.39, 4.16. Calculated exact mass for the protonated molecule (C\textsubscript{22}H\textsubscript{24}NO\textsubscript{3}): 350.1756; measured accurate mass (ESI): 350.1745. LC-MS purity: 97%, t\textsubscript{R} = 12.54 min.

\[
\text{4-(3-(t-Butylamino)propoxy)-N-(4-chlorophenyl)-3-methyl-benzofuran-2-carboxamide (97)}
\]
Prepared from a solution of compound 55 (10 mg, 0.03 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (6.1 µL, 0.037 mmol), PyBOP (19 mg, 0.037 mmol) and 4-chloroaniline (4.7 mg, 0.037 mmol), the brominated precursor was obtained. After that, the amination with \(t\)-butylamine (13.6 µL, 0.13 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (6.2 mg, 39% overall yield). \(^1\)H-NMR (CD\textsubscript{3}OD, 400 MHz): \(\delta\) 8.05 (d, J=7.2 Hz, 2H), 7.68 (t, J=7.2 Hz, 1H), 7.58 (t, J=7.2 Hz, 1H), 7.48 (t, J=8.4 Hz, 1H), 7.19 (d, J=8.4 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.31 (t, J=6.0 Hz, 2H), 3.43 (t, J=8.0 Hz, 2H), 2.91-2.85 (m, 1H), 2.83 (s, 3H), 2.38-2.28 (m, 2H), 1.03-0.96 (m, 2H), 0.94-0.89 (m, 2H). \(^{13}\)C-NMR (CD\textsubscript{3}OD, 100 MHz): \(\delta\) 187.45, 157.10, 156.97, 139.26, 138.74, 133.83, 130.82, 130.58, 129.46, 128.76, 119.90, 106.27, 105.59, 66.20, 46.91, 31.31, 27.11, 12.39, 4.16. Calculated exact mass for the protonated molecule (C\textsubscript{22}H\textsubscript{24}NO\textsubscript{3}): 350.1756; measured accurate mass (ESI): 350.1745. LC-MS purity: 97%, t\textsubscript{R} = 12.54 min.
Experimental section

MHz):  δ 7.74 (d, J=8.8 Hz, 2H), 7.41 (t, J=8.4 Hz, 1H), 7.36 (d, J=8.8 Hz, 2H), 7.21 (d, J=8.4 Hz, 1H), 6.82 (d, J=8.0 Hz, 1H), 4.28 (t, J=6.0 Hz, 2H), 3.28 (t, J=8.0 Hz, 2H), 2.81 (s, 3H), 2.31-2.24 (m, 2H), 1.43 (s, 9H).  

\[ \text{HNMR (CD}_3\text{OD, 100 MHz): } \delta 160.34, 156.37, 156.28, 142.58, 138.09, 130.52, 129.76, 129.69, 125.15, 123.59, 120.15, 106.05, 105.60, 66.00, 58.15, 40.06, 27.87, 25.82, 11.58. \]

Calculated exact mass for the protonated molecule (C\textsubscript{23}H\textsubscript{28}N\textsubscript{2}O\textsubscript{3}Cl): 415.1788; measured accurate mass (ESI): 415.1780. LC-MS purity: 100%, \( t_R = 12.77 \) min.

\[ \text{4-(3-(t-Butylamino)propoxy)-N-(4-methoxyphenyl)} \]
\[ \text{-3-methylbenzofuran-2-carboxamide (98)} \]

Prepared from a solution of compound 55 (10 mg, 0.03 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (6.1 \( \mu L \), 0.037 mmol), PyBOP (19 mg, 0.037 mmol) and 4-methoxy aniline (4.6 mg, 0.037 mmol), the brominated precursor was obtained. After that, the amination with \( t \)-butylamine (13.6 \( \mu L \), 0.13 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (2.9 mg, 19% overall yield). \( \text{HNMR (CD}_3\text{OD, 400 MHz): } \delta 7.61 (d, J=9.2 Hz, 2H), 7.41 (t, J=8.4 Hz, 1H), 7.21 (d, J=8.4 Hz, 1H), 6.96 (d, J=9.2 Hz, 2H), 6.83 (d, J=8.0 Hz, 1H), 4.29 (t, J=6.0 Hz, 2H), 3.82 (s, 3H), 3.29 (t, J=8.0 Hz, 2H), 2.82 (s, 3H), 2.31-2.24 (m, 2H), 1.44 (s, 9H). \)

\[ \text{13C-NMR (CD}_3\text{OD, 100 MHz): } \delta 160.38, 158.39, 156.33, 156.28, 142.89, 131.91, 129.49, 124.49, 124.23, 120.19, 114.98, 106.05, 105.57, 65.98, 58.16, 55.88, 40.08, 27.88, 25.82, 11.58. \]

Calculated exact mass for the protonated molecule (C\textsubscript{24}H\textsubscript{31}N\textsubscript{2}O\textsubscript{4}): 411.2284; measured accurate mass (ESI): 411.2301. LC-MS purity: 100%, \( t_R = 11.98 \) min.

\[ \text{4-(3-(t-Butylamino)propoxy)-N-(4-methoxyphenyl)} \]
\[ \text{-3-methyl-N-p-tolyll benzofuran-2-carboxamide (99)} \]

Prepared from a solution of compound 55 (10 mg, 0.03 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (6.1 \( \mu L \), 0.037 mmol), PyBOP (19 mg, 0.037 mmol) and 4-methyl aniline (4.0 mg, 0.037 mmol), the brominated
precursor was obtained. After that, the amination with t-butylamine (13.6 µL, 0.13 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (5.1 mg, 34% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.59 (d, J=8.4 Hz, 2H), 7.42 (t, J=8.4 Hz, 1H), 7.23-7.20 (m, 3H), 6.83 (d, J=8.0 Hz, 1H), 4.30 (t, J=6.0 Hz, 2H), 3.30 (t, J=8.4 Hz, 2H), 2.83 (s, 3H), 2.36 (s, 3H), 2.31-2.24 (m, 2H), 1.44 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.40, 156.34, 156.28, 142.88, 136.46, 135.60, 130.30, 129.53, 124.64, 122.49, 120.20, 106.08, 105.58, 65.98, 58.18, 40.08, 27.90, 25.83, 20.96, 11.59. Calculated exact mass for the protonated molecule (C$_{24}$H$_{31}$N$_2$O$_3$): 395.2335; measured accurate mass (ESI): 395.2334. LC-MS purity: 100%, t$_R$ = 12.52 min.

![Chemical Structure](image)

4-(3-(t-Butylamino)propoxy)-N-(3-chlorophenyl)-3-methyl-benzo-furan-2-carboxamide (100)

Prepared from a solution of compound 55 (10 mg, 0.03 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (6.1 µL, 0.037 mmol), PyBOP (19 mg, 0.037 mmol) and 3-chloro aniline (3.9 µL, 0.037 mmol), the brominated precursor was obtained. After that, the amination with t-butylamine (13.6 µL, 0.13 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as a white solid (6.3 mg, 40% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.94 (t, J=2 Hz, 1H), 7.64 (d, J=9.6 Hz, 1H), 7.42 (t, J=8.4 Hz, 1H), 7.36 (t, J=8.0 Hz, 1H), 7.22 (d, J=8.4 Hz, 1H), 7.17 (d, J=10.0 Hz, 1H), 6.83 (d, J=8.0 Hz, 1H), 4.29 (t, J=6.0 Hz, 2H), 3.30 (t, J=8.0 Hz, 2H), 2.83 (s, 3H), 2.32-2.25 (m, 2H), 1.44 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.39, 156.39, 156.31, 142.52, 140.75, 135.37, 131.08, 129.75, 125.35, 121.91, 120.20, 106.09, 105.63, 66.02, 58.18, 40.09, 27.89, 25.84, 11.60. Calculated exact mass for the protonated molecule (C$_{23}$H$_{28}$N$_2$O$_3$Cl): 415.1788; measured accurate mass (ESI): 415.1792. LC-MS purity: 97%, t$_R$ = 12.30 min.
Prepared from a solution of compound 55 (10 mg, 0.03 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (6.1 µL, 0.037 mmol), PyBOP (19 mg, 0.037 mmol) and N,N-dimethyl-p-phenylenediamine (5 mg, 0.037 mmol), the brominated precursor was obtained. After that, the amination with t-butylamine (13.6 µL, 0.13 mmol) in DMF (1 mL) was carried out according to the preparation of compound 59, to afford the title compound as an off-white solid (6.6 mg, 41% overall yield).

$^{1}H$-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.94 (d, J=9.2 Hz, 2H), 7.50 (d, J=8.4 Hz, 2H), 7.44 (t, J=8.4 Hz, 1H), 7.23 (d, J=8.0 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.31 (t, J=6.0 Hz, 2H), 3.30 (t, J=8.0 Hz, 2H), 3.26 (s, 6H), 2.84 (s, 3H), 2.32-2.25 (m, 2H), 1.44 (s, 9H).

$^{13}C$-NMR (CD$_3$OD, 100 MHz): $\delta$ 160.44, 156.42, 156.35, 142.53, 141.87, 138.51, 129.80, 125.38, 123.52, 120.36, 120.17, 106.06, 105.66, 66.04, 58.17, 45.90, 40.06, 27.87, 25.82, 11.59.

Calculated exact mass for the protonated molecule (C$_{25}$H$_{34}$N$_3$O$_3$): 424.2600; measured accurate mass (ESI): 424.2604. LC-MS purity: 100%, $t_r$ = 10.47 min.

4-(3-(t-butyloamino)propoxy)-N-(4-(dimethylamino)phenyl)-3-methylbenzofuran-2-carboxamide (101)

Prepared from a solution of 2,6-dihydroxyacetophenone (30 mg, 0.2 mmol), 2-Bromo-4'-chloroacetophenone (51 mg, 0.22 mmol) and potassium carbonate (82 mg, 0.6 mmol) in acetonitrile (1 mL), according to the preparation of compound 70, the benzofuran core was obtained. The resulting scaffold was then treated with 1,3-dibromopropane (14 µL, 0.14 mmol) and potassium carbonate (58 mg, 0.4 mmol) in DMF (1 mL), to afford the brominated precursor. After that, the amination with t-butylamine (15 µL, 0.14 mmol) in DMF (1 mL) was carried out according to the preparation of compound 72, to give the title compound as a white solid (1.3 mg, 2% overall yield). $^{1}H$-NMR (CD$_3$OD, 400 MHz): $\delta$ 8.05 (d, J=8.4 Hz, 2H), 7.59 (d, J=8.4 Hz, 2H), 7.48 (t, J=8.4 Hz, 1H), 7.20 (d, J=8.4 Hz, 1H), 6.86 (d, J=8.4 Hz, 1H), 4.32 (t, J=6.0 Hz, 2H), 3.32-3.28 (m, 2H), 2.84 (s, 3H), 2.32-2.25 (m, 2H), 1.44 (s, 9H).
1.43 (s, 9H). \(^{13}\text{C-NMR (CD}_3\text{OD, 100 MHz):} \delta 185.69, 157.07, 156.89, 148.20, 140.03, 137.69, 132.30, 129.70, 129.14, 119.86, 106.28, 105.60, 66.09, 58.18, 40.05, 27.88, 25.82, 12.37. Calculated exact mass for the protonated molecule (C\(_{23}\)H\(_{27}\)NO\(_3\)Cl): 400.1679; measured accurate mass (ESI): 400.1669. LC-MS purity: 95%, \(t_R = 13.46\) min.

**t-Butyl 4-hydroxypiperidine-1-carboxylate (103)**

4-hydroxypiperidine (1 g, 9.9 mmol) was dissolved in THF (15 mL) and 10% NaOH (4 mL) was added. Di-tert-butyl-dicarbonate (2.37 g, 10.9 mmol) was added dropwise over 15 minutes and the resulting mixture was stirred for 24 hours. After that, the reaction was diluted with CHCl\(_3\) (40 mL) and partitioned with water (30 mL). The aqueous layer was then extracted with CHCl\(_3\) (2 x 20 mL). The combined organic layers were washed with brine and dried over anhydrous sodium sulphate, concentrated in vacuo, to give the title compound as a white solid (2.17 g, quantitative yield). \(^1\text{H-NMR (CDCl}_3, 400 MHz):} \delta 3.88 \text{ (brs, 3H), 3.07 \text{ (brs, 2H), 1.89 \text{ (brs, 2H), 1.67-1.54 \text{ (m, 2H), 1.47 (s, 9H).}}}

**t-Butyl 3-hydroxypiperidine-1-carboxylate (104)**

Prepared from a solution of 3-hydroxypiperidine (0.5 g, 4.9 mmol) and Di-tert-butyl-dicarbonate (1.18 g, 5.4 mmol) in THF (10 mL) and 10% NaOH (2 mL), according to the preparation of compound 103, the title compound was obtained as a white solid (1.24 g, quantitative yield). \(^1\text{H-NMR (CDCl}_3, 400 MHz):} \delta 3.88 \text{ (brs, 3H), 3.07 \text{ (brs, 2H), 1.89 \text{ (brs, 2H), 1.67-1.54 \text{ (m, 2H), 1.47 (s, 9H).}}}

**t-Butyl N-(3-hydroxy-2,2-dimethylpropyl)carbamate (105)**

Prepared from a solution of 3-amino-2,2-dimethylpropan-1-ol (0.5 g, 4.8 mmol) and Di-tert-butyl-dicarbonate (1.16 g, 5.3 mmol) in THF (10 mL) and 10% NaOH (2 mL), according to the preparation of compound 103, the title compound was obtained as a white solid (1.01 g, quantitative yield). \(^1\text{H-NMR (CDCl}_3, 400 MHz):} \delta 4.93 \text{ (brs, 1H), 3.20 \text{ (s, 2H), 2.96 (d, J=6.0 Hz, 2H), 1.45 (s, 9H), 0.86 (s, 6H).}
**Experimental section**

**t-Butyl 4-[[2-(ethoxycarbonyl)-3-methyl-1-benzofuran-4-yl]oxy]piperidine-1-carboxylate (106)**

To a stirred solution of compound 3 (2 g, 9.1 mmol), compound 103 (4.56 g, 22.7 mmol) and triphenylphosphine (5.95 g, 22.7 mmol) in anhydrous THF (20 mL) was added DIAD (4.34 mL, 22.7 mmol) dropwise at room temperature. The resulting mixture was stirred at the same temperature for 4 hours, and then concentrated in vacuo. The residue was purified by column chromatography over silica gel, eluting with hexane: ethyl acetate, 5:1 to afford the title compound as colourless oil (3.52 g, 96% yield).

**1H-NMR (CDCl₃, 400 MHz): δ 7.32 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.0 Hz, 1H), 6.65 (d, J=8.0 Hz, 1H), 4.74-4.69 (m, 1H), 4.46 (q, J=7.2 Hz, 2H), 3.68-3.61 (m, 2H), 3.58-3.52 (m, 2H), 2.78 (s, 3H), 2.04-1.97 (m, 2H), 1.96-1.86 (m, 2H), 1.50 (s, 9H), 1.46 (t, J=7.2 Hz, 3H).**

Ethyl 3-methyl-4-(piperidin-4-yl)oxy)benzofuran-2-carboxylate (109) [188]

A mixture of compound 106 (10 mg, 0.025 mmol) and TFA (50 µL) in DCM (1 mL) was stirred at room temperature for 2 hours. The reaction mixture was evaporated under pressure to dryness, which was further purified by semi-preparative reverse phase HPLC to give the title compound as a white solid (9.8 mg, 94% yield).

**1H-NMR (CD₃OD, 400 MHz): δ 7.41 (t, J=8.4 Hz, 1H), 7.15 (d, J=8.4 Hz, 1H), 6.88 (d, J=8.0 Hz, 1H), 4.98-4.93 (m, 1H), 4.42 (q, J=7.2 Hz, 2H), 3.50-3.40 (m, 2H), 3.33-3.28 (m, 2H), 2.80 (s, 3H), 2.34-2.24 (m, 2H), 2.22-2.12 (m, 2H), 1.43 (t, J=7.2 Hz, 3H).**

**13C-NMR (CD₃OD, 100 MHz): δ 161.87, 157.38, 154.55, 141.09, 130.09, 127.02, 120.30, 106.50, 106.16, 69.95, 62.13, 42.01, 28.28, 14.63, 11.82.** Calculated exact mass for the protonated molecule (C₁₇H₂₂NO₄): 304.1549; measured accurate mass (ESI): 304.1561. LC-MS purity: 100%, tₘ = 11.39 min.
Ethyl-3-methyl-4-(piperidin-3-yloxy)benzofuran-2-carboxylate (110)

Prepared from a solution of compound 3 (20 mg, 0.09 mmol), compound 104 (46 mg, 0.22 mmol), triphenylphosphine (60 mg, 0.22 mmol) and DIAD (44 µL, 0.22 mmol) in anhydrous THF (2 mL), according to the preparation of compound 106, the N-Boc precursor was obtained. After that, the deprotection in DCM (1 mL) containing TFA (100 µL) was carried out according to the preparation of compound 109, the title compound was obtained as a white solid (17 mg, 45% overall yield). ¹H-NMR (CD₃OD, 400 MHz): δ 7.42 (t, J=8.4 Hz, 1H), 7.18 (d, J=8.4 Hz, 1H), 6.86 (d, J=8.0 Hz, 1H), 4.98-4.91 (m, 1H), 4.42 (q, J=7.2 Hz, 2H), 3.62-3.45 (m, 2H), 3.28-3.20 (m, 2H), 2.81 (s, 3H), 2.28-2.07 (m, 3H), 2.00-1.86 (m, 1H), 1.43 (t, J=7.2 Hz, 3H). ¹³C-NMR (CD₃OD, 100 MHz): δ 161.86, 157.39, 154.60, 141.18, 130.04, 127.22, 120.40, 106.65, 106.51, 70.48, 62.14, 46.79, 44.96, 28.36, 19.83, 14.63, 11.80. Calculated exact mass for the protonated molecule (C₁₇H₂₂NO₄): 304.1549; measured accurate mass (ESI): 304.1560. LC-MS purity: 96%, tᵣ = 11.44 min.

