Design and Synthesis of Inhibitors of Dihydroorotate Dehydrogenase as Novel Anti-infectives

Robert David Starley

Submitted in accordance with the requirements for the degree of Doctor of Philosophy

University of Leeds

School of Chemistry

February 2020

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

Firstly, I would like to thank my supervisor Professor Colin Fishwick for giving me the chance to work on such an interesting PhD project and for his advice throughout. I would also like to thank Professor Peter Johnson, Professor Philip Kocienski and Dr Martin McPhillie for their support and guidance during my PhD.

The other members of the Fishwick group have been instrumental in supporting me and helping me to enjoy me time in the lab. In particular, I would like to thank Dr Elisabeth Ruethlein for teaching me how to carry out solubility assays, Dr Christopher Fitzpatrick for assistance with the computational aspects of my project and my MChem students Kyle Orritt and Omar Allam for their hard work and the synthesis of compounds.

I would like to acknowledge the support I have received from the technicians in the School of Chemistry, most notably Dr Mark Howard and Simon Barrett for NMR and Martin Huscroft for HPLC.

I greatly appreciate the work of the many different collaborators who have performed the various assays needed to assess my compounds. They are the groups of: Professor Margaret Phillips at UT Southwestern, Professor Rima MacLeod at University of Chicago, Professor Sue Charman at Monash University and the Drug Discovery Unit at the University of Dundee.

I would also like to thank my family for their love and support, in particular my parents and Aunt who spent many evenings proof-reading my thesis.

Finally, I want to thank the University of Leeds for the Leeds Anniversary Research Scholarship which has funded my PhD.

Abstract

Malaria remains a large burden in many areas of the world, with millions of deaths caused by the *Plasmodium* parasite each year. There are several existing treatments for malaria but none are perfect, especially with the increasing prevalence of resistant strains.

Dihydroorotate dehydrogenase (DHODH) is an enzyme in the *de novo* pyrimidine biosynthesis pathway. It is an attractive target for *Plasmodium* parasites in particular, since these species lack a salvage pathway and therefore rely solely on the *de novo* pathway.

In this project, a potent and selective series of inhibitors based on a 1,8-naphthyridine scaffold has been developed. Once a reliable synthetic route to the desired 1,8-naphthyridines had been established, a library of compounds was synthesised, allowing the series to be optimised with special focus on pharmacokinetic properties.

Initial compounds showed excellent selectivity and potency against both DHODH and *Plasmodium* parasite but solubility in fasted-state simulated intestinal fluid (FaSSIF) was quite poor and metabolism by aldehyde oxidase was detected. Subsequently, compounds were made which overcame this metabolic liability while retaining excellent potency (IC_{50} < 20 nM on *P. falciparum* DHODH, EC_{50} < 10 nM on *Plasmodium* cells) and with an improvement in solubility (> 50 μM in FaSSIF). These lead compounds will be taken forward for further assays to assess whether they could be used as novel anti-malarial drugs.

Toxoplasma gondii, the causative agent of toxoplasmosis, was also targeted with this series of molecules. However, *T. gondii* DHODH has a mutation which results in lower levels of inhibition with these 1,8-naphthyridines. By using computational design, potency against *T. gondii* was increased, albeit not to as high a level as against *P. falciparum*, with the best inhibitors for this species having EC_{50} values around 500 nM. The tight binding site in this species made the design of potent inhibitors challenging.

List of Abbreviations

ADME – Absorption, distribution, metabolism and excretion

AO – Aldehyde oxidase

APDTC – Ammonium pyrrolidinedithiocarbamate

ATP – Adenosine triphosphate

$AUC_{0–inf}$ – Area under the concentration versus time curve from time zero to infinity

$AUC_{0–last}$ – Area under the concentration versus time curve from time zero to the last time point with measurable concentration

AUC_{IV} – $AUC_{0–inf}$ after IV administration

$AUMC_{IV}$ – Area under the first moment of the concentration versus time curve from time zero to infinity after IV administration

BA – Oral bioavailability

BOC – ^{t}Butyloxycarbonyl

Boc_2O – Di-*tert*-butyl decarbonate

bp – Base pairs

B/P – Blood to plasma partitioning ratio

br – Broad

CDMT – 2-Chloro-4,6-dimethoxy-1,3,5-triazine

CL_{blood} – Blood clearance value

CL_{int} – Intrinsic clearance value

d – Doublet

DAST – Diethylaminosulphur trifluoride

DCIP – 2,6-Dichloroindophenol

DCM – Dichloromethane

DFMS – Bis(((difluoromethyl)sulfinyl)oxy)zinc

DHODH – Dihydroorotate dehydrogenase

DMA – Dimethylacetamide

DMAP – 4-Dimethylaminopyridine

DMF – Dimethylformamide

DMSO – Dimethyl sulfoxide

EC_{50} – Half-maximal effective concentration

EDTA – Ethylenediaminetetraacetic acid

E_H – Hepatic extraction ratio

E_R – Efflux ratio

ES – Electron spray

FaSSGF – Fasted-state simulated gastric fluid

FaSSIF – Fasted-state simulated intestinal fluid

FBS – Foetal bovine serum

FMN – Flavin mononucleotide

FTIR – Fourier-transform infrared spectroscopy

HEPES – 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

HFF – Human foreskin fibroblasts

HMQC – Heteronuclear multiple quantum coherence

HPLC – High-performance liquid chromatography

HPMC – (Hydroxypropyl)methyl cellulose

HsDHODH – *Homo sapiens* dihydroorotate dehydrogenase

IC_{50} – Half-maximal inhibitory concentration

IMDM – Iscove's modified Dulbecco's medium

λ_z – Terminal elimination rate constant

LB – Lysogeny broth

LCMS – Liquid chromatography–mass spectrometry

MIC – Minimum inhibitory concentration

MRSA – Methicillin-resistant *Staphylococcus aureus*

NADPH – Nicotinamide adenine dinucleotide phosphate

NCS – *N*-Chlorosuccinimide

NIS – *N*-Iodosuccinimide

NMM – *N*-Methylmorpholine

NMR – Nuclear magnetic resonance

p – Pentet

PAGE – Polyacrylamide Gel Electrophoresis

P_{app} – Apparent permeability coefficient

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PDB – Protein data bank

PfDHODH – *Plasmodium falciparum* dihydroorotate dehydrogenase

PPA – Polyphosphoric acid

PvDHODH – *Plasmodium vivax* dihydroorotate dehydrogenase

q – Quartet

RFU – Relative fluorescence units

RT – Room temperature

s – Singlet

SAR – Structure–activity relationship

SD – Standard deviation

SP – Standard precision

SIPR – 1,3-Bis(2,6-diisopropylphenyl) imidazolin-2-ylidene

siRNA – Small interfering ribonucleic acid

t – Triplet

TBHP – ^{t}Butyl hydroperoxide

TFA – Trifluoroacetic acid

TFAA – Trifluoroacetic anhydride

TgDHODH – *Toxoplasma gondii* dihydroorotate dehydrogenase

THF – Tetrahydrofuran

TPP – Triphenylphosphine

UMP – Uridine monophosphate

UPLC – Ultra performance liquid chromatography

V_{ss} – Apparent volume of distribution at steady state

WST – Water-soluble tetrazolium salt

YFP – Yellow fluorescent protein

Amino acids:

Ala (A) – Alanine

Arg (R) – Arginine

Asn (N) – Asparagine

Asp (D) – Aspartate

Cys (C) – Cysteine

Gln (Q) – Glutamine

Glu (E) – Glutamic acid

Gly (G) – Glycine

His (H) – Histidine

Ile (I) – Isoleucine

Leu (L) – Leucine

Lys (K) – Lysine

Met (M) – Methionine

Phe (F) – Phenylalanine

Pro (P) – Proline

Ser (S) – Serine

Thr (T) – Threonine

Trp (W) – Tryptophan

Tyr (Y) – Tyrosine

Val (V) – Valine

Table of Contents

Acknowledgements **3**

Abstract **4**

List of Abbreviations **5**

Table of Contents **9**

Chapter 1 Introduction **12**

1.1 The need for novel anti-infectives 12

1.1.1 The rise in drug-resistant infections 12

1.1.2 Malaria 14

1.1.2.1 Treatment 14

1.1.3 Toxoplasmosis 15

1.1.3.1 Treatment 16

1.2 Dihydroorotate dehydrogenase 17

1.2.1 Inhibitors of human DHODH 20

1.2.2 DHODH as a target for anti-infectives 22

1.2.2.1 Antibacterial agents 23

1.2.2.2 Anti-parasitic agents 24

1.2.3 Inhibitors of DHODH as anti-malarials 26

1.2.4 1,8-Naphthyridines as DHODH inhibitors 34

1.2.4.1 Structure-activity relationships 35

1.2.4.2 Synthetic routes to 1,8-naphthyridines 37

1.3 Project goals 39

1.3.1 1,8-Naphthyridines as DHODH inhibitors for the treatment of malaria 39

1.3.2 DHODH inhibitors as treatments for other infectious diseases 40

Chapter 2 Synthesis and Evaluation of 6-Chloro-1,8-naphthyridines **41**

2.1 Development of a new synthetic route to 4-anilino-1,8-naphthyridines 41

2.1.1 Biological and pharmacokinetic evaluation of 2-difluoromethyl-4-anilino-1,8-naphthyridines 48

2.2 Investigating substituents other than anilines at the 4-position of the 1,8-naphthyridines 53

2.3 Investigating more polar aniline substituents 57

2.4 Full evaluation of front-runner compound **55** 64

2.4.1 Mammalian DHODH screening 64

2.4.2 Metabolic stability 64
2.4.3 Cytochrome P450 inhibition 67
2.4.4 Permeability 67
2.4.5 *In vivo* pharmacokinetics 68
Chapter 3 Investigating Aldehyde Oxidase Metabolism 73
3.1 Aldehyde oxidase 73
3.2 Investigating AO susceptibility of a quinoline series using the 'litmus test' 74
3.3 *In vitro* metabolism of the quinoline series 76
3.4 Quinoline series conclusions 78
Chapter 4 1,8-Naphthyridines with a Substituent at the 7-Position 79
4.1 7-Amino- derivatives 79
4.1.1 Synthesis 80
4.1.2 Biological evaluation of 7-amino-1,8,naphthyridines 81
4.2 7-Methylamino- and dimethylamino derivatives 83
4.2.1 Synthesis 84
4.2.2 Biological evaluation 86
4.3 7-Methyl-1,8-napthyridines 90
4.3.1 Attempted synthesis of 7-methyl-1,8-naphthyridines 90
4.4 7-Difluoromethyl-1,8-napthyridines 90
4.4.1 Synthesis 90
4.5 Summary of 7-substituted 1,8-naphthyridines 92
Chapter 5 1,8-Naphthyridines with Different Substituents at the 6-Position 93
5.1 6-Fluoro- and 6-(trifluoromethyl)-1,8-naphthyridines 94
5.1.1 Synthesis 94
5.1.2 Biological evaluation 95
5.2 6-(Difluoromethyl)-1,8-naphthyridines 101
5.2.1 Synthesis of 6-(difluoromethyl)-1,8-naphthyridines 101
5.1.2 Biological evaluation 106
Chapter 6 1,8-Naphthyridines as Inhibitors of *Toxoplasma gondii* DHODH 111
6.1 Investigating whether anti-malarial 1,8-naphthyridines also have efficacy against *T. gondii* 111
6.2 *In silico* design of 1,8-naphthyridines with improved binding to *T. gondii* DHODH 114

6.3 Synthesis of putative inhibitors of *T. gondii* DHODH 116
6.4 Assessment of potency against *T. gondii* 117
6.5 1,8-Naphthyridines as inhibitors of *T. gondii* DHODH – conclusions 120
Chapter 7 Conclusion 121
7.1 1,8-Naphthyridines as DHODH inhibitors for the treatment of malaria 121
7.2 1,8-Naphthyridines as DHODH inhibitors for the treatment of toxoplasmosis 123
Chapter 8 Experimental 124
8.1 Biological assays 124
8.1.1 DHODH enzyme assays 124
8.1.1.1 Protein expression 124
8.1.1.2 Enzymatic assay 126
8.1.2 *P. falciparum* asexual blood stage assay 127
8.1.3 *T. gondii* assays 129
8.1.3.1 Tachyzoite assays 129
8.1.3.2 Cytotoxicity assay 130
8.1.5 Cytochrome P450 inhibition 131
8.1.6 Permeability 132
8.1.7 *In vivo* pharmacokinetics 133
8.1.7.1 Mouse 133
8.1.7.2 Rat 134
8.2 Solubility assay 135
8.3 Computational 136
8.3.1 Generation of TgDHODH homology model 136
8.3.2 Glide docking 137
8.4 General information 137
8.5 Synthetic procedures 138
9 Bibliography 205

Chapter 1 Introduction

1.1 The need for novel anti-infectives

The development of several potent anti-infectives, along with vaccines and increased understanding of how better to control the spread of diseases, led Nobel Prize-winning virologist Sir Frank Macfarlane Burnet to state in 1962 that: “By the end of the Second World War it was possible to say that almost all of the major practical problems of dealing with infectious disease had been solved”.[1] However, there were 16 million deaths caused by pathogens and parasites in 1990 and 15 million in 2010, and such diseases are still the largest cause of death in less developed countries.[2] This shows how little progress has been made in 20 years, a problem aggravated by the increase in resistance of disease-causing organisms to currently available medications.