Ethyl-4-(3-amino-2,2-dimethylpropoxy)-3-methylbenzofuran-2-carboxylate (111)

Prepared from a solution of compound 3 (10 mg, 0.045 mmol), compound 105 (23 mg, 0.11 mmol), triphenylphosphine (30 mg, 0.11 mmol) and DIAD (22 µL, 0.11 mmol) in anhydrous THF (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM (1 mL) containing TFA (100 µL) was carried out according to the preparation of compound 110, to give the title compound as a white solid (2.7 mg, 14% yield). ¹H-NMR (CD₃OD, 400 MHz): δ 7.41 (t, J=8.0 Hz, 1H), 7.15 (d, J=8.4 Hz, 1H), 6.82 (d, J=8.0 Hz, 1H), 4.42 (q, J=7.2 Hz, 2H), 3.99 (s, 2H), 3.12 (s, 2H), 2.80 (s, 3H), 1.43 (t, J=7.2 Hz, 3H), 1.25 (s, 6H). ¹³C-NMR (CD₃OD, 100 MHz): δ 161.88, 157.12, 156.51, 140.97, 130.13, 126.86, 119.67, 106.12, 105.48, 74.97, 62.12, 47.90, 35.43, 22.53, 14.63, 12.16. Calculated exact mass for the protonated molecule (C₁₇H₂₄NO₄): 306.1705; measured accurate mass (ESI): 306.1708. LC-MS purity: 100%, tᵣ = 12.06 min.
Ethyl 3-methyl-4-(1-methylpiperidin-4-yloxy)benzofuran-2-carboxylate (112)

A mixture of compound 109 (30 mg, 0.07 mmol), formaldehyde (37% aqueous, 17.5 µL, 0.21 mmol), acetic acid (24 µL, 0.42 mmol) in THF/DMF (2 mL, 1:1) was stirred at room temperature for 2 hours, followed by the addition of sodium triacetoxyborohydride (74 mg, 0.35 mmol). The resulting mixture was further stirred at room temperature for 12 hours. After that, the reaction mixture was diluted with ethyl acetate (20 mL) and sequentially washed with 5% NaHCO₃, water and brine (each 15 mL). The organic layer was concentrated in vacuo and the residue was purified by semi-preparative reverse phase HPLC, with no TFA in the mobile phase, to give the title compound as colourless oil (17 mg, 77% yield).

$^1$H-NMR (CDCl₃, 400 MHz): $\delta$ 7.30 (t, $J=8.0$ Hz, 1H), 7.11 (d, $J=8.0$ Hz, 1H), 6.64 (d, $J=8.0$ Hz, 1H), 4.61-4.51 (m, 1H), 4.45 (q, $J=7.2$ Hz, 2H), 2.79 (s, 3H), 2.67 (brs, 2H), 2.42 (brs, 2H), 2.34 (s, 3H), 2.14-2.05 (m, 2H), 2.02-1.94 (m, 2H), 1.45 (t, $J=7.2$ Hz, 3H). $^{13}$C-NMR (CDCl₃, 100 MHz): $\delta$ 160.58, 155.94, 154.09, 139.61, 128.41, 126.75, 119.45, 104.93, 104.69, 71.56, 60.92, 52.31, 46.31, 30.54, 14.40, 11.61. Calculated exact mass for the protonated molecule (C₁₈H₂₄NO₄): 318.1705; measured accurate mass (ESI): 318.1718. LC-MS purity: 100%, $t_r$ = 11.25 min.

Ethyl 4-(1-ethypiperidin-4-yloxy)-3-methylbenzofuran-2-carboxylate (113)

Prepared from a solution of compound 109 (30 mg, 0.07 mmol), acetalaldehyde (11.8 µL, 0.21 mmol), acetic acid (24 µL, 0.42 mmol) and sodium triacetoxyborohydride (74 mg, 0.35 mmol) in THF/DMF (2 mL, 1:1), according to the preparation of compound 112, the title compound was obtained as colourless oil (18 mg, 78% yield). $^1$H-NMR (CDCl₃, 400 MHz): $\delta$ 7.30 (t, $J=8.0$ Hz, 1H), 7.11 (d, $J=8.0$ Hz, 1H), 6.65 (d, $J=8.0$ Hz, 1H), 4.63-4.53 (m, 1H), 4.45 (q, $J=7.2$ Hz, 2H), 2.78 (s, 3H), 2.72 (brs, 2H), 2.48 (q, $J=7.2$ Hz, 2H), 2.45 (brs, 2H), 2.16-2.06 (m, 2H), 2.04-1.94 (m, 2H), 1.45 (t, $J=7.2$ Hz, 3H), 1.14 (t, $J=7.2$ Hz, 3H). $^{13}$C-NMR (CDCl₃, 100 MHz): $\delta$ 160.60,
155.96, 154.13, 139.62, 128.42, 126.78, 119.47, 104.95, 104.67, 72.28, 60.92, 52.43, 49.85, 30.52, 14.41, 12.17, 11.60. Calculated exact mass for the protonated molecule (C_{19}H_{26}NO_{4}): 332.1862; measured accurate mass (ESI): 332.1865. LC-MS purity: 100%, \( t_R = 11.34 \) min.

**Ethyl 4-(1-isopropylpiperidin-4-ylloxy)-3-methylbenzofuran-2-carboxylate (114)**

Prepared from a solution of compound 109 (30 mg, 0.07 mmol), acetone (15.9 µL, 0.21 mmol), acetic acid (24 µL, 0.42 mmol) and sodium triacetoxyborohydride (74 mg, 0.35 mmol) in THF/DMF (2 mL, 1:1), according to the preparation of compound 112, the title compound was obtained as colourless oil (21 mg, 87% yield). \(^1\)H-NMR (CDCl\(_3\), 400 MHz): \( \delta \) 7.30 (t, \( J=8.0 \) Hz, 1H), 7.11 (d, \( J=8.0 \) Hz, 1H), 6.65 (d, \( J=8.0 \) Hz, 1H), 4.60-4.53 (m, 1H), 4.46 (q, \( J=7.2 \) Hz, 2H), 2.84-2.75 (m, 3H), 2.79 (s, 3H), 2.51 (brs, 2H), 2.14-2.07 (m, 2H), 2.02-1.93 (m, 2H), 1.45 (t, \( J=7.2 \) Hz, 3H), 1.10 (d, \( J=6.8 \) Hz, 6H). \(^13\)C-NMR (CDCl\(_3\), 100 MHz): \( \delta \) 160.61, 155.96, 154.16, 139.59, 128.43, 126.82, 119.46, 104.92, 104.60, 72.51, 60.92, 54.58, 45.32, 30.86, 18.37, 14.42, 11.55. Calculated exact mass for the protonated molecule (C_{20}H_{28}NO_{4}): 346.2019; measured accurate mass (ESI): 346.2019. LC-MS purity: 100%, \( t_R = 11.51 \) min.

**Ethyl 4-(1-benzylpiperidin-4-ylloxy)-3-methylbenzofuran-2-carboxylate (115)**

Prepared from a solution of compound 109 (20 mg, 0.048 mmol), benzaldehyde (15.2 µL, 0.15 mmol), acetic acid (16.5 µL, 0.29 mmol) and sodium triacetoxyborohydride (51 mg, 0.24 mmol) in THF/DMF (2 mL, 1:1), according to the preparation of compound 112, the title compound was obtained as colourless oil (11 mg, 58% yield). \(^1\)H-NMR (CDCl\(_3\), 400 MHz): \( \delta \) 7.36-7.29 (m, 6H), 7.11 (d, \( J=8.4 \) Hz, 1H), 6.64 (d, \( J=8.0 \) Hz, 1H), 4.62-4.53 (m, 1H), 4.47 (q, \( J=7.2 \) Hz, 2H), 3.59 (s, 2H), 2.77 (s, 3H), 2.74-2.67 (m, 2H), 2.51-2.39 (m, 2H), 2.14-2.03 (m, 2H), 2.03-1.91 (m, 2H), 1.47 (t, \( J=7.2 \) Hz, 3H). \(^13\)C-NMR (CDCl\(_3\), 100 MHz): \( \delta \) 160.63, 155.97, 154.18, 139.60,
Ethyl-4-((1-[(4-fluorophenyl)methyl]piperidin-4-yl)oxy)-3-methyl-1-benzofuran-2-carboxylate (116)

Prepared from a solution of compound 109 (50 mg, 0.12 mmol), 4-fluoro-benzaldehyde (38.6 µL, 0.36 mmol), acetic acid (41.2 µL, 0.72 mmol) and sodium triacetoxyborohydride (127 mg, 0.6 mmol) in THF/DMF (2 mL, 1:1), according to the preparation of compound 112, the title compound was obtained as colourless oil (26 mg, 48% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 7.38-7.28 (m, 3H), 7.11 (d, J=8.0 Hz, 1H), 7.07-7.01 (m, 2H), 6.64 (d, J=8.0 Hz, 1H), 4.62-4.52 (m, 1H), 4.47 (q, J=7.2 Hz, 2H), 3.54 (s, 2H), 2.77 (s, 3H), 2.08 (brs, 2H), 2.44 (brs, 2H), 2.13-1.90 (m, 4H), 1.46 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CDCl$_3$, 100 MHz): δ 163.25, 160.63, 155.97, 154.14, 139.62, 133.90, 130.64, 128.45, 126.79, 119.44, 115.03, 104.79, 62.34, 60.96, 50.01, 30.52, 14.42, 11.61. Calculated exact mass for the protonated molecule (C$_{24}$H$_{28}$NO$_4$F): 412.1924; measured accurate mass (ESI): 412.1940. LC-MS purity: 100%, $t_R$ = 12.19 min.

(3-Methyl-4-(piperidin-4-yloxy)benzofuran-2-yl)(phenyl)methanone (117)

Prepared from a solution of compound 70 (17 mg, 0.068 mmol), compound 103 (34 mg, 0.17 mmol), triphenylphosphine (44 mg, 0.17 mmol) and DIAD (32 µL, 0.17 mmol) in anhydrous THF (1 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 110, to afford the title compound as an off-white solid (17.3 mg, 84% overall yield). $^1$H-NMR (CD$_2$OD, 400 MHz): δ 8.01 (d, J=7.2 Hz, 2H), 7.66 (t, J=7.6 Hz, 1H), 7.56 (t, J=7.6 Hz, 2H), 7.46 (t, J=8.4 Hz, 1H), 7.18 (d, J=8.4 Hz, 2H), 7.01 (m, 3H), 4.62-4.52 (m, 1H), 4.47 (q, J=7.2 Hz, 2H), 3.54 (s, 2H), 2.77 (s, 3H), 2.44 (brs, 2H), 1.46 (t, J=7.2 Hz, 3H). $^{13}$C-NMR (CD$_2$OD, 100 MHz): δ 163.25, 160.63, 155.97, 154.14, 139.62, 133.90, 130.64, 128.45, 126.79, 119.44, 115.03, 104.79, 62.34, 60.96, 50.01, 30.52, 14.42, 11.61. Calculated exact mass for the protonated molecule (C$_{25}$H$_{27}$NO$_4$F): 421.1924; measured accurate mass (ESI): 421.1940. LC-MS purity: 100%, $t_R$ = 12.19 min.
Hz, 1H), 6.91 (d, J=8.4 Hz, 1H), 5.02-4.95 (m, 1H), 3.51-3.42 (m, 2H), 3.38-3.30 (m, 2H), 2.84 (s, 3H), 2.38-2.28 (m, 2H), 2.25-2.14 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 186.00, 155.91, 153.53, 147.07, 137.86, 132.42, 129.35, 129.19, 128.05, 127.20, 119.12, 105.23, 104.91, 68.60, 40.57, 26.84, 11.08. Calculated exact mass for the protonated molecule (C$_{21}$H$_{22}$NO$_3$): 336.1600; measured accurate mass (ESI): 336.1603. LC-MS purity: 100%, $t_R$ = 12.37 min.

(3-Methyl-4-(1-methylpiperidin-4-yloxy)benzofuran-2-yl)(phenyl) methanone (118)

Prepared from a solution of compound 117 (15 mg, 0.033 mmol), formaldehyde (37% aqueous, 8.1 µL, 0.1 mmol), acetic acid (11.5 µL, 0.2 mmol) and sodium triacetoxyborohydride (35 mg, 0.17 mmol) in THF/DMF (1 mL, 1:1), according to the preparation of compound 112, the title compound was obtained as colourless oil (9 mg, 78% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 8.06 (d, J=7.2 Hz, 2H), 7.61 (t, J=7.2 Hz, 1H), 7.53 (t, J=7.2 Hz, 2H), 7.36 (t, J=8.0 Hz, 1H), 7.10 (d, J=8.0 Hz, 1H), 6.68 (d, J=8.0 Hz, 1H), 4.66-4.56 (m, 1H), 2.84 (s, 3H), 2.69 (brs, 2H), 2.45 (brs, 2H), 2.36 (s, 3H), 2.15-2.08 (m, 2H), 2.05-1.95 (m, 2H). $^{13}$C-NMR (CDCl$_3$, 100 MHz): δ 185.87, 155.89, 154.54, 147.21, 138.15, 132.37, 129.64, 128.95, 128.23, 128.00, 119.74, 105.00, 104.65, 71.60, 52.25, 46.31, 30.50, 12.23. Calculated exact mass for the protonated molecule (C$_{22}$H$_{24}$NO$_3$): 350.1756; measured accurate mass (ESI): 350.1752. LC-MS purity: 100%, $t_R$ = 12.26 min.

(4-(1-Benzylpiperidin-4-yloxy)-3-methylbenzofuran-2-yl) (phenyl)-methanone (119)

Prepared from a solution of compound 117 (15 mg, 0.033 mmol), benaldehyde (10 µL, 0.1 mmol), acetic acid (11.5 µL, 0.2 mmol) and sodium triacetoxyborohydride (35 mg, 0.17 mmol) in THF/DMF (1 mL, 1:1), according to the preparation of compound 112, the title compound was obtained as colourless oil (7 mg, 50% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 8.06 (d, J=7.2 Hz, 2H), 7.62
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(t, J=7.2 Hz, 1H), 7.53 (t, J=7.2 Hz, 2H), 7.37-7.30 (m, 6H), 7.09 (d, J=8.0 Hz, 1H), 6.67 (d, J=8.0 Hz, 1H), 4.65-4.56 (m, 1H), 3.59 (s, 2H), 2.81 (s, 3H), 2.74 (brs, 2H), 2.46 (brs, 2H), 2.16-2.08 (m, 2H), 2.02-1.93 (m, 2H). 13C-NMR (CDCl3, 100 MHz): δ 185.89, 155.91, 154.64, 147.20, 138.19, 132.38, 129.67, 129.18, 128.97, 128.11, 127.10, 119.72, 104.95, 104.57, 72.40, 63.17, 50.08, 30.56, 12.21. Calculated exact mass for the protonated molecule (C28H28NO3): 426.2069; measured accurate mass (ESI): 426.2079. LC-MS purity: 100%, tR = 12.76 min.

4-({1-{[tert-butoxy]carbonyl}piperidin-4-yl}oxy)-3-methyl-1-benzofuran-2-carboxylic acid (120)

Prepared from a solution of compound 106 (1.85 g, 4.6 mmol) and LiOH·H2O (1.16 g, 27.5 mmol) in THF/MeOH (40 mL, 1:1) according to the preparation of compound 55, the title compound was obtained as a white solid (1.51 g, 88% yield). 1H-NMR (CD3OD, 400 MHz): δ 7.40 (t, J=8.0 Hz, 1H), 7.12 (d, J=8.0 Hz, 1H), 6.65 (d, J=8.0 Hz, 1H), 4.74-4.69 (m, 1H), 3.69-3.62 (m, 2H), 3.56-3.50 (m, 2H), 2.76 (s, 3H), 2.06-1.99 (m, 2H), 1.94-1.86 (m, 2H), 1.50 (s, 3H).

N-isopropyl-3-methyl-4-(piperidin-4-yl)oxybenzofuran-2-carboxamide (121)

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and isopropylamine (7.6 µL, 0.088 mmol) according to the preparation of compound 59, the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 109, to afford the title compound as a white solid (11.5 mg, 34% overall yield). 1H-NMR (CD3OD, 400 MHz): δ 7.37 (t, J=8.4 Hz, 1H), 7.16 (d, J=8.4 Hz, 1H), 6.87 (d, J=8.4 Hz, 1H), 4.98-4.93 (m, 1H), 4.30-4.96 (m, 1H), 3.46-3.40 (m, 2H), 3.32-3.29 (m, 2H), 2.80 (s, 3H), 2.32-2.25 (m, 2H), 2.21-2.13 (m, 2H), 1.29 (d, J=6.4 Hz, 6H). 13C-NMR (CD3OD, 100 MHz): δ 161.52, 156.45, 154.30, 143.11, 129.19, 123.31, 120.78, 106.62, 106.05, 69.78, 42.32, 41.92, 28.23, 22.58, 11.52. Calculated exact mass for the
protonated molecule \((C_{18}H_{25}N_{2}O_{3})\): 317.1865; measured accurate mass (ESI): 317.1867. LC-MS purity: 100%, \(t_R = 10.75\) min.