1.1.1 The rise in drug-resistant infections

There are increasing numbers of infectious agents that have evolved resistance to some or all of the drugs that were previously used to treat them. Well-known examples of antibiotic-resistant bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae* and *Mycobacterium tuberculosis*, which is now commonly seen in multi-drug resistant and even extensively-drug resistant forms.[3] However, resistance has also been seen in almost all pathogenic bacteria and many eukaryotic parasites too.[4]

The fast replication rates of many micro-organisms have enabled them to quickly evolve to meet the selection pressure of antibiotics introduced by humans. Thus novel scaffolds with new modes of action are highly sought after in drugs to minimise the chance of resistance mechanisms already being present in nature. Over-use of antibiotics has hastened the rate of resistance appearing, with thousands of tons of antibiotics being used in agriculture and the treatment of human diseases over the past 60 years.[5] Reducing the use of antibiotics would therefore reduce the rate of resistance spreading but would not ultimately prevent it. Hence, new antibiotics are urgently required but, despite this, the amount of research carried out by pharmaceutical companies on the treatment of infectious diseases has been declining due to their relatively low rate of return on investment and the need to reduce the use of any new antimicrobials that are developed.[6] With this problem of anti-microbial resistance being described as “dire” by the World Health

Organisation,[7] it has largely been left to smaller research organisations to try to develop new treatments for the infectious diseases that cause so much suffering across the globe.

This is an equally large problem for parasitic diseases as it is for those caused by bacteria, with many parasites showing resistance to several first-line treatments.

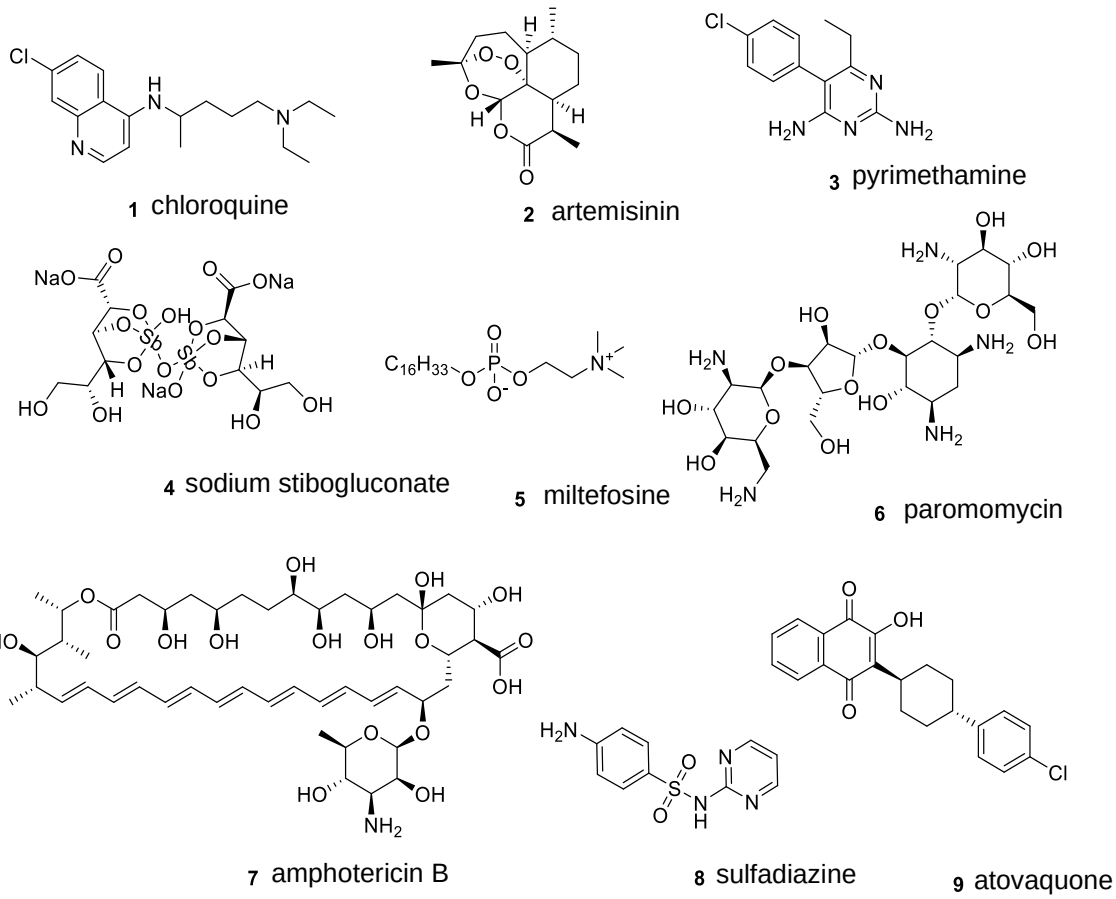


Figure 1.1 The structures of some anti-parasitics for which resistance has been observed

For example, *Plasmodium falciparum* now shows widespread resistance to chloroquine (**1**) and sulfadoxine–pyrimethamine treatments and increasingly to the current front-line artemisinin-based malaria treatments (**2**) too.[8] In this case the spread of resistance is thought to be due to the transmission of resistant parasites rather than frequent mutations occurring in the population.[9] Another parasite, *Leishmania*, is especially good at overcoming the effects of drugs: the extrachromosomal episomes found in these organisms contain genes which can quickly be up- or down-regulated as needed to combat a stressful environment.[10] The increased drug pressure on parasitic infections, coupled with poor quality mono-therapy treatments, has allowed for selection of resistant mutants and over time the effectiveness of drugs on the entire population of parasites has decreased significantly as multi-drug resistant

parasites have become more common. Multi-drug resistance has now been seen in parasites including *Plasmodia*, *Leishmania*, *Entamoeba*, *Trichomonas vaginalis*, schistosomes[11] and *Toxoplasma gondii*[12-14] against many different drugs (Fig. 1.1). Although combination therapies are reducing the rate at which resistance to a new drug spreads, there is a serious danger that these infections could soon become untreatable unless new anti-infectives can be developed.[15]

1.1.2 Malaria

Malaria is a disease that remains a major global problem, resulting in 219 million cases and 435000 deaths in 2017.[16] The cause of the infection in humans is by one of five different plasmodium species of parasite: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*; these are mostly spread through the bite of female *Anopheles* mosquitoes. The two most problematic are *P. falciparum*, which is found predominantly in Africa and causes the most deaths, and *P. vivax*, which can survive in a wider range of environmental conditions and hence is more widespread worldwide.[16] Malaria results in severe anaemia due to the bursting of erythrocytes by the parasite, eventually leading to death if not treated. The *P. vivax* species also has a dormant liver form (hypnozoite) which can activate at a later stage, resulting in a relapse of symptoms.[17]

Better prevention of malaria has largely contributed to the decrease in deaths, with vector control methods such as use of insecticide-treated mosquito nets shown to reduce the incidence of malaria by more than 50%.[18] Chemoprevention has also been largely successful, especially in pregnant women where sulfadoxine–pyrimethamine is commonly administered.