**N-cyclopropyl-3-methyl-4-(piperidin-4-yloxy)benzofuran-2-carboxamide (122)**

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and cyclopropylamine (6.2 µL, 0.088 mmol), the \(N\)-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (11 mg, 32% overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta \) 7.37 (t, \(J=8.4\) Hz, 1H), 7.14 (d, \(J=8.4\) Hz, 1H), 6.87 (d, \(J=8.0\) Hz, 1H), 4.98-4.93 (m, 1H), 3.46-3.40 (m, 2H), 3.32-3.29 (m, 2H), 2.88-2.82 (m, 1H), 2.81 (s, 3H), 2.32-2.25 (m, 2H), 2.21-2.13 (m, 2H), 0.86-0.82 (m, 2H), 0.71-0.67 (m, 2H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta \) 163.90, 156.51, 154.34, 142.95, 129.26, 123.51, 120.76, 106.65, 106.05, 69.82, 41.94, 28.25, 23.25, 11.51, 6.63. Calculated exact mass for the protonated molecule \((C_{18}H_{23}N_{2}O_{3})\): 315.1709; measured accurate mass (ESI): 315.1702. LC-MS purity: 100%, \(t_R = 10.25\) min.

**N-tert-butyl-3-methyl-4-(piperidin-4-yloxy)benzofuran-2-carboxamide (123)**

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and tert-butylamine (9.2 µL, 0.088 mmol), the \(N\)-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (13 mg, 37% overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta \) 7.37 (t, \(J=8.4\) Hz, 1H), 7.15 (d, \(J=8.4\) Hz, 1H), 6.86 (d, \(J=8.4\) Hz, 1H), 4.97-4.93 (m, 1H), 3.49-3.38 (m, 2H), 3.32-3.26 (m, 2H), 2.78 (s, 3H), 2.34-2.24 (m, 2H), 2.21-2.13 (m, 2H), 1.50 (s, 9H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta \) 161.83, 156.23, 154.27, 143.47.
129.10, 122.83, 120.82, 106.67, 106.06, 69.81, 52.64, 41.93, 29.12, 28.25, 11.52.

Calculated exact mass for the protonated molecule (C_{19}H_{27}N_{2}O_{3}): 331.2022; measured accurate mass (ESI): 331.2021. LC-MS purity: 100%, t_R = 11.75 min.

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\text{N-cyclohexyl-3-methyl-4-(piperidin-4-yl)benzofuran-2-carboxamide (124)}
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Prepared from a solution of compound 120 (50 mg, 0.13 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (26 µL, 0.15 mmol), PyBOP (76 mg, 0.15 mmol) and cyclohexylamine (17 µL, 0.15 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (13.4 mg, 22% overall yield). ^1H-NMR (CD_{3}OD, 400 MHz): δ 7.37 (t, J=8.4 Hz, 1H), 7.16 (d, J=8.4 Hz, 1H), 6.87 (d, J=8.4 Hz, 1H), 4.97-4.92 (m, 1H), 3.94-3.86 (m, 1H), 3.49-3.38 (m, 2H), 3.32-3.29 (m, 2H), 2.80 (s, 3H), 2.35-2.23 (m, 2H), 2.23-2.12 (m, 2H), 2.00-1.91 (m, 2H), 1.91-1.76 (m, 2H), 1.76-1.66 (m, 1H), 1.50-1.35 (m, 4H), 1.35-1.19 (m, 1H). ^13C-NMR (CD_{3}OD, 100 MHz): δ 161.46, 156.45, 154.30, 143.11, 129.18, 123.36, 120.80, 106.64, 106.05, 69.79, 49.72, 41.93, 33.76, 28.25, 26.57, 26.44, 11.54. Calculated exact mass for the protonated molecule (C_{21}H_{29}N_{2}O_{3}): 357.2178; measured accurate mass (ESI): 357.2162. LC-MS purity: 100%, t_R = 12.20 min.

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\text{N-adamantyl-3-methyl-4-(piperidin-4-yl)benzofuran-2-carboxamide (125)}
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Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and 1-adamantylamine (13.3 mg, 0.088 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (15 mg, 36% overall yield).
$^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.36 (t, J=8.4 Hz, 1H), 7.14 (d, J=8.4 Hz, 1H), 6.86 (d, J=8.0 Hz, 1H), 4.97-4.90 (m, 1H), 3.48-3.40 (m, 2H), 3.33-3.28 (m, 2H), 2.77 (s, 3H), 2.34-2.24 (m, 2H), 2.19 (brd, 7H), 2.14 (brd, 4H), 1.79 (brd, 6H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 161.48, 156.18, 154.26, 143.37, 129.12, 122.92, 120.82, 106.68, 106.05, 69.81, 53.52, 42.51, 41.92, 37.45, 30.99, 28.24, 11.54. Calculated exact mass for the protonated molecule (C$_{25}$H$_{33}$N$_2$O$_3$): 409.2491; measured accurate mass (ESI): 409.2497. LC-MS purity: 100%, t$_R$ = 13.40 min.

3-Methyl-N-phenyl-4-(piperidin-4-yl)oxybenzofuran-2-carboxamide (126)

Prepared from a solution of compound 120 (15 mg, 0.04 mmol) in DMF/DCM (1 mL, 1:1) was sequentially added DIPEA (7.4 µL, 0.044 mmol), PyBOP (23 mg, 0.044 mmol) and aniline (4 µL, 0.044 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (1 mL) was carried out according to the preparation of compound 121, to afford the title compound as an off-white solid (5 mg, 37% overall yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 8.32 (s, 1H, CONH), 7.72 (d, J=7.2 Hz, 2H), 7.40 (t, J=7.2 Hz, 2H), 7.33 (t, J=8.0 Hz, 1H), 7.17 (t, J=7.2 Hz, 1H), 7.09 (d, J=8.0 Hz, 1H), 6.69 (d, J=8.0 Hz, 1H), 4.67-4.58 (m, 1H), 3.23-3.17 (m, 2H), 2.89 (s, 3H), 2.87-2.81 (m, 2H), 2.15-2.08 (m, 2H), 1.89-1.81 (m, 2H). $^{13}$C-NMR (CDCl$_3$, 100 MHz): δ 158.18, 154.82, 154.17, 141.00, 137.59, 129.08, 128.13, 124.89, 124.39, 119.91, 105.44, 104.11, 73.08, 43.53, 31.89, 11.20. Calculated exact mass for the protonated molecule (C$_{21}$H$_{23}$N$_2$O$_3$): 351.1709; measured accurate mass (ESI): 351.1696. LC-MS purity: 92%, t$_R$ = 11.74 min.

3-Methyl-4-(piperidin-4-yl)oxy-N-(1,2,4-trimethyl-1H-imidazol-5-yl)benzofuran-2-carboxamide (127)

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and 4-amino-1,3,5-
trimethylpyrazole (11 mg, 0.088 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (29 mg, 73% overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 7.43 (t, J=8.4 Hz, 1H), 7.21 (d, J=8.0 Hz, 1H), 6.91 (d, J=8.0 Hz, 1H), 4.97-4.91 (m, 1H), 3.81 (s, 3H), 3.5-3.39 (m, 2H), 3.38-3.30 (m, 2H), 2.84 (s, 3H), 2.37-2.27 (m, 2H), 2.25 (s, 3H), 2.21 (s, 3H), 2.21-2.15 (m 2H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 161.78, 156.73, 154.49, 144.74, 142.68, 139.07, 129.71, 125.02, 120.75, 115.88, 106.79, 106.11, 69.96, 41.94, 36.26, 28.26, 11.62, 10.86, 9.30. Calculated exact mass for the protonated molecule (C\(_{21}\)H\(_{27}\)N\(_4\)O\(_3\)): 383.2083; measured accurate mass (ESI): 383.2068. LC-MS purity: 100%, \(t_R = 10.03\) min.

\[
\text{N-benzyl-3-methyl-4-(piperidin-4-yloxy)benzofuran-2-carboxamide(128)}
\]

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DiPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and benzylamine (9.6 µL, 0.088 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (12.5 mg, 33% overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 7.40-7.32 (m, 5H), 7.26 (t, J=7.2 Hz, 1H), 7.14 (d, J=8.4 Hz, 1H), 6.85 (d, J=8.4 Hz, 1H), 4.92-4.87 (m, 1H), 4.58 (s, 2H), 3.45-3.39 (m, 2H), 3.32-3.28 (m, 2H), 2.80 (s, 3H), 2.34-2.24 (m, 2H), 2.20-2.12 (m, 2H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \(\delta\) 162.32, 156.54, 154.34, 142.97, 140.18, 129.55, 129.33, 128.51, 128.20, 123.78, 120.76, 106.64, 106.06, 69.79, 43.54, 41.90, 28.22, 11.56. Calculated exact mass for the protonated molecule (C\(_{22}\)H\(_{23}\)N\(_2\)O\(_3\)): 365.1865; measured accurate mass (ESI): 365.1875. LC-MS purity: 100%, \(t_R = 11.55\) min.
3-Methyl-N-(naphthalen-1-yl)-4-(piperidin-4-yloxy)benzofuran-2-carboxamide (129)

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and 1-naphthylamine (12.6 mg, 0.088 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as an off-white solid (13.8 mg, 34% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.06-8.04 (m, 1H), 7.97-7.95 (m, 1H), 7.88 (d, J=8.4 Hz, 1H), 7.10 (d, J=7.2 Hz, 1H), 7.60-7.54 (m, 3H), 7.45 (t, J=8.4 Hz, 1H), 7.28 (d, J=8.4 Hz, 1H), 6.92 (d, J=8.0 Hz, 1H), 4.98-4.94 (m, 1H), 3.47-3.40 (m, 2H), 3.31-3.28 (m, 2H), 2.87 (s, 3H), 2.34-2.26 (m, 2H), 2.22-2.15 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 161.59, 156.76, 154.48, 142.98, 135.81, 133.58, 130.71, 129.42, 128.19, 127.28, 126.53, 125.07, 124.86, 123.62, 120.89 106.81, 106.26, 69.92, 41.96, 28.27, 11.71. Calculated exact mass for the protonated molecule (C$_{25}$H$_{25}$N$_2$O$_3$): 401.1865; measured accurate mass (ESI): 401.1859. LC-MS purity: 100%, t$_R$ = 12.21 min.

3-Methyl-N-(naphthalen-1-ylmethyl)-4-(piperidin-4-yloxy)benzofuran-2-carboxamide (130)

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and 1-naphthylmethylamine (12.9 µL, 0.088 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (20.7 mg, 49% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.18 (d, J=8.4 Hz, 1H), 7.90 (d, J=8.4 Hz, 1H), 7.82 (d, J=8.0 Hz, 1H), 7.58-7.44 (m, 4H), 7.33 (t, J=8.4 Hz, 1H), 7.10 (d, J=8.4 Hz, 1H), 6.80 (d, J=8.4 Hz, 1H), 5.05 (s, 2H), 4.89-4.84 (m, 1H), 3.43-3.37 (m, 2H), 3.32-3.26 (m, 2H), 2.80 (s, 3H), 2.28-2.21 (m, 2H), 2.18-2.10 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ
162.23, 156.51, 154.29, 142.92, 135.33, 134.98, 132.62, 129.79, 129.31, 129.13, 127.36
126.86, 126.61, 126.44, 124.31, 123.85, 120.72, 106.61, 106.06, 69.75, 41.87, 41.59,
28.19, 11.59. Calculated exact mass for the protonated molecule (C$_{26}$H$_{27}$N$_{2}$O$_{3}$): 415.2022;
measured accurate mass (ESI): 415.2020. LC-MS purity: 100%, $t_R = 12.50$ min.

![Chemical structure](image)

(3,4-Dihydroisoquinolin-2(1H)-yl)(3-methyl-4-(piperidin-4-yloxy)-benzofuran-2-yl)methanone (131)

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and 1,2,3,4-tetrahydroisoquinoline (11 µL, 0.088 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (17.6 mg, 44% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.37 (t, J=8.4 Hz, 1H), 7.22 (brd, 4H), 7.17 (d, J=8.0 Hz, 1H), 6.89 (d, J=8.0 Hz, 1H), 4.97-4.92 (m, 1H), 4.85 (s, 2H), 4.00-3.80 (m, 2H), 3.46-3.40 (m, 2H), 3.33-3.28 (m, 2H), 3.02 (t, J=5.6 Hz, 2H), 2.61 (s, 3H), 2.32-2.25 (m, 2H), 2.21-2.13 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 163.18, 162.04, 161.75, 156.77, 154.02, 144.07, 135.63, 129.75, 128.80, 127.97, 127.63, 122.11, 120.10, 106.76, 106.06, 69.80, 46.20, 41.91, 30.69, 29.22, 28.25, 11.47. Calculated exact mass for the protonated molecule (C$_{24}$H$_{29}$N$_{2}$O$_{3}$): 391.2022; measured accurate mass (ESI): 391.2029. LC-MS purity: 100%, $t_R = 12.40$ min.

![Chemical structure](image)

(4-Benzylpiperazin-1-yl)(3-methyl-4-(piperidin-4-yloxy)-benzofuran-2-yl)methanone (132)

Prepared from a solution of compound 120 (30 mg, 0.08 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (15 µL, 0.088 mmol), PyBOP (46 mg, 0.088 mmol) and 1-benzylpiperazine (15.3 µL, 0.088 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the
preparation of compound 121, to afford the title compound as a white solid (27.2 mg, 62% overall yield). ¹H-NMR (CD₃OD, 400 MHz): δ 7.58-7.54 (m, 2H), 7.54-7.52 (m, 3H), 7.39 (t, J=8.4 Hz, 1H), 7.15 (d, J=8.4 Hz, 1H), 6.90 (d, J=8.4 Hz, 1H), 4.97-4.92 (m, 1H), 4.44 (s, 2H), 3.46-3.40 (m, 6H), 3.32-3.29 (m, 2H), 2.64 (s, 3H), 2.33-2.26 (m, 2H), 2.20-2.12 (m, 2H).

¹³C-NMR (CD₃OD, 100 MHz): δ 162.34, 156.67, 154.17, 142.73, 132.40, 131.43, 130.45, 129.93, 129.38, 124.61, 120.05, 106.85, 106.04, 69.96, 61.66, 52.60, 49.06, 41.94, 28.26, 11.63. Calculated exact mass for the protonated molecule (C₁₉H₁₇N₃O₃): 434.2444; measured accurate mass (ESI): 434.2423. LC-MS purity: 100%, tᵣ = 8.83 min.

1-(TriphenyImethyl)piperidin-4-ol (138)

4-Hydroxypiperidine (0.30 g, 3.0 mmol) and triphenylmethyl chloride (0.84 g, 3.0 mmol) were dissolved in DCM (5 mL). Triethylamine (0.84 mL, 6.0 mmol) was added dropwise and the resulting mixture was stirred for 24 hours at room temperature. After that, the reaction was diluted with CHCl₃ (20 mL) and partitioned with 5% NaHCO₃ solution (30 mL). The aqueous layer was then extracted with CHCl₃ (2 x 15 mL). The combined organic layers were washed with brine and dried over anhydrous sodium sulphate, concentrated in vacuo, to give the title compound as a white solid (1.05 g, quantitative yield). ¹H-NMR (CDCl₃, 400 MHz): δ 7.49 (brs, 5H), 7.34-7.26 (m, 7H), 7.20-7.16 (m, 3H), 2.91 (brs, 1H), 2.03-1.85 (m, 2H), 1.71-1.60 (m, 2H), 1.60-1.24 (m, 4H).

Ethyl 3-methyl-4-[[1-(triphenylimethyl)piperidin-4-yl]oxy]-1-benzofuran-2-carboxylate (139)

Prepared from compound 3 (100 mg, 0.45 mmol) and compound 138 (386 mg, 1.1 mmol), triphenylphosphine (294 mg, 1.1 mmol) and DIAD (214 µL, 1.1 mmol) in anhydrous THF (4 mL) according to the preparation of compound 106, the title compound was obtained as a white solid (160 mg, 65% yield).

¹H-NMR (CDCl₃, 400 MHz): δ 7.63-7.42 (m, 5H), 7.38-7.29 (m, 7H), 7.26-7.14 (m, 4H), 7.05 (d, J=8.0 Hz, 1H), 6.55 (d, J=8.0 Hz, 1H), 4.46 (q, J=7.2 Hz, 2H), 2.28-2.13 (m, 3H), 2.12-1.98 (m, 3H), 1.64-1.53 (m, 2H), 1.47 (t, J=7.2 Hz, 3H).
Prepared from compound 139 (160 mg, 0.29 mmol) and LiAlH₄ (17 mg, 0.44 mmol) in anhydrous THF according to the preparation of compound 79, the title compound was obtained as colourless oil (150 mg, quantitative yield). ¹H-NMR (CDCl₃, 400 MHz): δ 7.68-7.41 (m, 5H), 7.38-7.30 (m, 6H), 7.25-7.14 (m, 4H), 7.10 (t, J=8.0 Hz, 1H), 6.98 (d, J=8.0 Hz, 1H), 6.54 (d, J=8.0 Hz, 1H), 4.70 (s, 2H), 4.06-3.94 (m, 1H), 2.24-2.11 (m, 2H), 2.12-1.97 (m, 4H), 1.91-1.83 (m, 2H).

Prepared from compound 140 (5 mg, 0.001 mmol) in DCM (1 mL), containing 0.1% of TFA and 0.2% of water, was stirred at room temperature for 12 hours. After that, the reaction mixture was concentrated in vacuo and the residue was purified by semi-preparative reverse phase HPLC, with no TFA in the mobile phases, to give the title compound as colourless oil (1.7 mg, 59% yield). ¹H-NMR (CD₃OD, 400 MHz): δ 7.21 (t, J=8.0 Hz, 1H), 7.07 (d, J=8.0 Hz, 1H), 6.79 (d, J=8.0 Hz, 1H), 4.89-4.87 (m, 2H), 4.65 (s, 2H), 3.46-3.40 (m, 2H), 3.31-3.26 (m, 2H), 2.46 (s, 3H), 2.31-2.22 (m, 2H), 2.20-2.11 (m, 2H). ¹³C-NMR (CD₃OD, 125 MHz): δ 157.36, 153.12, 152.29, 126.29, 120.88, 113.50, 106.26, 105.82, 69.51, 55.27, 41.98, 28.53, 10.42. Calculated exact mass for the protonated molecule (C₁₅H₂₀N₅O₃): 262.1443; measured accurate mass (ESI): 262.1455. LC-MS purity: 89% (somewhat decomposition under weakly acidic or LC-MS conditions), tᵣ = 9.29 min.

Prepared from compound 140 (30 mg, 0.06 mmol), phenol (14.1 mg, 0.15 mmol), triphenylphosphine (39 mg, 0.15 mmol) and DIAD (29 µL, 0.15 mmol) in anhydrous THF (2 mL) according to the preparation of compound 80, the N-trityl precursor was obtained. After that, the
deprotection in DCM (1mL) containing 0.1% of TFA and 0.2% of water was carried out according to the preparation of compound 141, to afford the title compound as colourless oil (0.4 mg, 2% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.32-7.28 (m, 2H), 7.24 (t, J=8.0 Hz, 1H), 7.09 (d, J=8.0 Hz, 1H), 7.04 (d, J=8.0 Hz, 2H), 6.98 (t, J=8.0 Hz, 1H), 6.81 (d, J=8.0 Hz, 1H), 5.15 (s, 2H), 4.96-4.89 (m, 1H), 3.43-3.38 (m, 2H), 3.30-3.26 (m, 2H), 2.48 (s, 3H), 2.30-2.22 (m, 2H), 2.20-2.11 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 159.96, 153.18, 148.70, 138.03, 130.54, 126.75, 122.40, 120.63, 116.18, 115.81, 106.33, 105.89, 69.55, 61.90, 41.98, 28.34, 10.50. Calculated exact mass for the protonated molecule (C$_{21}$H$_{24}$NO$_3$): 338.1756; measured accurate mass (ESI): 338.1772. LC-MS purity: 100%, $t_R$ = 12.44 min.

4-[[2-(Methoxymethyl)-3-methyl-1-benzofuran-4-yl]oxy]piperidine (143)

A solution of compound 140 (30 mg, 0.06 mmol) and sodium hydride (60% w/w, 7.2 mg, 0.18 mmol) in anhydrous DMF was stirred at room temperature for 30 minutes, then treated with methyl iodide (9.4 µL, 0.15 mmol). The resulting mixture was further stirred at room temperature for 12 hours. After that, the reaction mixture was quenched with 100 µL of cold water, diluted with EtOAc (20 mL) and sequentially washed with water and brine (each for 20 mL). The organic layer was concentrated in vacuo and the residue was purified by column chromatography over silica gel, eluting with hexane:ethyl acetate, 8:1, to give the N-trityl precursor. The compound was further deprotected in DCM (1mL), containing 0.1% of TFA and 0.2% of water, according to the preparation of compound 141, to afford the title compound as yellow oil (7.8 mg, 28% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.22 (t, J=8.0 Hz, 1H), 7.06 (d, J=8.0 Hz, 1H), 6.80 (d, J=8.0 Hz, 1H), 4.90-4.87 (m, 1H), 4.55 (s, 2H), 3.46-3.37 (m, 2H), 3.40 (s, 3H), 3.32-3.27 (m, 2H), 2.45 (s, 3H), 2.31-2.23 (m, 2H), 2.20-2.12 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 157.42, 153.16, 149.55, 126.61, 120.58, 115.58, 106.25, 105.78, 69.53, 65.16, 58.07, 41.92, 28.31, 10.49. Calculated exact mass for the protonated molecule (C$_{16}$H$_{22}$NO$_3$): 276.1600; measured accurate mass (ESI):
276.1606. LC-MS purity: 100%, \( t_R = 10.96 \) min.

![4-[[2-(Ethoxymethyl)-3-methyl-1-benzofuran-4-yl]oxy]piperidine (144)](image)

Prepared from a solution of compound 140 (20 mg, 0.04 mmol), sodium hydride (60% w/w, 4.8 mg, 0.12 mmol) and ethyl iodide (6.4 \( \mu \)L, 0.08 mmol) in anhydrous DMF, the \( N \)-trityl precursor was obtained. After that, the deprotection in DCM (1mL) containing 0.1% of TFA and 0.2% of water was carried out according to the preparation of compound 143, to afford the title compound as yellow oil (1.7 mg, 15% overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \( \delta \) 7.22 (t, \( J=8.0 \) Hz, 1H), 7.07 (d, \( J=8.0 \) Hz, 1H), 6.81 (d, \( J=8.0 \) Hz, 1H), 4.88-4.85 (m, 1H), 4.60 (s, 2H), 3.61 (q, \( J=7.2 \) Hz, 2H), 3.46-3.40 (m, 2H), 3.32-3.25 (m, 2H), 2.47 (s, 3H), 2.32-2.23 (m, 2H), 2.23-2.11 (m, 2H), 1.23 (t, \( J=7.2 \) Hz, 3H). \(^{13}\)C-NMR (CD\(_3\)OD, 100 MHz): \( \delta \) 157.41, 153.14, 149.92, 126.55, 120.64, 115.19, 106.26, 105.82, 69.51, 66.77, 63.42, 41.94, 28.34, 15.38, 10.51. Calculated exact mass for the protonated molecule (C\(_{17}\)H\(_{24}\)NO\(_3\)): 290.1756; measured accurate mass (ESI): 290.1761. LC-MS purity: 87% (somewhat decomposition under weakly acidic or LC-MS conditions), \( t_R = 11.39 \) min.

![4-[[2-[[Benzyl(oxy)methyl]-3-methyl-1-benzofuran-4-yl]oxy]-piperidine (145)](image)

Prepared from a solution of compound 140 (30 mg, 0.06 mmol), sodium hydride (60% w/w, 7.2 mg, 0.18 mmol) and benzyl bromide (10.7 \( \mu \)L, 0.09 mmol) in anhydrous DMF, the \( N \)-trityl precursor was obtained. After that, the deprotection in DCM (1mL) containing 0.1% of TFA and 0.2% of water was carried out according to the preparation of compound 143, to afford the title compound as colourless oil (2 mg, 10% overall yield). \(^1\)H-NMR (CD\(_3\)OD, 400 MHz): \( \delta \) 7.40-7.28 (m, 5H), 7.23 (t, \( J=8.0 \) Hz, 1H), 7.09 (d, \( J=8.0 \) Hz, 1H), 6.81 (d, \( J=8.0 \) Hz, 1H), 4.91-4.85 (m, 1H), 4.64 (s, 2H), 4.60 (s, 2H), 3.46-3.39 (m, 2H), 3.30-3.27 (m, 2H), 2.43 (s, 3H), 2.30-2.22 (m, 2H), 2.20-2.12 (m, 2H). \(^{13}\)C-NMR (CD\(_3\)OD, 125 MHz): \( \delta \) 156.06, 151.72,
148.30, 137.87, 128.02, 127.62, 127.45, 127.45, 125.19, 119.22, 114.14, 104.84, 104.45, 71.65, 68.06, 61.44, 40.53, 26.90, 9.14. Calculated exact mass for the protonated molecule \((\text{C}_{22}\text{H}_{26}\text{N}3)\): 352.1913; measured accurate mass (ESI): 352.1909. LC-MS purity: 100%, \(t_R = 12.34\) min.

**t-Butyl 4-[(2-formyl-3-methyl-1-benzofuran-4-yl)oxy]piperidine-1-carboxylate (153)**

To a solution of compound 135 (50 mg, 0.13 mmol) in anhydrous DCM was added activated manganese dioxide (560 mg, 6.5 mmol) at room temperature, and the resulting mixture was stirred at the same temperature for 24 hours. After that, the reaction mixture was filtered over a celite pad and the filtrate was concentrated in vacuo, to afford the title compound as yellow oil (37 mg, 77% yield).

\[^1\text{H-}NMR\ (\text{CDCl}_3, 400\text{ MHz}): \delta 9.96\ (s, 1\text{H}), 7.38\ (t, J=8.0\ Hz, 1\text{H}), 7.11\ (d, J=8.0\ Hz, 1\text{H}), 6.66\ (d, J=8.0\ Hz, 1\text{H}), 4.74-4.69\ (m, 1\text{H}), 3.69-3.62\ (m, 2\text{H}), 3.56-3.50\ (m, 2\text{H}), 2.76\ (s, 3\text{H}), 2.06-1.99\ (m, 2\text{H}), 1.94-1.86\ (m, 2\text{H}), 1.50\ (s, 3\text{H}).**

**Benzyl([3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-yl]methyl)-amine (154)**

A mixture of compound 153 (18 mg, 0.05 mmol), benzylamine (6.2 µL, 0.055 mmol) and acetic acid (18 µL, 0.3 mmol) in THF (1 mL) was stirred at room temperature for 2 hours, followed by the addition of sodium triacetoxyborohydride (53 mg, 0.25 mmol). The resulting mixture was further stirred at room temperature for 12 hours. After that, the reaction mixture was diluted with ethyl acetate (20 mL) and sequentially washed with 5% NaHCO3, water and brine (each 15 mL). The organic layer was concentrated in vacuo, to give the \(N\)-Boc protected title compound without further purification. After that, the deprotection in DCM (1 mL) containing 5% TFA was carried out according to the preparation of compound 109, to afford the title compound as a yellow solid (5.5 mg, 24% overall yield).

\[^1\text{H-}NMR\ (\text{CD}_3\text{OD}, 400\text{ MHz}): \delta 7.54-7.46\ (m, 5\text{H}), 7.31\ (t, J=8.0\ Hz, 1\text{H}), 7.14\ (d, J=8.0\ Hz,
1H), 6.87 (d, J=8.0 Hz, 1H), 4.92-4.86 (m, 1H), 4.44 (s, 2H), 4.31 (s, 2H), 3.47-3.40 (m, 2H), 3.32-3.28 (m, 2H), 2.47 (s, 3H), 2.33-2.25 (m, 2H), 2.19-2.11 (m, 2H). \(^{13}\text{C-NMR}\) (CD\(_3\)OD, 125 MHz): \(\delta\) 157.81, 153.48, 142.85, 132.31, 131.00, 130.75, 130.33, 127.81, 120.08, 118.86, 106.74, 105.85, 69.88, 51.81, 42.13, 42.01, 28.36, 10.62. Calculated exact mass for the protonated molecule (C\(_{22}\)H\(_{27}\)N\(_2\)O\(_2\)): 351.2073; measured accurate mass (ESI): 351.2061. LC-MS purity: 100%, \(t_R = 8.87\) min.

\([3\text{-Methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-yl}]\text{methyl}\)-(naphthalen-1-ylmethyl)amine (155)

Prepared from a solution of compound 153 (18 mg, 0.05 mmol), 1-naphthylmethylamine (8.1 µL, 0.055 mmol), acetic acid (18 µL, 0.3 mmol) and sodium triacetoxyborohydride (53 mg, 0.25 mmol) in THF (1 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM (1 mL) containing 5% TFA was carried out according to the preparation of compound 154, to afford the title compound as a white solid (14.5 mg, 56% overall yield). \(^1\text{H-NMR}\) (CD\(_3\)OD, 400 MHz): \(\delta\) 8.02-7.97 (m, 3H), 7.69 (d, J=6.8 Hz, 1H), 7.62-7.54 (m, 3H), 7.33 (t, J=8.0 Hz, 1H), 7.16 (d, J=8.0 Hz, 1H), 6.89 (d, J=8.0 Hz, 1H), 4.91-4.85 (m, 1H), 4.78 (s, 2H), 4.57 (s, 2H), 3.46-3.40 (m, 2H), 3.32-3.28 (m, 2H), 2.48 (s, 3H), 2.33-2.26 (m, 2H), 2.19-2.11 (m, 2H). \(^{13}\text{C-NMR}\) (CD\(_3\)OD, 125 MHz): \(\delta\) 157.81, 153.52, 142.77, 135.46, 132.64, 131.70, 130.17, 128.43, 128.26, 127.91, 127.60, 126.49, 123.59, 120.10, 119.24, 106.79, 105.88, 69.90, 48.31, 42.53, 42.01, 28.37, 10.62. Calculated exact mass for the protonated molecule (C\(_{26}\)H\(_{29}\)N\(_2\)O\(_4\)): 401.2229; measured accurate mass (ESI): 401.2221. LC-MS purity: 100%, \(t_R = 10.02\) min.

\([3\text{-Methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-yl}]\text{methanamine}\) (156)

A solution of compound 154 (10 mg, 0.022 mmol), palladium on carbon (10% w/w, 7 mg, 0.006 mmol) and 1,4-cyclohexadiene (50 µL) in ethanol (1 mL) was stirred at 80 °C for 6 hours. After that, the reaction mixture
was concentrated in vacuo and the residue was purified by semi-preparative reverse phase HPLC to give the title compound as yellow oil (3.1 mg, 38% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.28 (t, J=8.0 Hz, 1H), 7.12 (d, J=8.0 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.87-4.85 (m, 1H), 4.30 (s, 2H), 3.46-3.40 (m, 2H), 3.30-3.27 (m, 2H), 2.49 (s, 3H), 2.32-2.25 (m, 2H), 2.18-2.10 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 157.00, 153.43, 144.54, 127.52, 120.20, 117.07, 106.70, 105.81, 69.89, 42.07, 35.06, 28.42, 10.43. Calculated exact mass for the protonated molecule (C$_{15}$H$_{21}$N$_2$O$_4$): 261.1603; measured accurate mass (ESI): 261.1606. LC-MS purity: 100%, $t_R$ = 1.17 min.

3-Methyl-N-(2-phenylethyl)-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxamide (157)

Prepared from a solution of compound 120 (50 mg, 0.13 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (26 µL, 0.15 mmol), PyBOP (76 mg, 0.15 mmol) and 2-phenethylamine (18.4 µL, 0.15 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 121, to afford the title compound as a white solid (25.9 mg, 41% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.36 (t, J=8.0 Hz, 1H), 7.34-7.32 (m, 4H), 7.23-7.19 (m, 1H), 7.12 (d, J=8.0 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.93-4.89 (m, 1H), 3.60 (t, J=7.6 Hz, 2H), 3.46-3.39 (m, 2H), 3.31-3.28 (m, 2H), 2.93 (t, J=7.6 Hz, 2H), 2.79 (s, 3H), 2.32-2.24 (m, 2H), 2.20-2.12 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 162.31, 156.46, 154.31, 143.01, 140.44, 129.82, 129.51, 129.24, 127.38, 123.44, 120.74, 106.64, 105.99, 69.80, 41.89, 41.78, 36.76, 28.21, 11.50. Calculated exact mass for the protonated molecule (C$_{23}$H$_{27}$N$_2$O$_3$): 379.2022; measured accurate mass (ESI): 379.2022. LC-MS purity: 100%, $t_R$ = 11.91 min.
3-Methyl-N-(3-phenylpropyl)-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxamide (158)

Prepared from a solution of compound 120 (50 mg, 0.13 mmol) in DMF/DCM (2 mL, 1:1) was sequentially added DIPEA (26 µL, 0.15 mmol), PyBOP (76 mg, 0.15 mmol) and 3-phenylpropylamine (20.8 µL, 0.15 mmol), the N-Boc precursor was obtained. After that, the deprotection in DCM (2 mL) containing 5% TFA was carried out according to the preparation of compound 121, to afford the title compound as a white solid (35.1 mg, 54% overall yield).

$^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.36 (t, J=8.0 Hz, 1H), 7.28-7.21 (m, 4H), 7.17-7.13 (m, 1H), 7.14 (d, J=8.0 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.92-4.88 (m, 1H), 3.45-3.38 (m, 2H), 3.42 (t, J=7.2 Hz, 2H), 3.32-3.27 (m, 2H), 2.79 (s, 3H), 2.70 (t, J=7.2 Hz, 2H), 2.31-2.24 (m, 2H), 2.19-2.12 (m, 2H), 1.98-1.91 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 162.35, 156.44, 154.30, 143.05, 143.00, 129.39, 129.20, 126.88, 123.34, 120.76, 106.64, 106.01, 69.81, 41.88, 39.86, 34.36, 32.45, 28.21, 11.52. Calculated exact mass for the protonated molecule (C$_{24}$H$_{29}$N$_2$O$_3$): 393.2178; measured accurate mass (ESI): 393.2166. LC-MS purity: 100%, t$_R$ = 12.27 min.

Methyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (160)

A mixture of compound 120 (30 mg, 0.08 mmol), EDCI (16.8 mg, 0.088 mmol), hydroxybenzotriazole (13.6 mg, 0.11 mmol) in anhydrous acetonitrile (3 mL) was stirred at room temperature for 30 minutes, and then treated with methanol (3.6 µL, 0.088 mmol) and DIPEA (28 µL, 0.16 mmol). The resulting mixture was further stirred at room temperature for another 12 hours. After that, the solution was evaporated to dryness in vacuo. The residue was re-dissolved in ethyl acetate (20 mL) and washed with brine, 5% NaHCO$_3$ and water (each 20 mL). The organic layer was dried over anhydrous sodium sulphate and concentrated in vacuo, to produce the N-Boc precursor without further purification. This compound was further deprotected in DCM (2 mL) containing 5% TFA according to the preparation of
compound 109, to afford the title compound as a white solid (16.1 mg, 50% overall yield).

$^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.40 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.0 Hz, 1H), 6.87 (d, J=8.0 Hz, 1H), 4.96-4.94 (m, 1H), 3.95 (s, 3H), 3.48-3.41 (m, 2H), 3.41-3.33 (m, 2H), 2.78 (s, 3H), 2.36-2.24 (m, 2H), 2.23-2.11 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 162.27, 157.39, 154.56, 140.89, 130.15, 127.20, 120.22, 106.48, 106.08, 69.95, 52.32, 41.96, 37.87, 28.25, 11.82. Calculated exact mass for the protonated molecule (C$_{16}$H$_{20}$NO$_4$): 290.139; measured accurate mass (ESI): 290.1395. LC-MS purity: 100%, $t_R = 10.76$ min.

Phenyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate(161)

Prepared from a mixture of compound 120 (44 mg, 0.12 mmol), EDCI (25 mg, 0.13 mmol), hydroxybenzotriazole (20.5 mg, 0.13 mmol), phenol (12.1 mg, 0.13 mmol) and DIPEA (41 µL, 0.24 mmol) in anhydrous acetonitrile (3 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM (2 mL) containing 5% TFA was carried out according to the preparation of compound 160, to afford the title compound as a white solid (28.1 mg, 51% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.50-7.43 (m, 3H), 7.32 (t, J=7.6 Hz, 1H), 7.26 (d, J=7.6 Hz, 2H), 7.18 (d, J=8.0 Hz, 1H), 6.90 (d, J=8.0 Hz, 1H), 4.94-4.90 (m, 1H), 3.48-3.41 (m, 2H), 3.35-3.29 (m, 2H), 2.85 (s, 3H), 2.35-2.27 (m, 2H), 2.22-2.14 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 160.02, 157.66, 154.69, 151.71, 140.26, 130.67, 130.62, 129.16, 127.21, 122.79, 120.29, 106.62, 106.19, 70.09, 41.99, 28.26, 12.05. Calculated exact mass for the protonated molecule (C$_{21}$H$_{22}$NO$_4$): 352.1549; measured accurate mass (ESI): 352.1545. LC-MS purity: 100%, $t_R = 12.17$ min.

Benzyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate(162)

Prepared from a mixture of compound 120 (15 mg, 0.04 mmol), EDCI (8.4 mg, 0.044 mmol), hydroxybenzotriazole (6.8 mg, 0.05 mmol), benzyl alcohol (4.5 µL, 0.044 mmol) and DIPEA (14 µL, 0.08 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the
deprotection in DCM (2 mL) containing 5% TFA was carried out according to the preparation of compound 160, to afford the title compound as a white solid (8.5 mg, 44% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.50 (d, J=8.0 Hz, 2H), 7.44-7.35 (m, 4H), 7.14 (d, J=8.0 Hz, 1H), 6.86 (d, J=8.0 Hz, 1H), 5.40 (s, 2H), 4.95-4.91 (m, 1H), 3.45-3.39 (m, 2H), 3.31-3.27 (m, 2H), 2.78 (s, 3H), 2.33-2.23 (m, 2H), 2.15-2.10 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta$ 161.59, 157.47, 154.57, 140.88, 137.29, 130.21, 129.67, 129.46, 127.48, 120.29, 106.53, 106.20, 69.95, 67.70, 41.98, 28.25, 24.21, 11.94. Calculated exact mass for the protonated molecule (C$_{22}$H$_{24}$NO$_4$): 366.1705; measured accurate mass (ESI): 366.1708. LC-MS purity: 100%, $t_R$ = 12.38 min.

Cyclohexylmethyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (163)

Prepared from a mixture of compound 120 (20 mg, 0.055 mmol), EDCI (11.3 mg, 0.06 mmol), hydroxybenzotriazole (9.4 mg, 0.07 mmol), cyclohexanemethanol (7.3 µL, 0.06 mmol) and DIPEA (18.5 µL, 0.11 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM (2 mL) containing 5% TFA was carried out according to the preparation of compound 160, to afford the title compound as a white solid (2.7 mg, 10% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta$ 7.42 (t, J=8.0 Hz, 1H), 7.16 (d, J=8.0 Hz, 1H), 6.88 (d, J=8.0 Hz, 1H), 5.00-4.94 (m, 1H), 4.19 (d, J=6.4 Hz, 2H), 3.50-3.41 (m, 2H), 3.32-3.27 (m, 2H), 2.81 (s, 3H), 2.35-2.26 (m, 2H), 2.24-2.12 (m, 2H), 1.93-1.69 (m, 6H), 1.42-1.25 (m, 3H), 1.21-1.07 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): $\delta$ 161.99, 157.46, 154.56, 141.14, 130.10, 126.94, 120.31, 106.52, 106.20, 71.15, 69.94, 41.99, 38.63, 30.83, 28.27, 27.45, 26.83, 11.96. Calculated exact mass for the protonated molecule (C$_{22}$H$_{30}$NO$_4$): 372.2175; measured accurate mass (ESI): 372.2167. LC-MS purity: 100%, $t_R$ = 12.46 min.
Chapter 6 – Experimental section

Naphthalen-1-ylmethyl 3-methyl-4-(piperidin-4-yloxy)-1-benzo-furan-2-carboxylate (164)

Prepared from a mixture of compound 120 (15 mg, 0.04 mmol), EDCI (8.4 mg, 0.044 mmol), hydroxybenzotriazole (6.8 mg, 0.05 mmol), 1-naphthalenemethanol (7 mg, 0.044 mmol) and DIPEA (14 µL, 0.08 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA was carried out according to the preparation of compound 160, to afford the title compound as a white solid (4.3 mg, 20% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.19 (d, J=8.0 Hz, 1H), 7.96 (t, J=7.2 Hz, 2H), 7.69 (d, J=7.2 Hz, 1H), 7.63-7.51 (m, 3H), 7.38 (t, J=8.0 Hz, 1H), 7.12 (d, J=8.0 Hz, 1H), 6.83 (d, J=8.0 Hz, 1H), 4.92-4.86 (m, 1H), 3.42-3.35 (m, 2H), 3.31-3.25 (m, 2H), 2.71 (s, 3H), 2.30-2.21 (m, 2H), 2.17-2.10 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 161.62, 157.50, 154.53, 140.90, 135.34, 132.61, 130.18, 128.96, 127.42, 126.36, 124.70, 120.25, 106.47, 106.20, 69.84, 66.01, 41.92, 28.19, 11.92. Calculated exact mass for the protonated molecule (C$_{26}$H$_{26}$NO$_4$): 416.1862; measured accurate mass (ESI): 416.1854. LC-MS purity: 99%, $t_R = 13.10$ min.

3-Methyl-4-(piperidin-4-yloxy)-1-benzo-furan-2-carboxylic acid (165)

Prepared from a solution of compound 120 (10 mg, 0.027 mmol) in DCM containing 5% TFA (1 mL), according to the method of compound 109, the title compound was obtained as a white solid (2.3 mg, 22% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.38 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.0 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 4.94-4.92 (m, 1H), 3.48-3.38 (m, 2H), 3.30-3.24 (m, 2H), 2.78 (s, 3H), 2.33-2.23 (m, 2H), 2.15-2.11 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 163.47, 157.39, 154.52, 129.88, 126.64, 120.47, 106.45, 106.20, 69.88, 42.02, 28.29, 11.92. Calculated exact mass for the protonated molecule (C$_{15}$H$_{18}$NO$_4$): 276.1236; measured accurate mass (ESI): 276.1234. LC-MS purity: 100%, $t_R = 9.54$ min.
2-Phenylethyl-3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (166)

Prepared from a mixture of compound 120 (20 mg, 0.055 mmol), EDCI (11.3 mg, 0.06 mmol), hydroxybenzotriazole (9.4 mg, 0.07 mmol), phenethyl alcohol (7.1 µL, 0.06 mmol) and DIPEA (18.5 µL, 0.11 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as a white solid (2.4 mg, 9% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.41 (t, J=8.0 Hz, 1H), 7.34-7.30 (m, 4H), 7.27-7.22 (m, 1H), 7.14 (d, J=8.0 Hz, 1H), 6.87 (d, J=8.0 Hz, 1H), 5.02-4.92 (m, 1H), 4.59 (t, J=6.8 Hz, 2H), 3.49-3.40 (m, 2H), 3.32-3.25 (m, 2H), 3.11 (t, J=6.8 Hz, 2H), 2.71 (s, 3H), 2.35-2.26 (m, 2H), 2.23-2.10 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 161.79, 157.46, 154.57, 140.99, 139.22, 130.14, 130.00, 129.56, 127.64, 127.14, 120.27, 106.50, 106.16, 69.97, 66.71, 42.03, 36.09, 28.28, 11.87. Calculated exact mass for the protonated molecule (C$_{23}$H$_{26}$NO$_4$): 380.1862; measured accurate mass (ESI): 380.1856. LC-MS purity: 100%, $t_R$ = 12.78 min.

3-Phenylpropyl-3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (167)

Prepared from a mixture of compound 120 (20 mg, 0.055 mmol), EDCI (11.3 mg, 0.06 mmol), hydroxybenzotriazole (9.4 mg, 0.07 mmol), 3-phenyl-1-propanol (8 µL, 0.06 mmol) and DIPEA (18.5 µL, 0.11 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% trifluoroacetic acid (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as an off-white solid (2.3 mg, 8% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.43 (t, J=8.0 Hz, 1H), 7.32-7.19 (m, 5H), 7.17 (d, J=8.0 Hz, 1H), 6.89 (d, J=8.0 Hz, 1H), 5.01-4.92 (m, 1H), 4.37 (t, J=6.4 Hz, 2H), 3.50-3.39 (m, 2H), 3.32-3.28 (m, 2H), 2.82 (t, J=7.6 Hz, 2H), 2.82 (s, 3H), 2.36-2.25 (m, 2H), 2.25-2.07 (m, 4H). $^{13}$C-NMR (CD$_3$OD, 125
(4-Chlorophenyl) methyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (173)

Prepared from a mixture of compound 120 (15 mg, 0.04 mmol), EDCI (8.4 mg, 0.044 mmol), hydroxybenzotriazole (6.8 mg, 0.05 mmol), 4-chloro-benzyl alcohol (6.3 mg, 0.044 mmol) and DIPEA (14 µL, 0.08 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as a white solid (1.5 mg, 7% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.51 (d, J=8.4 Hz, 2H), 7.44 (d, J=8.4 Hz, 2H), 7.42 (t, J=8.0 Hz, 1H), 7.16 (d, J=8.0 Hz, 1H), 6.88 (d, J=8.0 Hz, 1H), 5.40 (s, 2H), 4.96-4.94 (m, 1H), 3.46-3.40 (m, 2H), 3.31-3.26 (m, 2H), 2.80 (s, 3H), 2.34-2.25 (m, 2H), 2.16-2.13 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 160.04, 156.11, 153.17, 138.28, 134.75, 129.69, 128.88, 128.39, 126.28, 113.40, 105.15, 104.84, 68.55, 65.40, 40.61, 26.87, 22.79, 10.52. Calculated exact mass for the protonated molecule (C$_{22}$H$_{23}$NO$_4$Cl): 400.1316; measured accurate mass (ESI): 400.1300. LC-MS purity: 100%, $t_R = 12.91$ min.

(4-Methylphenyl) methyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (174)

Prepared from a mixture of compound 120 (15 mg, 0.04 mmol), EDCI (8.4 mg, 0.044 mmol), hydroxybenzotriazole (6.8 mg, 0.05 mmol), 4-methyl-benzyl alcohol (5.4 mg, 0.044 mmol) and DIPEA (14 µL, 0.08 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as a white solid (4 mg, 21%
overall yield). $^1$H-NMR (CD$_2$OD, 400 MHz): $\delta$ 7.43-7.37 (m, 3H), 7.24 (d, $J$=8.0 Hz, 2H), 7.15 (d, $J$=8.0 Hz, 1H), 6.87 (d, $J$=8.0 Hz, 1H), 5.36 (s, 2H), 4.96-4.94 (m, 1H), 3.46-3.39 (m, 2H), 3.31-3.28 (m, 2H), 2.78 (s, 3H), 2.38 (s, 3H), 2.32-2.24 (m, 2H), 2.22-2.13 (m, 2H). $^{13}$C-NMR (CD$_2$OD, 100 MHz): $\delta$ 161.65, 157.46, 154.55, 140.94, 139.48, 134.24, 130.25, 130.18, 129.63, 127.36, 120.29, 106.51, 106.20, 69.91, 67.66, 41.97, 28.24, 24.21, 21.23, 11.94. Calculated exact mass for the protonated molecule (C$_{23}$H$_{26}$NO$_4$): 380.1862; measured accurate mass (ESI): 380.1857. LC-MS purity: 100%, $t_R$ = 12.83 min.

![Diagram of compound 175](image1.png)

(3-Chlorophenyl) methyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (175)

Prepared from a mixture of compound 120 (40 mg, 0.11 mmol), EDCI (22.6 mg, 0.12 mmol), hydroxybenzotriazole (18.8 mg, 0.14 mmol), 3-chloro-benzyl alcohol (13.9 µL, 0.12 mmol) and DIPEA (37 µL, 0.22 mmol) in anhydrous acetonitrile (3 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as a yellow solid (23.6 mg, 42% overall yield). $^1$H-NMR (CD$_2$OD, 400 MHz): $\delta$ 7.51 (s, 1H), 7.43-7.35 (m, 4H), 7.11 (d, $J$=8.0 Hz, 1H), 6.82 (d, $J$=8.0 Hz, 1H), 5.36 (s, 2H), 4.91-4.88 (m, 1H), 3.45-3.39 (m, 2H), 3.32-3.28 (m, 2H), 2.75 (s, 3H), 2.32-2.24 (m, 2H), 2.20-2.12 (m, 2H). $^{13}$C-NMR (CD$_2$OD, 100 MHz): $\delta$ 159.96, 156.05, 153.17, 139.22, 138.22, 134.08, 129.86, 128.90, 128.07, 127.89, 126.41, 126.28, 118.83, 105.10, 104.75, 68.56, 65.31, 40.53, 26.82, 10.57. Calculated exact mass for the protonated molecule (C$_{22}$H$_{23}$NO$_4$Cl): 400.1316; measured accurate mass (ESI): 400.1319. LC-MS purity: 100%, $t_R$ = 13.26 min.

![Diagram of compound 176](image2.png)

(3-Methylphenyl) methyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (176)

Prepared from a mixture of compound 120 (20 mg, 0.055 mmol), EDCI (11.3 mg, 0.06 mmol), hydroxybenzotriazole (9.4 mg, 0.07 mmol), 3-methyl-benzyl alcohol (7.1 µL, 0.06 mmol) and DIPEA (18.5 µL,
0.11 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as an off-white solid (9.4 mg, 35% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.40 (t, J=8.0 Hz, 1H), 7.33-7.28 (m, 3H), 7.22-7.17 (m, 2H), 7.14 (d, J=8.0 Hz, 1H), 6.86 (d, J=8.0 Hz, 1H), 5.36 (s, 2H), 4.96-4.91 (m, 1H), 3.46-3.40 (m, 2H), 3.33-3.28 (m, 2H), 2.78 (s, 3H), 2.39 (s, 3H), 2.33-2.25 (m, 2H), 2.21-2.13 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.22, 156.05, 153.16, 139.48, 138.11, 135.77, 128.79, 128.71, 128.17, 126.04, 125.14, 118.88, 105.10, 104.77, 68.53, 66.35, 40.55, 26.83, 20.00, 10.54. Calculated exact mass for the protonated molecule (C$_{23}$H$_{26}$NO$_4$): 380.1862; measured accurate mass (ESI): 380.1858. LC-MS purity: 100%, $t_R$ = 13.10 min.

(3-Methoxyphenyl) methyl 3-methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-carboxylate (177)

Prepared from a mixture of compound 120 (20 mg, 0.055 mmol), EDCI (11.3 mg, 0.06 mmol), hydroxybenzotriazole (9.4 mg, 0.07 mmol), 3-methoxyl-benzyl alcohol (7.4 µL, 0.06 mmol) and DIPEA (18.5 µL, 0.11 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as an off-white solid (10.9 mg, 39% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.40 (t, J=8.0 Hz, 1H), 7.33 (t, J=8.0 Hz, 1H), 7.13 (d, J=8.0 Hz, 1H), 7.07-7.05 (m, 2H), 6.95-6.92 (m, 1H), 6.85 (d, J=8.0 Hz, 1H), 5.37 (s, 2H), 4.94-4.91 (m, 1H), 3.83 (s, 3H), 3.46-3.40 (m, 2H), 3.33-3.28 (m, 2H), 2.78 (s, 3H), 2.32-2.25 (m, 2H), 2.20-2.13 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.17, 159.98, 156.06, 153.17, 139.44, 137.33, 129.34, 128.81, 126.10, 120.10, 118.87, 113.54, 113.42, 105.11, 104.77, 68.54, 66.16, 54.32, 40.55, 26.83, 10.56. Calculated exact mass for the protonated molecule (C$_{23}$H$_{26}$NO$_5$): 396.1811; measured accurate mass (ESI): 396.1798. LC-MS purity: 100%, $t_R$ = 12.65 min.
(2-Chlorophenyl) methyl 3-methyl-4-(piperidin-4-ylxy)-1-benzofuran-2-carboxylate (178)

Prepared from a mixture of compound 120 (40 mg, 0.11 mmol), EDCI (22.6 mg, 0.12 mmol), hydroxybenzotriazole (18.8 mg, 0.14 mmol), 2-chloro-benzyl alcohol (16.8 mg, 0.12 mmol) and DIPEA (37 µL, 0.22 mmol) in anhydrous acetonitrile (3 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as a white solid (25.2 mg, 45% overall yield). 

$^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.62-7.59 (m, 1H), 7.50-7.48 (m, 1H), 7.41-7.36 (m, 3H), 7.13 (d, J=8.0 Hz, 1H), 6.84 (d, J=8.0 Hz, 1H), 5.49 (s, 2H), 4.96-4.91 (m, 1H), 3.46-3.40 (m, 2H), 3.33-3.28 (m, 2H), 2.76 (s, 3H), 2.33-2.25 (m, 2H), 2.21-2.13 (m, 2H). 

$^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 159.94, 156.10, 153.19, 139.26, 133.55, 133.34, 130.26, 129.80, 129.30, 128.89, 126.93, 126.36, 118.84, 105.12, 104.77, 68.55, 63.60, 40.53, 26.82, 10.56. Calculated exact mass for the protonated molecule (C$_{22}$H$_{23}$NO$_4$Cl): 400.1316; measured accurate mass (ESI): 400.1317. LC-MS purity: 100%, $t_R$ = 13.15 min.