1.1.2.1 Treatment

For the treatment of uncomplicated *P. falciparum* malaria, artemisinin-based combination therapy is commonly used and is highly effective.[16] This involves the partnering of artemisinin (**2**) with one of several partner drugs. This is thought to reduce the chance of resistance to artemisinin occurring, as has happened with many previously used malarial drugs such as chloroquine. The short half-life is also thought to help prevent resistant parasites from evolving.[19] Nevertheless, resistance has been detected in five countries.[16]

This more expensive therapy is increasingly being used against *P. vivax* as well, now that resistance to chloroquine is emerging in this species too.[20] The only drug that can successfully treat the latent hypnozoite stage, however, is primaquine but this has some common side effects such as gastrointestinal

upset[21] and the drug cannot be given to pregnant or glucose-6-phosphate dehydrogenase-deficient people, as this can cause acute haemolysis. There are also some strains that are tolerant or resistant to primaquine.[22]

In conclusion, malaria continues to present challenges to medicinal chemists because of the constant need for new drugs to overcome resistance. There is also a need to improve on some of the limitations of current therapies, especially those targeting *P. vivax*.

1.1.3 Toxoplasmosis

Another example of an infection that remains a major global problem is that which leads to the disease toxoplasmosis, caused by the *Toxoplasma gondii* parasite that infects an estimated 30–50% of the world's population.[23] *T. gondii* is a protozoan, part of the phylum *Apicomplexa*, with three stages to its life cycle: sporozoites which are protected by oocysts in the environment and hence enable transmission to new hosts via ingestion, invasive tachyzoites which divide quickly, and bradyzoites which divide slowly but can remain in human cells for a long time (Fig. 1.2).[24]

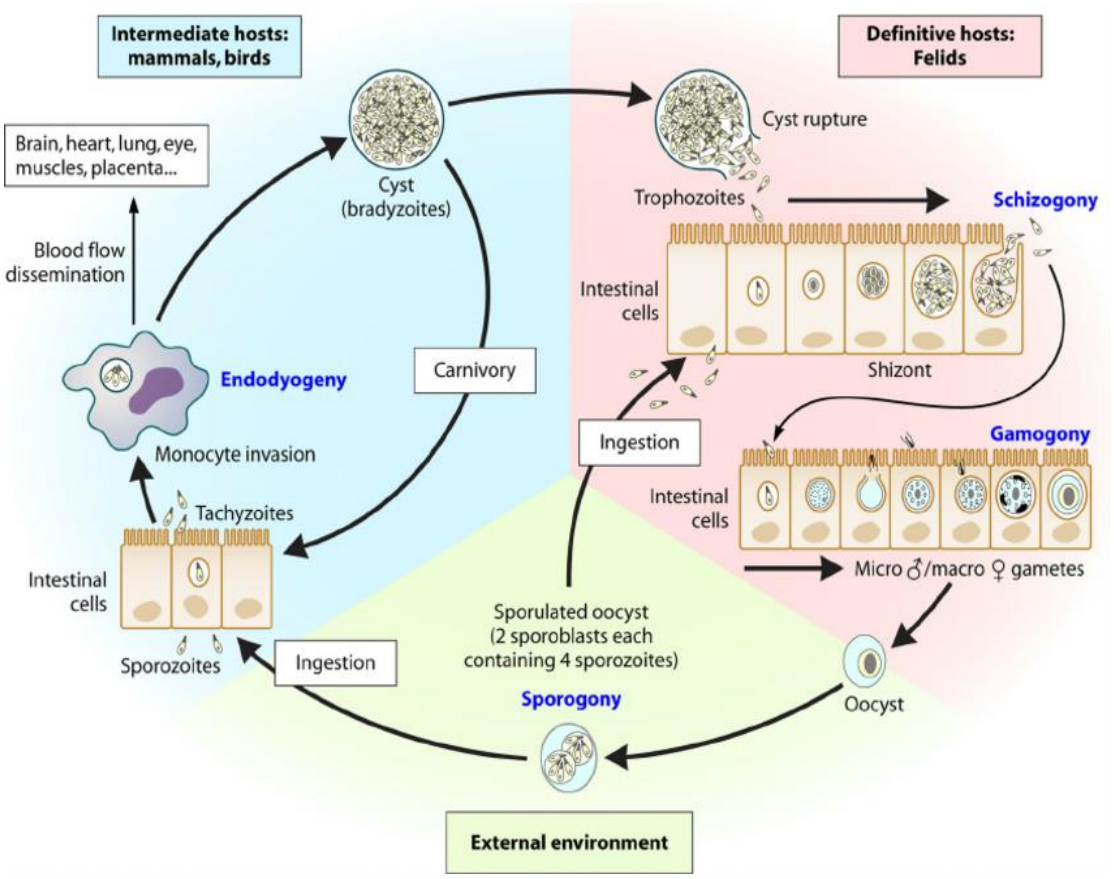


Figure 1.2 The life cycle of *T. gondii*. Reproduced from Robert-Gangneux and Dardé, 2012[24], with permission.

Humans can become infected by consumption of contaminated food or water. The tachyzoite form of the parasite occurs in acute infection and can infect any nucleated cell in the body. However, in the majority of cases, the immune system can fight off the infection before it results in illness. Nevertheless, there are some cases where infection can cause serious problems such as toxoplasmic retinochoroiditis, which is the leading cause of blindness in Brazil[25], one of the countries where toxoplasmosis is most prevalent.[26] People with weakened immune systems, such as those with AIDS or undergoing chemotherapy, are most susceptible, and an infection can be fatal for these patients if untreated. It is also possible for a pregnant mother to transfer the disease to her baby (congenital transmission) if she contracts an acute infection during pregnancy, even if this is asymptomatic. Infection can cause spontaneous abortion, hydrocephaly, mental retardation, hearing loss, recurrent retinochoroiditis or other defects.[27]

1.1.3.1 Treatment

First discovered by Eyles and Coleman[28] over 60 years ago, the most commonly used treatment for toxoplasmosis is still the combination of pyrimethamine (**3**) with sulfadiazine (**8**) which inhibits folic acid metabolism and hence DNA synthesis.[29]

Unfortunately these drugs, originally designed to treat malaria, can induce side effects including nausea, vomiting and diarrhoea as well as rashes[30], and only treat an acute infection, not the underlying bradyzoite stage. Although this latent stage has long been considered asymptomatic, there is evidence that infection can alter the psychology of the host. A striking example is in mice that lose their fear of cats, increasing the rate at which the parasite returns into a cat to complete its life cycle.[31] In humans there is some evidence of a possible link with mental disorders like schizophrenia[32] and bipolar disorder[33] and neurodegenerative disorders such as Parkinson's[34] and Alzheimer's[35], although this has been disputed.[36, 37] Atovaquone (**9**), another repurposed anti-malarial which acts against the cytochrome bc_1 complex of the parasite, can also be used for the treatment of toxoplasmosis.[13] This drug shows some activity against the chronic stage of the infection, albeit only at much higher doses than those needed for tachyzoites.

Strains of *T. gondii* that are resistant to either atovaquone[13, 38] or sulfadiazine have been observed[39, 40] and a mitochondrial protein, TgPRELID, has recently been discovered that is thought to help this parasite develop resistance to multiple drugs simultaneously.[12] Therefore, new inhibitors acting against novel targets are needed to solve this problem of drug resistance as well as the

issues with the existing drugs of side effects and lack of efficacy against bradyzoites.

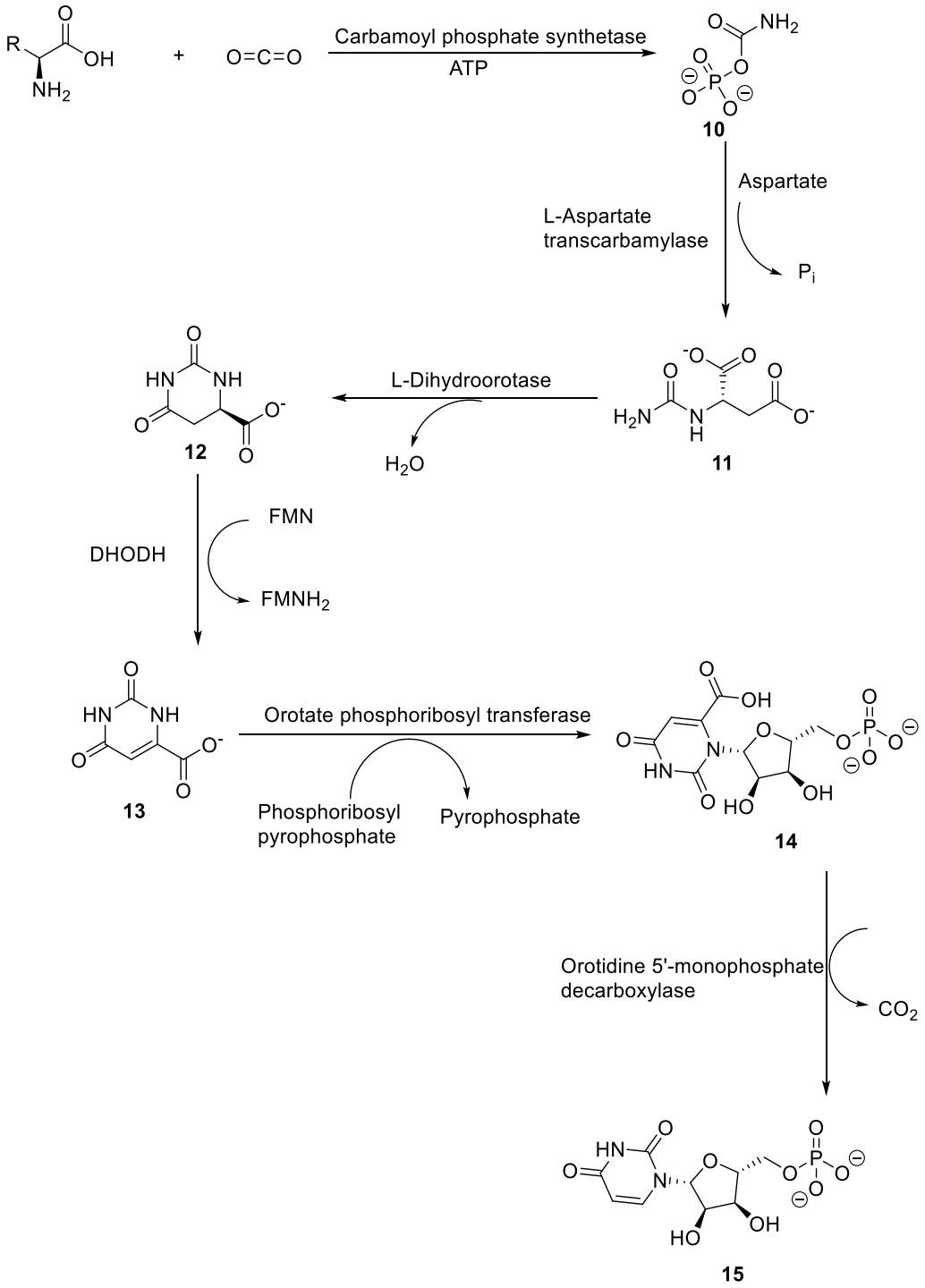
1.2 Dihydroorotate dehydrogenase

Dihydroorotate dehydrogenase (DHODH) is the fourth enzyme in the *de novo* pyrimidine nucleoside biosynthetic pathway (Scheme 1) in which uridine 5′-monophosphate (**15**) is made from an amino acid and can then be converted to the other necessary pyrimidines.

Many organisms also have a salvage pathway, enabling them to make UMP from any pyrimidine nucleotides taken up from food or the environment. Therefore the *de novo* pathway may not be essential for the survival of parasites, meaning that the salvage pathway may also have to be blocked in any potential treatment involving the inhibition of this enzyme.

In the fourth step of the *de novo* pathway, DHODH catalyses the FMN-mediated oxidation of dihydroorotate (**12**) to orotate (**13**). There are two different classes of this enzyme, determined by sequence alignment, that are further divided into subclasses depending on the oxidant used.[41] Class 1 DHODHs are found in the cytosol and use cysteine as the general base needed to catalyse the reaction (Fig. 1.3). This class is split into subclasses 1A and 1B; 1A uses fumarate to accept electrons and is homodimeric,[42] whereas 1B uses NAD^+ as an electron acceptor and forms heterodimers including class 1A-like subunits as well as different ones containing an iron–sulphur cluster and FAD.[43]