(2-Methylphenyl) methyl 3-methyl-4-(piperidin-4-ylxy)-1-benzofuran-2-carboxylate (179)

Prepared from a mixture of compound 120 (20 mg, 0.055 mmol), EDCI (11.3 mg, 0.06 mmol), hydroxybenzotriazole (9.4 mg, 0.07 mmol), 2-methyl-benzyl alcohol (7.2 mg, 0.06 mmol) and DIPEA (18.5 µL, 0.11 mmol) in anhydrous acetonitrile (2 mL), the N-Boc precursor was obtained. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the preparation of compound 160, to afford the title compound as an off-white solid (8.4 mg, 31% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.44 (d, J=7.2 Hz, 1H), 7.40 (t, J=8.0 Hz, 1H), 7.31-7.21 (m, 3H), 7.14 (d, J=8.0 Hz, 1H), 6.85 (d, J=8.0 Hz, 1H), 5.44 (s, 2H), 4.97-4.91 (m, 1H), 3.46-3.39 (m, 2H), 3.31-3.28 (m, 2H), 2.77 (s, 3H), 2.45 (s, 3H), 2.32-2.24 (m, 2H), 2.20-2.12 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 160.19, 156.07, 153.16, 139.48, 137.03, 133.69, 130.04, 129.26, 128.81, 128.45, 126.02, 125.71, 118.88, 105.12, 104.79,
68.52, 64.74, 40.54, 26.83, 17.61, 10.51. Calculated exact mass for the protonated molecule (C_{23}H_{26}NO_{4}): 380.1862; measured accurate mass (ESI): 380.1855. LC-MS purity: 100%, t_R = 13.02 min.

Benzyl 3-methyl-4-[[1-(propan-2-yl) piperidin-4-yl]oxy]-1-benzofuran-2-carboxylate (180)

Prepared from a mixture of compound 162 (20 mg, 0.042 mmol), acetone (10 µL, 0.13 mmol), acetic acid (15 µL, 0.26 mmol) and sodium triacetoxyborohydride (45 mg, 0.21 mmol) in THF (2 mL), according to the preparation of compound 112, the title compound was obtained as colourless oil (11 mg, 64% yield). 1H-NMR (CDCl_3, 400 MHz): δ 7.50 (d, J=7.2 Hz, 2H), 7.44-7.35 (m, 3H), 7.31 (t, J=8.0 Hz, 1H), 7.10 (d, J=8.0 Hz, 1H), 6.64 (d, J=8.0 Hz, 1H), 5.44 (s, 2H), 4.60-4.51 (m, 1H), 2.84-2.74 (m, 3H), 2.78 (s, 3H), 2.50 (brs, 2H), 2.14-2.04 (m, 2H), 2.01-1.90 (m, 2H), 1.09 (d, J=6.8 Hz, 6H). 13C-NMR (CDCl_3, 100 MHz): δ 160.43, 156.09, 154.22, 139.38, 135.81, 128.62, 128.36, 127.33, 125.88, 119.44, 104.94, 104.63, 72.52, 66.52, 54.59, 45.34, 30.85, 18.40, 11.65. Calculated exact mass for the protonated molecule (C_{25}H_{30}NO_{4}): 408.2175; measured accurate mass (ESI): 408.2168. LC-MS purity: 100%, t_R = 12.41 min.

Benzyl-4-[3-[(t-butylamino)propoxy]-3-methyl-1-benzofuran-2-carboxylate (181)

From a mixture of compound 55 (40 mg, 0.13 mmol), EDCI (27 mg, 0.14 mmol), hydroxybenzotriazole (22 mg, 0.17 mmol) benzyl alcohol (14.6 µL, 0.14 mmol) and DIPEA (45 µL, 0.26 mmol) in anhydrous acetonitrile (3 mL) according to the preparation of compound 160, the brominated precursor was obtained. This compound was further reacted with t-butylamine (11.5 µL, 0.11 mmol) in DMF (1 mL) according to the preparation of compound 1, to afford the title compound as an off-white solid (5.7 mg, 11% overall yield). 1H-NMR (CD_3OD, 400 MHz): δ 7.50 (d, J=7.2 Hz, 2H), 7.45-7.36 (m, 4H), 7.14 (d, J=8.0 Hz, 1H), 6.80 (d, J=8.0 Hz, 1H), 5.40 (s, 2H), 4.27 (t,
J=6.0 Hz, 2H), 3.26 (t, J=8.0 Hz, 2H), 2.75 (s, 3H), 2.30-2.23 (m, 2H), 1.42 (s, 9H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 161.68, 157.19, 156.49, 140.69, 137.21, 130.30, 129.67, 129.46, 127.59, 119.57, 106.05, 105.42, 67.72, 66.02, 58.09, 39.97, 27.78, 25.80, 11.82.

Calculated exact mass for the protonated molecule (C$_{24}$H$_{30}$NO$_4$): 396.2175; measured accurate mass (ESI): 396.2190. LC-MS purity: 97%, $t_R = 12.68$ min.

![Methyl-4-(piperidin-4-yloxy)benzofuran-2-yl]methyl benzoate (182)

Prepared from a mixture of benzoic acid (7.3 mg, 0.06 mmol), EDCI (12.6 mg, 0.066 mmol), hydroxybenzotriazole (10.5 mg, 0.08 mmol), compound 140 (30 mg, 0.06 mmol) and DIPEA (21 µL, 0.12 mmol) in anhydrous acetonitrile (3 mL) according to the preparation of compound 160, the N-trityl precursor was obtained. This compound was further deprotected in DCM containing 0.1% of trifluoroacetic acid and 0.2% of water (1 mL), according to the preparation of compound 141, to afford the title compound as colourless oil (2.3 mg, 11% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.05 (d, J=7.2 Hz, 2H), 7.63 (t, J=7.2 Hz, 1H), 7.50 (t, J=7.2 Hz, 2H), 7.26 (t, J=8.0 Hz, 1H), 7.10 (d, J=8.0 Hz, 1H), 6.82 (d, J=8.0 Hz, 1H), 5.48 (s, 2H), 4.96-4.86 (m, 1H), 3.46-3.40 (m, 2H), 3.32-3.28 (m, 2H), 2.56 (s, 3H), 2.30-2.23 (m, 2H), 2.21-2.13 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 166.15, 156.22, 151.86, 146.33, 133.02, 129.68, 129.18, 128.23, 125.62, 119.07, 115.27, 104.96, 104.47, 68.12, 56.47, 40.53, 26.89, 9.19. Calculated exact mass for the protonated molecule (C$_{22}$H$_{24}$NO$_4$): 366.1705; measured accurate mass (ESI): 366.1707. LC-MS purity: decompose under weakly acidic or LC-MS conditions.

4-[[3-Methyl-2-(2-phenylethyl)-1-benzofuran-4-yl]oxy] piperidine(186)

A mixture of compound 189 (13 mg, 0.039 mmol), palladium on carbon (10% w/w, 5 mg, 0.008 mmol) and 1,4-cyclohexadiene (70 µL) in ethanol (1 mL) was stirred at 80 °C for 4 hours. The reaction mixture was then concentrated in vacuo and the residue was purified by semi-
preparative reverse phase HPLC to give the title compound as colourless oil (6.6 mg, 38% yield). $^1$H-NMR (CD$_3$OD, 500 MHz): δ 7.23 (t, J=8.0 Hz, 2H), 7.18-7.11 (m, 4H), 7.03 (d, J=8.0 Hz, 1H), 6.74 (d, J=8.0 Hz, 1H), 4.85-4.81 (m, 1H), 3.40-3.35 (m, 2H), 3.30-3.25 (m, 2H), 3.00 (brs, 4H), 2.25-2.19 (m, 2H), 2.15-2.09 (m, 2H), 2.11 (s, 3H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 157.06, 153.11, 152.38, 142.37, 129.55, 129.31, 127.08, 125.06, 121.20, 111.15, 106.34, 105.55, 69.41, 41.90, 35.44, 29.07, 28.31, 10.17. Calculated exact mass for the protonated molecule (C$_{22}$H$_{26}$NO$_2$): 336.1964; measured accurate mass (ESI): 336.1964. LC-MS purity: 100%, $t_R$ = 13.30 min.

4-({3-Methyl-2-[(E)-2-phenylethenyl]-1-benzofuran-4-yl}oxy)piperidine(188)

To a mixture of compound 153 (50 mg, 0.14 mmol) and benzyltriphenyl phosphonium bromide (65 mg, 0.15 mmol) in DCM/water (2 mL, 1:1), 10M of NaOH solution (50 µL) was added. The resulting mixture was stirred at room temperature for 1 hour. After that, the reaction mixture was diluted with DCM (20 mL) and washed sequentially with water and brine (15 mL each). The organic layer was concentrated to give the mixture of the cis/trans N-Boc precursors. After that, the deprotection in DCM containing 5% TFA (2 mL) was carried out according to the method of ICL41, to isolate the title compound as the 1st major fraction from preparative HPLC (white solid, 4.9 mg, 8% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.41 (d, J=8.0 Hz, 2H), 7.34-7.26 (m, 3H), 7.17 (t, J=8.0 Hz, 1H), 6.89 (d, J=8.0 Hz, 1H), 6.78 (d, J=8.0 Hz, 1H), 6.72 (d, J=12.4 Hz, 1H), 6.51 (d, J=12.4 Hz, 1H), 4.89-4.85 (m, 1H), 3.44-3.37 (m, 2H), 3.31-3.26 (m, 2H), 2.54 (s, 3H), 2.32-2.22 (m, 2H), 2.19-2.11 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 156.93, 152.96, 150.05, 138.72, 132.03, 130.13, 128.88, 128.52, 127.58, 126.68, 120.99, 116.34, 106.39, 105.56, 69.57, 41.95, 38.32, 11.06. Calculated exact mass for the protonated molecule (C$_{22}$H$_{24}$NO$_2$): 334.1807; measured accurate mass (ESI): 334.1813. LC-MS purity: 96% (trans:cis = 3:1), $t_R$ = 13.22 min.
4-((3-methyl-2-((Z)-2-phenylethenyl)-1-benzofuran-4-yl)oxy)piperidine (189)

The title compound was isolated as the 2\textsuperscript{nd} major fraction from above preparative HPLC (off-white solid, 14.6 mg, 23\% overall yield). \textsuperscript{1}H-NMR (CD\textsubscript{3}OD, 400 MHz): \(\delta 7.58\) (d, \(J=7.2\) Hz, 2H), \(7.38\) (t, \(J=8.0\) Hz, 2H), \(7.27\) (t, \(J=7.2\) Hz, 1H), \(7.23-7.18\) (m, 3H), \(7.07\) (d, \(J=8.0\) Hz, 1H), \(6.78\) (d, \(J=8.0\) Hz, 1H), \(4.90-4.82\) (m, 1H), \(3.46-3.40\) (m, 2H), \(3.32-3.27\) (m, 2H), \(2.53\) (s, 3H), \(2.35-2.22\) (m, 2H), \(2.21-2.12\) (m, 2H). \textsuperscript{13}C-NMR (CD\textsubscript{3}OD, 100 MHz): \(\delta 157.20\), \(153.02\), \(150.95\), \(138.43\), \(129.81\), \(129.62\), \(128.88\), \(127.58\), \(126.80\), \(121.67\), \(115.10\), \(114.66\), \(106.55\), \(105.43\), \(69.62\), \(41.94\), \(28.34\), \(10.52\). Calculated exact mass for the protonated molecule (C\textsubscript{22}H\textsubscript{24}NO\textsubscript{2}): 334.1807; measured accurate mass (ESI): 334.1799. LC-MS purity: 97\%, \(t_R = 13.64\) min.

1-[3-Methyl-4-(piperidin-4-yloxy)-1-benzofuran-2-yl]-3-phenylpropan-1-one (190)

Prepared from a solution of compound 193 (8 mg, 0.017 mmol), palladium on carbon (10\% w/w, 7 mg, 0.004 mmol), and 1,4-cyclohexadiene (60 \(\mu\)L) in ethanol (1 mL), according to the preparation of compound 186, the title compound was obtained as a white solid (3.3 mg, 41\% yield). \textsuperscript{1}H-NMR (CD\textsubscript{3}OD, 400 MHz): \(\delta 7.44\) (t, \(J=8.0\) Hz, 1H), \(7.29-7.28\) (m, 4H), \(7.22-7.18\) (m, 1H), \(7.17\) (d, \(J=8.0\) Hz, 1H), \(6.88\) (d, \(J=8.0\) Hz, 1H), \(4.98-4.95\) (m, 1H), \(3.46-3.38\) (m, 2H), \(3.33-3.27\) (m, 2H), \(3.30\) (t, \(J=7.6\) Hz, 2H), \(3.04\) (t, \(J=7.6\) Hz, 2H), \(2.82\) (s, 3H), \(2.34-2.25\) (m, 2H), \(2.23-2.12\) (m, 2H). \textsuperscript{13}C-NMR (CD\textsubscript{3}OD, 100 MHz): \(\delta 194.25\), \(157.00\), \(154.95\), \(148.16\), \(142.45\), \(130.56\), \(129.42\), \(127.10\), \(126.01\), \(125.94\), \(120.65\), \(106.52\), \(106.33\), \(69.94\), \(42.50\), \(42.00\), \(30.75\), \(28.27\), \(11.90\). Calculated exact mass for the protonated molecule (C\textsubscript{23}H\textsubscript{26}NO\textsubscript{3}): 364.1913; measured accurate mass (ESI): 364.1904. LC-MS purity: 100\%, \(t_R = 13.10\) min.
Prepared from compound 71 (190 mg, 1.0 mmol), compound 103 (500 mg, 2.5 mmol), triphenylphosphine (657 mg, 2.5 mmol) and DIAD (0.48 mL, 2.5 mmol) in anhydrous THF according to the preparation of compound 106, the title compound was obtained as a light yellow solid (318 mg, 84% yield). 

\[^1\text{H-NMR}\, (\text{CDCl}_3, 400 \text{ MHz}): \delta\, 7.35 \, (t, \, J=8.0 \, \text{Hz}, \, 1\text{H}), \, 7.08 \, (d, \, J=8.0 \, \text{Hz}, \, 1\text{H}), \, 6.65 \, (d, \, J=8.0 \, \text{Hz}, \, 1\text{H}), \, 4.77-4.66 \, (m, \, 1\text{H}), \, 3.71-3.62 \, (m, \, 2\text{H}), \, 3.56-3.50 \, (m, \, 2\text{H}), \, 2.79 \, (s, \, 3\text{H}), \, 2.60 \, (s, \, 3\text{H}), \, 2.06-1.96 \, (m, \, 2\text{H}), \, 1.95-1.86 \, (m, \, 2\text{H}), \, 1.50 \, (s, \, 9\text{H}).\]

To a mixture of compound 192 (50 mg, 0.13 mmol) and benzaldehyde (15 µL, 0.15 mmol) in ethanol (1 mL) was added 0.25M NaOH solution (1 mL). The resulting mixture was stirred at room temperature for 3 hours. The reaction mixture was then diluted with 20 mL EtOAc, followed by the wash with water and brine (20 mL each). The organic layer was dried over anhydrous sodium sulphate and concentrated in vacuo. And the residue was purified by column chromatography over silica gel, eluting with hexane:ethyl acetate, 6:1, to give the N-Boc precursor. This compound was further deprotected in DCM containing 5% TFA (2 mL) according to the preparation of compound 109, to afford the title compound as a yellow solid (14.7 mg, 24% overall yield). 

\[^1\text{H-NMR}\, (\text{CD}_3\text{OD}, 400 \text{ MHz}): \delta\, 7.86-7.75 \, (m, \, 4\text{H}), \, 7.51-7.45 \, (m, \, 4\text{H}), \, 7.24 \, (d, \, J=8.0 \, \text{Hz}, \, 1\text{H}), \, 6.89 \, (d, \, J=8.0 \, \text{Hz}, \, 1\text{H}), \, 5.02-4.93 \, (m, \, 1\text{H}), \, 3.50-3.39 \, (m, \, 2\text{H}), \, 3.33-3.27 \, (m, \, 2\text{H}), \, 2.88 \, (s, \, 3\text{H}), \, 2.36-2.24 \, (m, \, 2\text{H}), \, 2.24-2.12 \, (m, \, 2\text{H}).\]

\[^{13}\text{C-NMR}\, (\text{CD}_3\text{OD}, 100 \text{ MHz}): \delta\, 183.19, \, 157.14, \, 154.95, \, 148.89, \, 145.04, \, 136.18, \, 131.91, \, 130.81, \, 130.14, \, 129.73, \, 127.50, \, 123.26, \, 120.94, \, 106.64, \, 106.38, \, 70.04, \, 41.99, \, 28.26, \, 12.17.\]

Calculated exact mass for the protonated molecule (C_{23}H_{24}NO_3): 362.1756; measured accurate mass (ESI): 362.1750. LC-MS purity: 97%, t_R = 13.17 min.
To a solution of 2, 6-dihydroxyacetophenone (0.5 g, 3.3 mmol) in ethanol/water (8 mL, 1:1), ethyl carbazate (340 mg, 3.3 mmol) was added. The resulting mixture was refluxed for 24 hours and allowed to stay overnight in fridge. The precipitate was filtered off, washed with diethyl ether and dried under vacuum, to afford the title compound as a yellow solid (180 mg, 24% yield).

\[ \text{N'-(2,6-dihydroxyphenyl)ethylidene-ethoxycarbohydrazide} \]

\[ (197) \]

To the solution of compound \( 197 \) (200 mg, 0.84 mmol) in DMF (2 mL) was added thionyl chloride (1.2 mL) at the temperature of \(-20^\circ C\). After the liberation of gas ceased, the reaction mixture was warmed up to room temperature and maintained for a further 3 hours. The excess of thionyl chloride and DMF were removed under pressure. The residue was re-dissolved by EtOAc (20 mL) and partitioned with 5% NaHCO\(_3\) (20 mL). The aqueous layer was further extracted by EtOAc (2 x 15 mL). The combined organic layers were sequentially washed by water and brine, dried over anhydrous sodium sulphate, concentrated in vacuo, to afford the title compound without further purification as a yellow solid (87 mg, 54% yield).

\[ \text{H-NMR (CD}_3\text{SOCD}_3, 400 MHz):} \]

\[ \delta 10.52 \text{ (brs, 2H), 9.77 (s, 1H), 7.20 (t, J=8.0 Hz, 1H), 6.64 (d, J=8.0 Hz, 2H). m/z (ESI)} \]

\[ 195.0 \text{ (M+H}^+) \].