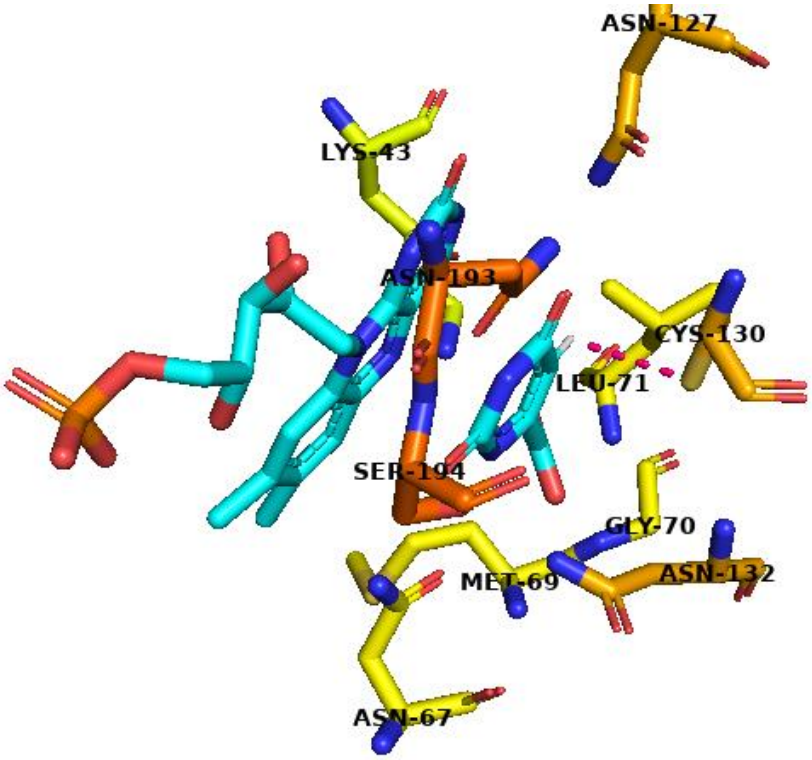
Class 2 enzymes differ in using serine as their base and coenzyme Q or ubiquinone as the oxidant (Fig. 1.3). They are also membrane-bound rather than free in the cytosol, being found in the inner mitochondrial membrane in eukaryotes[44] or the cell membrane in Gram-negative bacteria like *E. coli*.[45] Another difference between the two classes is seen in the mechanism of the reaction: the two C–H bonds breaking in this oxidation do so in a concerted manner in class 1 enzymes, as determined by kinetic isotope effect studies,[46] whereas a stepwise mechanism with enol formation followed by tautomerisation into orotic acid is preferred by class 2 enzymes.[47] In each case there are several conserved active-site residues, four asparagines and a threonine (or serine in class 1A), that are important for hydrogen bonding to the substrate (Fig. 1.3), and mutation of any of these results in a large decrease in rate constant.[48]



Scheme 1.1 The *de novo* biosynthetic pathway to pyrimidines in which an amino acid is converted into UMP in 6 steps catalysed by different enzymes

L. lactis

(Class 1)



E. coli

(Class 2)

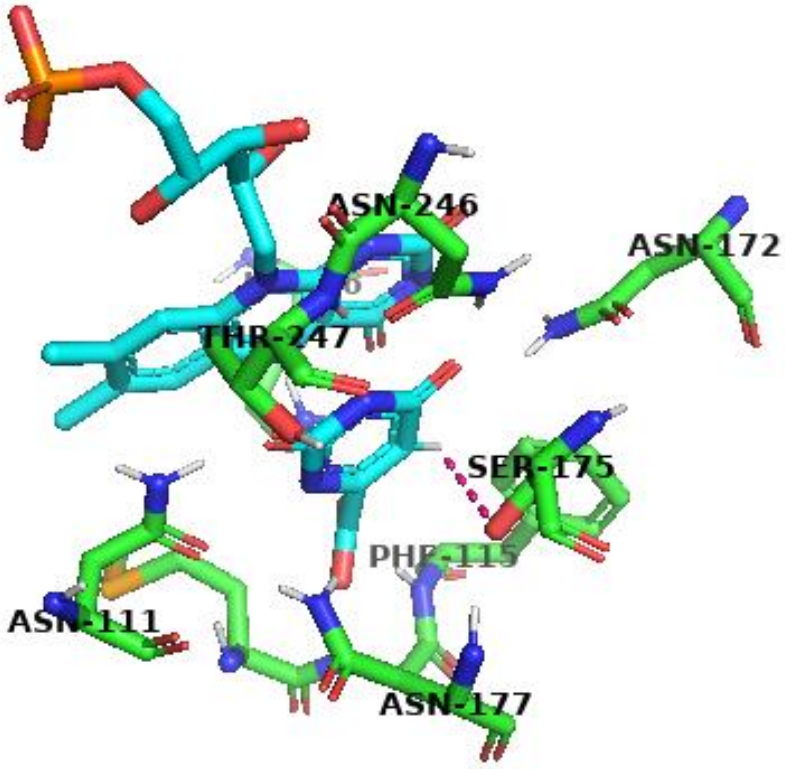
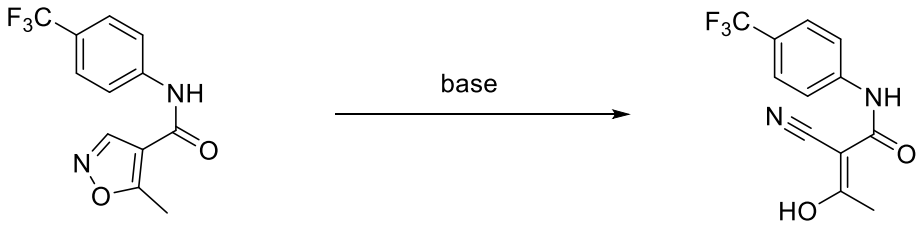


Figure 1.3 Substrate binding site of DHODH from an X-ray diffraction crystal structure of a class 1A enzyme from *L. lactis* (top) and a class 2 enzyme from *E. coli* (bottom). The orotic acid stacks above FMN and C-5 can be seen to be just below the catalytic base, with the interaction between the base and the hydrogen it removes shown as a pink dashed line. There are 5 conserved active site residues (4 asparagines and a serine or threonine) that form hydrogen bonds to the substrate. Image created from structures 2DOR and 1F76 in the PDB.

1.2.1 Inhibitors of human DHODH

Human DHODH is non-essential in most cells, which are able to obtain enough pyrimidines by the salvage pathway. Hence there has now been substantial work done to try to discover inhibitors of DHODH, especially for medicinal purposes. The first to be used in human medicine was leflunomide (**16**), for the treatment of rheumatoid arthritis.[49] This is a prodrug which undergoes a base-mediated ring-opening in the plasma (Scheme 1.2) to form the active drug, teriflunomide (**17**).



16 **17**

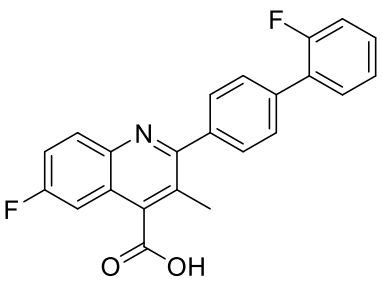
Scheme 1.2 The spontaneous *in vivo* ring-opening of leflunomide to produce the active drug molecule teriflunomide.