A mixture of the compound \( 199 \) (50 mg, 0.26 mmol), benzyl chloride (33 µL, 0.28 mmol) and potassium carbonate (54 mg, 0.38 mmol) in dry acetone (3 mL) was stirred at 60 °C for 6 hours. After that, the reaction mixture was evaporated under pressure to dryness and the residue was purified by column chromatography over silica gel, eluting with hexane:ethyl acetate 91:9, 89:11,
86:14, to afford the title compound as a white solid (15 mg, 24% yield). $^1$H-NMR (CD$_3$COCD$_3$, 400 MHz): δ 7.34-7.23 (m, 5H), 7.14 (t, J=8.0 Hz, 1H), 7.01 (d, J=8.0 Hz, 1H), 6.86 (s, 1H), 6.68 (d, J=8.0 Hz, 1H), 4.23 (s, 2H). m/z (ESI) 257.1 (M+H$^+$)

**t-Butyl 4-[[2-(benzylsulfanyl)-1-benzofuran-4-yl]oxy]piperidine-1-carboxylate (205)**

Prepared from a solution of compound 200 (9 mg, 0.035 mmol), compound 103 (18 mg, 0.088 mmol), triphenylphosphine (24 mg, 0.088 mmol) and DIAD (17 µL, 0.088 mmol) in anhydrous THF (1 mL), according to the preparation of compound 106, the title compound was obtained as colourless oil (6 mg, 40% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 7.33-7.25 (m, 5H), 7.21 (t, J=8.0 Hz, 1H), 7.11 (d, J=8.0 Hz, 1H), 6.79 (s, 1H), 6.68 (d, J=8.0 Hz, 1H), 4.67-4.56 (m, 1H), 4.16 (s, 2H), 3.71-3.65 (m, 2H), 3.44-3.38 (m, 2H), 2.00-1.89 (m, 2H), 1.89-1.77 (m, 2H), 1.51 (s, 9H).

**Prepared from a solution of compound 205 (10 mg, 0.023 mmol) in DCM containing 5% TFA (1 mL), according to the preparation of compound 109, the title compound was obtained as colourless oil (9.7 mg, 93% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.27-7.18 (m, 6H), 7.14 (d, J=8.0 Hz, 1H), 6.84 (d, J=8.0 Hz, 1H), 6.79 (s, 1H), 4.87-4.79 (m, 1H), 4.17 (s, 2H), 3.47-3.37 (m, 2H), 3.29-3.19 (m, 2H), 2.26-2.16 (m, 2H), 2.12-2.04 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 157.90, 149.48, 148.69, 137.54, 128.54, 128.04, 126.91, 125.25, 119.76, 108.89, 106.51, 104.41, 68.78, 40.37, 38.59, 26.95. Calculated exact mass for the protonated molecule (C$_{20}$H$_{22}$NO$_2$S): 340.1371; measured accurate mass (ESI): 340.1369. LC-MS purity: 100%, t$_R$ = 12.80 min.
4-[[2-(Phenylmethane)sulfonyl]-1-benzofuran-4-yl]oxy)piperidine(209)

A mixture of compound 205 (20 mg, 0.046 mmol) and 3-chloroperbenzoic acid (77% w/w, 16 mg, 0.07 mmol) in DCM (1 mL) was stirred at room temperature for 1 hour. The reaction mixture was concentrated in vacuo, to give the N-Boc precursor without further purification. This compound was further deprotected in DCM containing 5% TFA (2 mL), to afford the title compound as pink oil (8.0 mg, 36% overall yield). ¹H-NMR (CD₃OD, 400 MHz): δ 7.52 (t, J=8.0 Hz, 1H), 7.46 (s, 1H), 7.35-7.21 (m, 6H), 6.99 (d, J=8.0 Hz, 1H), 4.98-4.92 (m, 1H), 4.72 (s, 2H), 3.48-3.39 (m, 2H), 3.29-3.19 (m, 2H), 2.28-2.18 (m, 2H), 2.14-2.07 (m, 2H). ¹³C-NMR (CD₃OD, 100 MHz): δ 157.59, 151.61, 148.00, 130.60, 129.39, 128.55, 128.22, 127.77, 117.35, 112.47, 106.75, 105.11, 68.95, 60.60, 40.31, 26.78. Calculated exact mass for the protonated molecule (C₂₀H₂₂NO₄S): 372.1270; measured accurate mass (ESI): 372.1263. LC-MS purity: 100%, tᵣ = 10.91 min.

4-[[3-Methyl-2-(3-phenyl-1,2,4-oxadiazol-5-yl)-1-benzofuran-4-yl]oxy]piperidine (213)

A mixture of compound 120 (25 mg, 0.066 mmol), EDCI (14 mg, 0.073 mmol), hydroxybenzotriazole (12 mg, 0.086 mmol) in anhydrous acetonitrile (2 mL) was stirred at room temperature for 30 minutes, and then treated with N'-hydroxybenzenecarboximidamide (10 mg, 0.073 mmol) and DIPEA (24 µL, 0.13 mmol). The resulting mixture was further stirred at room temperature for 12 hours. After that, the solution was evaporated to dryness in vacuo. The residue was re-dissolved in ethyl acetate (20 mL) and washed with brine, 5% NaHCO₃ and water (each 20 mL). The organic layer was dried over anhydrous sodium sulphate and concentrated in vacuo. The residue was then dissolved in dry toluene over 4Å molecular sieve and the resulting mixture was stirred at 110 °C for 4 hours. After that, the reaction mixture was filtered off and the filtration was concentrated in vacuo, to give the N-Boc precursor without further purification. This compound was further deprotected in DCM
Chapter 6 – Experimental section

containing 5% TFA (1 mL) according to the preparation of compound 109, to afford the title compound as a white solid (6.3 mg, 20% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 8.14 (d, J=7.6 Hz, 2H), 7.62-7.53 (m, 3H), 7.43 (t, J=8.0 Hz, 1H), 7.22 (d, J=8.0 Hz, 1H), 6.90 (d, J=8.0 Hz, 1H), 4.99-4.95 (m, 1H), 3.50-3.44 (m, 2H), 3.37-3.31 (m, 2H), 2.94 (s, 3H), 2.38-2.26 (m, 2H), 2.26-2.13 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 170.00, 169.76, 158.24, 154.51, 137.38, 132.64, 130.37, 130.10, 128.46, 127.83, 126.01, 120.24, 106.94, 106.12, 70.09, 42.02, 28.30, 11.78. Calculated exact mass for the protonated molecule (C$_{22}$H$_{22}$N$_3$O$_3$): 376.1661; measured accurate mass (ESI): 376.1659. LC-MS purity: 100%, $t_R = 13.33$ min.

4-{{[2-(3-Benzyl-1,2,4-oxadiazol-5-yl)-3-methyl-1-benzofuran-4-yl]oxy}piperidine (215)

Prepared from a mixture of compound 120 (30 mg, 0.08 mmol), EDCI (16.9 mg, 0.088 mmol), hydroxybenzotriazole (14 mg, 0.1 mmol), N'-hydroxybenzyl carboximidamide (13.2 mg, 0.088 mmol) and DIPEA (28 µL, 0.16 mmol) in anhydrous acetonitrile (2 mL), the ester was formed, followed by the thermal dehydration under refluxing toluene conditions to give the N-Boc precursor. This compound was further deprotected in DCM containing 5% TFA (1 mL) according to the preparation of compound 213, to afford the title compound as a white solid (11.6 mg, 29% overall yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 7.46-7.24 (m, 6H), 7.18 (d, J=8.0 Hz, 1H), 6.86 (d, J=8.0 Hz, 1H), 4.99-4.90 (m, 1H), 4.17 (s, 2H), 3.49-3.38 (m, 2H), 3.33-3.26 (m, 2H), 2.83 (s, 3H), 2.36-2.26 (m, 2H), 2.19-2.09 (m, 2H). $^{13}$C-NMR (CD$_3$OD, 100 MHz): δ 171.17, 169.92, 158.18, 154.46, 137.34, 137.04, 130.30, 130.06, 129.72, 128.16, 125.85, 120.21, 106.91, 106.09, 70.04, 41.97, 32.86, 28.25, 11.67. Calculated exact mass for the protonated molecule (C$_{23}$H$_{24}$N$_3$O$_3$): 390.1818; measured accurate mass (ESI): 390.1819. LC-MS purity: 100%, $t_R = 12.86$ min.
6.2 Biology

6.2.1 General Methods

The purities of all the molecules used for biological assays were at least 95% (except for three ether compounds), which were determined by analytical LC-MS (Section 6.1.1). The stock solutions for test molecules were either 50 mM or 10 mM in anhydrous DMSO.

The enzymes PfNMT and LdNMT were expressed by Dr James Brannigan at University of York and stored as 0.98 mg/mL 0.58 mg/mL respectively, in a solution of 25% glycerol, 40 mM Tris pH 7.5, 1.25 mM NaCl, 2 mM dithiothreitol (DTT), 2 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM ethylenediaminetetraacetic acid (EDTA). The enzymes HsNMT1 and HsNMT2 were expressed by Dr Franziska Meier, a past member of our group, and stored as 9.8 mg/mL and 1.33 mg/mL respectively, in a solution of 20% glycerol, 30 mM Tris pH 7.4, 2.5 mM DTT, 0.5 mM EGTA and 0.5 mM EDTA. The peptide substrate GLYVSRLFNRLFQKK(Biotin)-NH₂ was synthesized by Dr Paul Bowyer, another past member of our group, and stored as 20 mM in anhydrous DMSO. Both [9,10(n)⁻³H]myristoyl CoA (3.51 µM @ 57 Ci/mmol in 0.01 M sodium acetate, pH 5.6:ethanol (1:1) solution) and streptavidin PVT beads were purchased from GE Healthcare (The radioactive department now belongs to Perkin Elmer). All the other chemicals were obtained from Sigma-Aldrich. Enzyme assays were carried out in white opaque 96-well microplates, which were purchased from Perkin Elmer.

*Plasmodium falciparum* inhibition assays were performed by Dr David Moss at the National Institute of Medical Research (NIMR). Hydroethidine (HE) fluorescent stain was obtained from Polyscience. 2% Haematocrit are 2% red blood cells in media, i.e. 2 mL erythrocytes in 100 mL media. 70% Percoll was purchased from Amersham Biosciences (formerly known as GE Healthcare). Albumax was purchased from GIBCO and Giesma from BDH.
Leishmania Donovani ex-vivo amastigote inhibition and macrophage toxicity assays were carried out by Dr Daniel Paape at University of York. Alama blue was purchased from Trek Diagnostics; DMEM medium was obtained from Invitrogen (catalogue number: 41966029); All the other chemicals, including RPMI1640 medium, were purchased from Sigma Aldrich.

6.2.2 Biological Assays

6.2.2.1 Standard procedure for enzyme inhibition assay

Enzyme inhibition assay (in total 100 µL for each well) for NMTs contained variable amounts of an inhibitor, 9.4 ng of purified PfNMT (4.8 ng for LdNMT, 5.1 ng for HsNMT1 and 20.8 ng for HsNMT2), 62.5 nM \(^3\)H-Myristoyl-CoA (8 Ci/mmol) and 500 nM of peptide substrate in 4% DMSO buffer 1 (Table 6.1). Incubation was allowed to proceed for 30 min at 37 °C, followed by the addition of 100 µL bead-stop solution (buffer 3, Table 6.1) to terminate the reaction. Overnight settling of the beads resulted in an accurate reading, with signal-to-noise ratio at least 20. The instrument used for counting is a Plate Chameleon multilabel reader (Hidex Oy, Finland). Final concentration of 100 µM and 10 µM of inhibitors are used for the single-point assay. The readout of \(^3\)H-myristoylated peptide in the reaction lacking an inhibitor was defined as 100% activity (positive control), and that from the reaction with no enzyme was defined as 0% (negative control). The effect of an inhibitor at the single concentration was calculated as a percentage of the enzyme activity left (% activity left = (readout-negative) / (positive-negative) X 100%). In terms of the IC\(_{50}\) determination, the inhibitory activity of an inhibitor was measured according to the method described above with the concentrations ranging from 500 µM to 2 nM and the 50% inhibitory concentration (IC\(_{50}\)) of an inhibitor was calculated by a nonlinear regression analysis using GraFit 7.0.1 version (Erithacus Software Limited, UK). Single point assays were performed in triplicate and IC\(_{50}\) determination assays were carried out in duplicate.
Table 6.1 Components of the assay buffer system

<table>
<thead>
<tr>
<th>Buffer 1</th>
<th>Buffer 2</th>
<th>Buffer 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mM Tris</td>
<td>0.2 M phosphoric acid</td>
<td>Streptavidin PVT beads in PBS/0.05% NaN₃ (50 mg/mL); diluted 50 times</td>
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<tr>
<td>0.5 mM EGTA</td>
<td>Adjust to pH 4.0 with NaOH</td>
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<tr>
<td>0.5 mM EDTA</td>
<td>0.75 mM MgCl₂</td>
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<td>2.5 mM DTT</td>
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<td>Adjust to pH 7.4</td>
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<tr>
<td>0.1% Triton X-100</td>
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</tr>
</tbody>
</table>

6.2.2.2 $K_m$ determination of substrates in enzyme assays

The $K_m$ for myristoyl-CoA was evaluated by varying the concentration of $[^3]H$-myristoyl-CoA between 500 nM and 0.23 nM, at a fixed 500 nM concentration of the assay peptide. The $K_m$ with respect to the peptide was evaluated by varying its concentration over the range 10-0.04 µM, at a fixed concentration of $[^3]H$-myristoyl-CoA of 62.5 nM. The amount of beads added in stop solution was proportional to the amount of the assay peptide. Other substrates were kept the same to the inhibition assay described above except that there was no inhibitor involved. The reactions were quenched after 1, 3 and 5 min, and the initial reaction velocities were derived. Data were analyzed by nonlinear regression (Michaelis-Menten) and linear regression of a double-reciprocal plot (Lineweaver-Burk) using GraFit 7.0.1 version (Erithacus Software Limited, UK). Assays were performed once due to safety and cost considerations.

6.2.2.3 *P. falciparum* inhibition assay

*P. falciparum* parasites were routinely cultured in 2% haematocrit at 37 °C in RPMI 1640 containing albumax and glutamine. To synchronise parasites, heterogeneous populations containing late stages were first centrifuged onto a 70% Percoll cushion to separate late trophozoite/schizont from rings. Late trophozoite/schizonts were then harvested from the Percoll cushion, pelleted and washed twice in media to remove remaining Percoll. These parasites were re-cultured for 3-5 hours with fresh red blood
cells, allowing invasion to occur, and assessed using Giemsa-stained smears. Following invasion the culture was pelleted and incubated in a final concentration of 90% sorbitol at 37 °C for 10 min to remove remaining schizonts. Post sorbitol treatment, the culture was pelleted and washed twice in fresh media prior to re-suspension in media to 2% haematocrit for culturing.

For each assay, a 90 µL culture was placed into a well of a 96-well plate. And for each compound, a 10x serial dilution was made using a 10x stock of DMSO media (5% DMSO) so that in each case the final DMSO concentration was maintained at 0.5%. To each well 10 µL of this 10x stock was added to above 90 µL culture. Therefore, the final assay volume was 100 µL in each well.

Synchronous cultures of late trophozoite-stage parasites (1% parasitemia in 2% haematocrit) were grown with experimental inhibitors at different concentrations, with the range of 20-0.62 µM. Cultures were incubated at 37 °C for another cycle, which is 48 hours. Aliquots of 50 µL were removed from each well then, and added to 500 µL freshly diluted HE (1:200 dilution of 10 mg/mL DMSO stock in PBS) and incubated for 20 min at 37 °C. Samples were then diluted with 1 mL PBS to enable appropriate counts (5000 counts/s). Staining was stopped by storing samples on ice. Parasites cultured in medium in the absence of an inhibitor and non-infected red blood cells were used as positive and negative controls respectively.

Parasitemia was calculated using a FACSCalibur flow cytometer. When expressed as a histogram of HE intensity vs. number of erythrocytes, a distinct peak representing late trophozoite/schizont infected erythrocytes is evident and can be used to calculate parasitemia of cultures. And the growth inhibition at each concentration is calculated as % inhibition = [1-(readout-negative)/(positive-negative)] x 100%. All assays were carried out in triplicate.
6.2.2.4 L. donovani ex vivo amastigote inhibition assay

L. donovani amastigotes (5 X 10^5/well of 96-well plate) were incubated with test compounds, with the concentrations ranging from 75 μM to 0.034 μM in 200 μL RPMI 1640 medium supplemented with 20% heat-inactivated FCS, 100 μM adenine, 20 mM 2-[N-morpholino]ethanesulphonic acid (pH 5.5), 5 μM hemin, 3 μM biopterin, 1 μM biotin, 100 U penicillin and 100 μg streptomycin. After 72 hour incubation at 26 °C, alamar blue (10% v/v) was added to each well and the resulting mixture was incubated for additional 4 hours prior to measuring the fluorescence (excitation wavelength was 560 nm and emission was recorded with a 590nm±10nm bandpass filter, POLARstar Optima). Parasites cultured in medium alone were used as a positive control and medium without cells containing corresponding concentration of a compound was used as a medium control. Growth inhibition at each concentration was calculated as % inhibition of normalized data = [1-(readout-medium)/(positive-medium)] x 100%. Assays were carried out in triplicate.

6.2.2.5 Macrophage toxicity test

Bone marrow-derived macrophage (BMDM, 4.2 X 10^4/well of 96-well plate) were incubated with the same concentrations of test compounds as mentioned above in DMEM medium containing 4% L929 cell and 10% FCS (fetal calf serum). Cells were incubated at 37°C and 5% CO_2 for 72 hours. Aalamar blue (10% v/v) was added to each well and the resulting mixture was incubated for additional 4 hours prior to measuring the fluorescence. Medium without cells containing the corresponding concentration of a compound was used as a medium control. Assays were performed in duplicate.
6.2.3 Crystallography

Protein-ligand complex crystallisation

45 µL PvNMT (6 mg/mL in 10 mM Tris pH 7.0, 125 mM NaCl) was incubated overnight at 4°C with 5 µL of S-(2-oxo)pentadecyl-CoA (10 mM in 50% DMSO) and 1 mg of solid ligand. Crystals were grown by the vapour diffusion method. Hanging drops consisting of 1 µL protein mixture (after centrifugation to pellet any solid material) and 1 µL of reservoir solution containing 0.2 M ammonium sulphate, 25% (w/v) PEG 3350, 5% (v/v) DMF in 0.1 M Bis-Tris buffer, pH 6.0 were equilibrated against 0.8 mL reservoir solution at 20°C. Preliminary X-ray analysis established that the crystals belong to the orthorhombic space group \(P2_1_2_1_2_1\) with cell dimensions \(a = 57.36 \, \text{Å}, b = 121.63 \, \text{Å}, c = 179.05 \, \text{Å}\). With three molecules per asymmetric unit, the Matthew’s coefficient is 2.08 Å\(^3\)/Da corresponding to a solvent content of 41 %. Crystals were flash-vitrified directly in liquid nitrogen before storage.

Data collection, processing and structure solution

X-ray diffraction data were collected on synchrotron beamline I04-1 (\(\lambda = 0.9173 \, \text{Å}\)) at the Diamond Light Source (Oxford) and processed using HKL2000\(^{[190]}\) implemented in the CCP4 suite\(^{[191]}\). All data were used in molecular replacement calculations in the program MOLREP\(^{[192]}\) using the coordinate set corresponding to a monomer of PvNMT (without associated waters) as the search model. The output trimeric model found by molecular replacement was refined using maximum likelihood methods implemented in REFMACS\(^{[193]}\). 5% of the data were excluded for \(R\)-free calculations. The model was refined by iterative cycles of REFMACS using anisotropic temperature factors, interspersed with manual modelling and adjustments carried out in COOT\(^{[194]}\). Refinement was concluded with an \(R\)-factor of 19.9 % (\(R\)\(_{\text{free}}\) 26.9 %) for data in the range 30 - 2.2 Å.
### Appendix – Full biological data of test compounds

<table>
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<tr>
<th>Reference Number</th>
<th>Structure</th>
<th>IC$_{50}$ against NMTs</th>
<th>P. falciparum EC$_{50}$</th>
<th>L. donovani ex-vivo amastigote EC$_{50}$</th>
<th>Toxicity$^d$</th>
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</table>
| 1                | ![Structure 1](image) | Pf: 51.4 µM$^a$  
Ld: (2%)$^b$  
Hs1: (0%)  
Hs2: (1.3%) |  |  |  |
| 13               | ![Structure 13](image) | Pf: (31.0%)  
Ld: (60.0%) |  |  |  |
| 14               | ![Structure 14](image) | Pf: (0%)  
Ld: (0%) |  |  |  |
| 15               | ![Structure 15](image) | Pf: 9.0 µM  
Ld: (37.5%)  
Hs1: (0%)  
Hs2: (37.5%) |  | 16.9 ± 2.7 µM; prediction$^c$ |  |
| 16               | ![Structure 16](image) | Pf: 8.5 µM  
Ld: (44.1%)  
Hs1: (0%)  
Hs2: (34.3%) |  | 6.8 ± 0.7 µM; Hill slope: 4.4 |  |
| 17               | ![Structure 17](image) | Pf: 54.5 µM  
Ld: (61.6%)  
Hs1: (0%)  
Hs2: (0%) |  |  |  |
| 18               | ![Structure 18](image) | Pf: 84.4 µM  
Ld: (20.4%) |  |  |  |
| 19               | ![Structure 19](image) | Pf: (48.1%)  
Ld: (46.6%) |  |  |  |
| 20               | ![Structure 20](image) | Pf: (48.4%)  
Ld: (0%) |  |  |  |
| 21               | ![Structure 21](image) | Pf: (21.2%)  
Ld: (0%) |  |  |  |
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## Appendix – Full biological data

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<th>Ld (%)</th>
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*Note: Pf and Ld values indicate the percentage of Pf3D7 and L. donovani strains killed, respectively.*
### Appendix – Full biological data

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<thead>
<tr>
<th></th>
<th>Structure</th>
<th>Pf: (0%)</th>
<th>Ld: (44.3%)</th>
<th>92% parasite death at 5 µM</th>
<th>2.6 ± 0.2 µM; Hill slope: 1.1</th>
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<tr>
<td>64</td>
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<td>Ld: (21.8%)</td>
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<td>Hs2: (60.9%)</td>
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<td><img src="image" alt="Structure 65" /></td>
<td>Pf: (13.5%)</td>
<td>Ld: (39.1%)</td>
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<tr>
<td>72</td>
<td><img src="image" alt="Structure 72" /></td>
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<td>Ld: 32.6 µM</td>
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<td>Hs2: 81.9 µM</td>
<td>99% parasite death at 10 µM; 43% parasite death at 5 µM</td>
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<tr>
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<td><img src="image" alt="Structure 73" /></td>
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<td>1.3 ± 0.5 µM; Prediction c</td>
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<tr>
<td>81</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>98.3 µM</td>
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<td>83% parasite death at 10 µM; 44% parasite death at 5 µM</td>
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<tr>
<td>82</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>2.3 µM</td>
<td>27.8 µM</td>
<td>Hs1: 35.7 µM; Hs2: 41.6 µM</td>
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<td>Hs1: (0%); Hs2: (28.3%)</td>
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<tr>
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<td>Hs2</td>
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<tr>
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<td>&gt;100 µM</td>
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<td>50.6 µM</td>
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<tr>
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<td><img src="image" alt="Structure" /></td>
<td>6.8 µM</td>
<td>(58.4%)</td>
<td>(0%)</td>
<td>(52.0%)</td>
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<tr>
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<td>(0%)</td>
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<tr>
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<td>(0%)</td>
<td>(24.8%)</td>
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<td>Pf IC50 (µM)</td>
<td>Ld IC50 (µM)</td>
<td>Hs1 (IC50)</td>
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<td>(12.7%)</td>
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<td>112</td>
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<td>50.0</td>
<td>(51.5%)</td>
<td>16.6 ± 0.4</td>
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<td>(46.4%)</td>
<td>23.7 ± 1.4</td>
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<td>114</td>
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<td>8.0</td>
<td>117</td>
<td>17% parasite death at 20 µM</td>
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<tr>
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<td>(32.5%)</td>
<td>(0%)</td>
<td>4.1 ± 0.2</td>
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<tr>
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<td></td>
<td></td>
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<td>(14.7%)</td>
<td>(0%)</td>
<td></td>
</tr>
<tr>
<td>117</td>
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<td>22.0</td>
<td>8.6</td>
<td>5.3 ± 0.6</td>
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<tr>
<td>118</td>
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<td>(43.0%)</td>
<td>68.8</td>
<td>75% parasite death at 10 µM; 0% parasite death at 5 µM</td>
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<tr>
<td>119</td>
<td></td>
<td></td>
<td></td>
<td>(0%)</td>
<td>(0%)</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>121</td>
<td></td>
<td></td>
<td></td>
<td>(19.6%)</td>
<td>(31.7%)</td>
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### Appendix – Full biological data

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<th>ID</th>
<th>Chemical Structure</th>
<th>Pf (folding)</th>
<th>Ld (folding)</th>
<th>IC₅₀ (µM)</th>
<th>Additional Information</th>
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<tr>
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<td>(38.3%)</td>
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<tr>
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<td>(57.8%)</td>
<td>4.0 ± 0.7 µM; prediction c</td>
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<td>5.3 ± 1.1 µM; prediction c</td>
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<tr>
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<td>(28.8 µM)</td>
<td>(94.8 µM)</td>
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<td>(19.1%)</td>
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<tr>
<td>127</td>
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<td>(23.9%)</td>
<td>(29.3%)</td>
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<tr>
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<td>(13.0 µM)</td>
<td>(13.5 µM)</td>
<td>(30.5%)</td>
<td>(40.5%)</td>
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<tr>
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<td><img src="image" alt="Chemical Structure" /></td>
<td>(100 µM)</td>
<td>(48.8 µM)</td>
<td>(48.9%)</td>
<td>(68.1%)</td>
</tr>
<tr>
<td>130</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>(2.3 µM)</td>
<td>(5.3 µM)</td>
<td>(63.2%)</td>
<td>(50.0 µM)</td>
</tr>
<tr>
<td>131</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>(21.0 µM)</td>
<td>(7.5 µM)</td>
<td>(50.7%)</td>
<td>(61.5%)</td>
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<tr>
<td>132</td>
<td><img src="image" alt="Molecule Structure" /></td>
<td>Pf: (42.1%) Ld: (56.3%)</td>
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<tr>
<td>141</td>
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<td>Pf: (3.9%) Ld: (29.4%)</td>
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<tr>
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<td>Pf: 6.6 µM Ld: 2.6 µM Hs1: (39.6%) Hs2: (52.1%)</td>
<td>1.9 ± 0.1 µM; prediction (^c) Between 8.3 and 25 µM</td>
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<td></td>
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<tr>
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<tr>
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<tr>
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<td>Pf: 7.1 µM Ld: 2.5 µM Hs1: (53.6%) Hs2: (53.0%)</td>
<td>4.5 ± 1.9 µM; Hill slope: 2.1 11.0 ± 2.0 µM; prediction (^c) Between 8.3 and 25 µM</td>
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<tr>
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<td><img src="image" alt="Molecule Structure" /></td>
<td>Pf: (44.6%) Ld: 9.0 µM Hs1: (35.4%) Hs2: (25.0%)</td>
<td>44% parasite death at 25 µM Between 8.3 and 25 µM</td>
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<tr>
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<td>Pf: 50.0 µM Ld: 5.3 µM Hs1: (48.8%) Hs2: (62.9%)</td>
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<tr>
<td>156</td>
<td><img src="image" alt="Molecule Structure" /></td>
<td>Pf: (0%) Ld: (15.8%)</td>
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<tr>
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<td>Pf: 37.2 µM Ld: 13.5 µM</td>
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<td></td>
</tr>
</tbody>
</table>
### Appendix – Full biological data

| 158 | ![Chemical Structure](image1) | Pf: (40.9%) | Ld: 64.0 µM |  |  |
| 160 | ![Chemical Structure](image2) | Pf: (50.6%) | Ld: (40.8%) |  |  |
| 161 | ![Chemical Structure](image3) | Pf: 24.4 µM | Ld: 19.8 µM | 34% parasite death at 10 µM | 4.9 ± 1.1 µM; Hill slope: 0.9 | Between 25 and 75 µM |
| 162 | ![Chemical Structure](image4) | Pf: 0.27 µM | Ld: 4.7 µM | 1.2 ± 0.2 µM; prediction; Hill slope: 1.1 | Between 8.3 and 25 µM |
| 163 | ![Chemical Structure](image5) | Pf: 9.7 µM | Ld: 15.6 µM | 3.2 ± 0.8 µM; Hill slope: 2.5 | 3.6 ± 0.7 µM; Hill slope: 5.1 | Between 2.8 and 8.3 |
| 164 | ![Chemical Structure](image6) | Pf: 1.4 µM | Ld: 1.9 µM | 1.5 ± 0.2 µM; Hill slope: 1.5 | Between 2.8 and 8.3 µM |
| 165 | ![Chemical Structure](image7) | Pf: (18.4%) | Ld: (2.7%) |  |  |
| 166 | ![Chemical Structure](image8) | Pf: 4.1 µM | Ld: 12.6 µM |  |  |
| 167 | ![Chemical Structure](image9) | Pf: 36.2 µM | Ld: 81.7 µM |  |  |
| 173 | ![Chemical Structure](image10) | Pf: 5.6 µM | Ld: 16.4 µM | 89% parasite death at 5 µM; 18% parasite death at 2.5 µM | 100% parasite death at 8.3 µM; 30% parasite death at 2.8 µM | Between 8.3 and 25 µM |
## Appendix – Full biological data

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<thead>
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<th>Compound</th>
<th>Pf</th>
<th>Ld</th>
<th>Hs1 (%)</th>
<th>Hs2 (%)</th>
<th>Effect at 5 μM</th>
<th>Effect at 2.5 μM</th>
<th>IC50 (μM)</th>
<th>Hill slope</th>
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<td>174</td>
<td>2.1 μM</td>
<td>21.1 μM</td>
<td>(42.4%)</td>
<td>(57.8%)</td>
<td>93% parasite death</td>
<td>34% parasite death</td>
<td>93% parasite death at 5 μM; 34% parasite death at 2.5 μM</td>
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<tr>
<td>175</td>
<td>0.99 μM</td>
<td>4.1 μM</td>
<td>(55.8%)</td>
<td>(56.5%)</td>
<td></td>
<td></td>
<td>7.4 ± 2.9 μM; Hill slope: 1.2</td>
<td>Between 8.3 and 25 μM</td>
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<tr>
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<td>0.62 μM</td>
<td>2.8 μM</td>
<td>(50.1%)</td>
<td>(52.1%)</td>
<td></td>
<td></td>
<td>4.7 ± 0.2 μM; Hill slope: 4.5</td>
<td>Between 8.3 and 25 μM</td>
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<tr>
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<td>0.68 μM</td>
<td>2.2 μM</td>
<td>&gt;100 μM</td>
<td>182 μM</td>
<td>75% parasite death at 25 μM; 0% parasite death at 8.3 μM</td>
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<td></td>
</tr>
<tr>
<td>178</td>
<td>0.98 μM</td>
<td>7.9 μM</td>
<td>(31.2%)</td>
<td>(40.4%)</td>
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<td>1.9 μM</td>
<td>6.1 μM</td>
<td>(29.5%)</td>
<td>(57.6%)</td>
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<tr>
<td>180</td>
<td>1.4 μM</td>
<td>12.5 μM</td>
<td>(53.4%)</td>
<td>(55.2%)</td>
<td>4.7 ± 0.2 μM; Hill slope: 4.5</td>
<td>1.8 ± 0.4 μM; Hill slope: 0.9</td>
<td>Between 8.3 and 25 μM</td>
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<tr>
<td>181</td>
<td>1.5 μM</td>
<td>14.6 μM</td>
<td>37.6 μM</td>
<td>34.0 μM</td>
<td>89% parasite death at 5 μM; 25% parasite death at 2.5 μM</td>
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<tr>
<td>182</td>
<td>9.6 μM</td>
<td>3.3 μM</td>
<td>(49.1%)</td>
<td>(54.2%)</td>
<td>75% parasite death at 25 μM; 0% parasite death at 8.3 μM</td>
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<td>Between 25 and 75 μM</td>
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<td>186</td>
<td>131 μM</td>
<td>30.9 μM</td>
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<td></td>
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</table>
| 188 | ![Chemical Structure] | Pf: 26.1 µM  
Ld: 20.8 µM |  |  |
| 189 | ![Chemical Structure] | Pf: 72.0 µM  
Ld: 68.8 µM |  |  |
| 190 | ![Chemical Structure] | Pf: 10.2 µM  
Ld: 18.2 µM  
Hs1: (15.9%)  
Hs2: (45.7%)  
4.0 ± 0.3 µM;  
Hill slope: 4.7  
99% parasite death at 8.3 µM;  
8% parasite death at 2.8 µM  
Between 8.3 and 25 µM |  |  |
| 193 | ![Chemical Structure] | Pf: 3.5 µM  
Ld: 9.7 µM  
Hs1: 43.9 µM  
Hs2: 39.5 µM  
2.5 ± 0.1 µM;  
Hill slope: 3.6  
1.3 ± 0.02 µM;  
Hill slope: 4.4  
Between 8.3 and 25 µM |  |  |
| 208 | ![Chemical Structure] | Pf: 38.2 µM  
Ld: 25.1 µM |  |  |
| 209 | ![Chemical Structure] | Pf: (44.0%)  
Ld: (63.1%) |  |  |
| 213 | ![Chemical Structure] | Pf: 17.9 µM  
Ld: 26.8 µM |  |  |
| 215 | ![Chemical Structure] | Pf: 2.0 µM  
Ld: 10.2 µM  
Hs1: (36.9%)  
Hs2: (40.2%)  
2.1 ± 0.1 µM;  
Hill slope: 4.8  
1.5 ± 0.02 µM;  
Hill slope: 4.2  
Between 8.3 and 25 µM |  |  |

* The IC\textsubscript{50} values were averaged from two independent dose-response curves; the variation was generally <15%.  
\textsuperscript{b} The numbers in parentheses were the percentage inhibition values of compounds at 100 µM in triplicate; the variation was generally <10%.  
\textsuperscript{c} The value was calculated from the two single points of inhibitory data, using GraFit (version 7.0.1).  
\textsuperscript{d} Bone marrow derived macrophage was used in the assay.
References

6. MMV Annual report 2009, Medicine for malaria venture.


References


References


References


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