This can then inhibit DHODH by binding to a hydrophobic channel found only in class 2 enzymes and hence block the production of UMP by the *de novo* pathway.

Since activated autoimmune lymphocytes require eight times the normal amount of UMP for their cell cycle progression, they can be selectively inhibited over other cells which can obtain enough pyrimidines by the salvage pathway.[50] Thus immunomodulation to reduce the symptoms of arthritis is possible with few side effects seen at clinical dosage levels.

Leflunomide has also been used to treat multiple sclerosis[51] and has been suggested as an immunosuppressive which acts as an antiviral against cytomegalovirus, a cause of transplant failure.[52]

Several other inhibitors of human DHODH have been developed, including the anti-cancer compound brequinar (**18**) (Fig. 1.4).[53, 54]



18

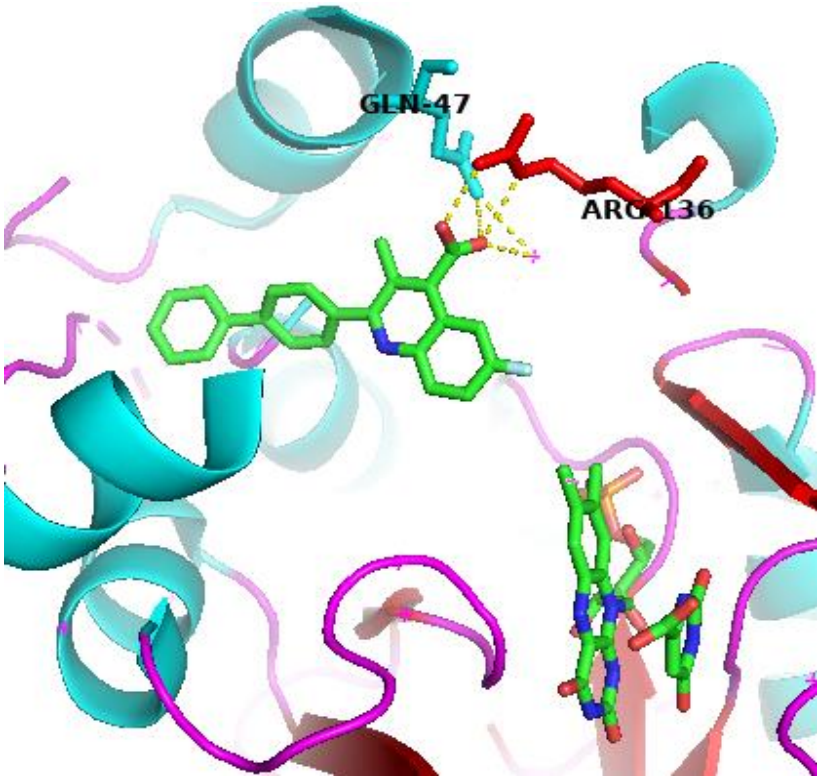
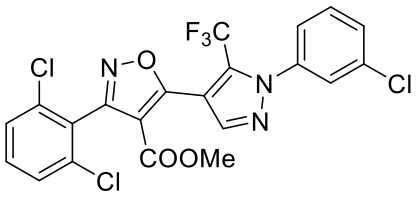


Figure 1.4 Structure of human DHODH with brequinar bound in the hydrophobic channel. Orotate and FMN are shown bound in the narrow polar region. Hydrogen bonds between brequinar and arginine and glutamine residues are shown by yellow dashes. Image created from structure 1D3G in the PDB.

Inhibition of host pyrimidine biosynthesis could also be a good way to treat infections caused by a virus due to the reliance of the virus on its host for the supply of such molecules. In one such example, brequinar had also been shown to inhibit dengue virus by lowering the intracellular concentration of pyrimidines[55] and so inhibitors of human DHODH were developed in the hope of finding a new treatment for dengue, a mosquito-borne disease resulting in an estimated 96 million symptomatic infections annually.[56] This used a cell-based high-throughput screening approach to find an inhibitor, **19**, with high potency against a cell culture assay (EC_{90} of 5.2 nM) that could be reversed by adding uridine.[57]



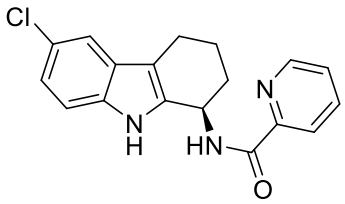
19

Unfortunately the compound was not effective in mouse models, thought to be due to the failure of the compound to significantly reduce pyrimidine levels

inside cells because of uptake from the diet via the salvage pathway. Another possibility is that the compound does not get into cells at high enough concentration due to its high level of binding to plasma proteins.[57]

Nevertheless, such a molecule could potentially be used as part of a combination therapy along with a nucleoside analogue to block viral RNA synthesis.[58]

Another example is a GSK compound (**20**) which has been shown to have broad antiviral activity by inhibiting DHODH,[59] with EC_{50} values of 10–40 nM against cells infected with human T-cell lymphotropic virus type 1, Simian virus 40, adenovirus type 5, Epstein-Barr virus or human papillomaviruses.[60]



20

1.2.2 DHODH as a target for anti-infectives

DHODH has been validated as a potential drug target in several species that cause infectious diseases. The high similarity between human DHODH and the enzyme found in these target organisms (Fig. 1.5) makes achieving selectivity challenging but nevertheless this has been achieved to a good level in many cases.

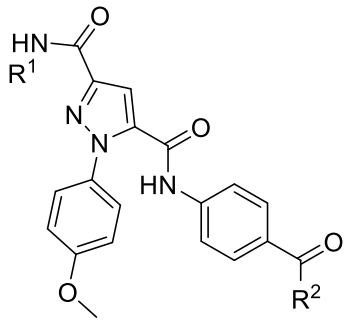
Figure 1.5 Sequence alignment of DHODH proteins from *Homo sapiens*, *Helicobacter pylori*, *Plasmodium falciparum*, *Toxoplasma gondii* and *Trypanosoma brucei*. Red shows highly conserved amino acids and blue those that are less conserved. Protein sequence data obtained from NCBI database.

1.2.2.1 Antibacterial agents

One bacterial organism for which DHODH has been targeted is the Gram-negative bacterium *Helicobacter pylori* which causes gastritis and gastric ulcers, with 25–75% of people infected.[61] The lack of many enzymes of the pyrimidine salvage pathway suggests that this bacterium should be dependent on the *de novo* pathway and this has been confirmed to be the only way for *H. pylori* to increase its pyrimidine levels.[62] As other bacteria still have salvage pathways present, good selectivity should be achievable by targeting its class 2 DHODH which would reduce the side effects seen with previous

treatments which indiscriminately kill gastrointestinal flora. *H. pylori* is capable of genetic exchange with other bacteria so selectively targeting this species could also reduce the chance of resistance occurring. There are some significant differences with human DHODH, especially in the coenzyme Q binding pocket, and inhibitors such as brequinar were shown to have no effect on Gram-negative bacterial DHODH (*H. pylori* enzyme expressed in *E. coli*) at concentrations up to 100 µM.[63]

Following on from this, a high-throughput screen against the *H. pylori* enzyme yielded pyrazole compounds of general structure **21** with K_i values as low as 12 nM for R^1 = benzyl, R^2 = N-pyrrolidine.[63] This inhibitor was shown to bind competitively with respect to coenzyme Q and uncompetitively with respect to dihydroorotate, providing evidence for binding in the coenzyme Q binding pocket. Further evidence was provided by a photolysable cross-link introduced into an analogue of **21** which showed irreversible inhibition of the enzyme that was reduced by increased concentrations of coenzyme Q_o.



21

These molecules did not inhibit human DHODH (K_i > 100 µM for all tested variants) but did inhibit the *E. coli* version of the enzyme to a similar degree to *H. pylori*. However, in a cell-based assay, the minimum inhibitory concentration (MIC) was 1–16 µg/mL for *H. pylori* compared to more than 50 µg/mL for all other tested bacteria and human cells,[63] suggesting that the presence of a salvage pathway in these organisms could be the reason for the observed selectivity.

1.2.2.2 Anti-parasitic agents

Trypanosomes, a complex of single-cell protozoan parasites, are another organism in which the validity of DHODH as a drug target has been investigated. These blood-borne organisms must reproduce constantly in order to evade the immune system so nucleotide biosynthesis is potentially a good target. Because of the lack of a purine biosynthesis pathway, the purine salvage pathway was the first choice to be blocked, but the great redundancy in this system means that targeting any transporter[64] or enzyme in this

pathway had little effect on the survival of the parasite.[65] Therefore the enzymes of the *de novo* pyrimidine biosynthetic pathway, such as DHODH, have been investigated for their validity as drug targets.

This enzyme had already been shown to be vital in *T. cruzi*, with interruption of the gene leading to non-viability of cells which was not rescued by the addition of pyrimidines to the growth medium.[66] Therefore, this target, a class 1A DHODH, was studied in *T. brucei*, the parasite which causes African sleeping sickness. With several differences in sequence from the human enzyme, especially in the FMN binding site which has only 25% sequence identity (Fig. 1.5) and two of the five residues forming hydrophobic interactions being different, there is potential to design a selective inhibitor of the parasite enzyme. The dimethylbenzene part of FMN is also solvent accessible in the *T. brucei* enzyme but blocked by the N-terminal domain in class 2 DHODHs, further increasing the possibility of creating selective inhibitors.

When siRNA was used to knock down the gene to less than 5% of normal levels, there was little change to the growth rate in normal medium; it was only when using dialysed serum without any pyrimidines that growth was significantly reduced.[67] This suggests that the salvage pathway is able to provide enough pyrimidines from the medium so the experiment was repeated in the presence of 5-fluorouracil, an inhibitor of pyrimidine uptake.[68] Only then was significant growth inhibition observed in a standard medium, with an IC_{50} value of 0.64 μM reported with siRNA compared with 10.4 μM without,[67] possibly due to increased uptake of 5-fluorouracil in response to the inhibition of the *de novo* pathway. Thus a potential combination therapy for African sleeping sickness could use a DHODH inhibitor and 5-fluorouracil, which is already an approved drug.

Although *T. gondii* has a functional salvage pathway, the *de novo* pyrimidine biosynthesis pathway has been shown to be necessary for virulence. Knocking out the uracil phosphoribosyltransferase gene required for the salvage pathway did not affect growth of tachyzoites, suggesting that the *de novo* pathway is used for growth. Mutants lacking carbamoyl phosphate synthetase II cannot replicate in cell culture without added uracil and were shown to not be virulent in mice, reinforcing the theory that the *de novo* pyrimidine biosynthetic pathway could be targeted for drug discovery.[69]

The DHODH gene in *T. gondii* has been sequenced and shown to have greatest sequence homology with the class 2 enzymes and to locate to the mitochondria as expected for this class. Attempts have been made to delete it but this has not been possible, indicating that it is an essential gene.[70]

Deletion was only possible when replaced with a catalytically deficient version of the enzyme by mutation of Asn-365 or Asn-468 to alanine in order to remove the hydrogen bonds analogous to those formed by Asn-172 and Asn-246 in *E.coli* (Fig. 1.3). These mutants showed significant increases in K_m and decreases in K_{cat}, and were shown to be uracil auxotrophs. This suggests a second, pyrimidine-independent, function of DHODH in *T. gondii* that is required for mitochondrial function, and so inhibitors of this enzyme could potentially block both pyrimidine biosynthesis and this other essential mitochondrial role, making it an interesting target for drug discovery.[71]

Inhibitors of mammalian DHODH have also been shown to be poor inhibitors of the *T. gondii* enzyme[70] so good selectivity could be achievable. 1-Hydroxyquinolones, similar in structure to ubiquinone, have been shown to competitively inhibit *T. gondii* DHODH with high nanomolar potency, competing with coenzyme Q. Since co-expression of the ubiquinone independent *S. cerevisiae* DHODH led to partial 1-hydroxyquinolone resistance, this suggests that the mechanism of action is by both pyrimidine depletion and lack of ATP by stopping the transfer of electrons to the mitochondrial electron transport chain.[72] This acts as proof-of-concept for using small molecules to inhibit *T. gondii* DHODH and hence develop new treatments for toxoplasmosis. Now that recombinant enzyme is available[70] it is hoped that an X-ray crystal structure of *T. gondii* DHODH will soon be obtained in order to aid with structure-based drug design for this target.

However, it is as an anti-malarial target that most work has been done on DHODH. This is because *P. falciparum* has been sequenced and found to have a complete *de novo* pyrimidine biosynthetic pathway[73, 74] but no salvage pathway. Studies using siRNA to block expression of DHODH saw the expected inhibition of *P. falciparum* growth.[75] Therefore, this pathway has been validated as a drug target for new antimalarials.[76] This has led to multiple efforts to find inhibitors for the class 2 *P. falciparum* DHODH.

1.2.3 Inhibitors of DHODH as anti-malarials

The first *de novo* designed inhibitors of *P. falciparum* DHODH were reported in 2006 by a group at the University of Leeds. This involved the application of computational design to the X-ray crystal structure of DHODH to look for molecules that would form hydrogen bonds to two highly conserved residues in class 2 DHODHs, His-185 and Arg-265, in the ubiquinone binding site, a channel leading to the active site (Fig. 1.6).[74]

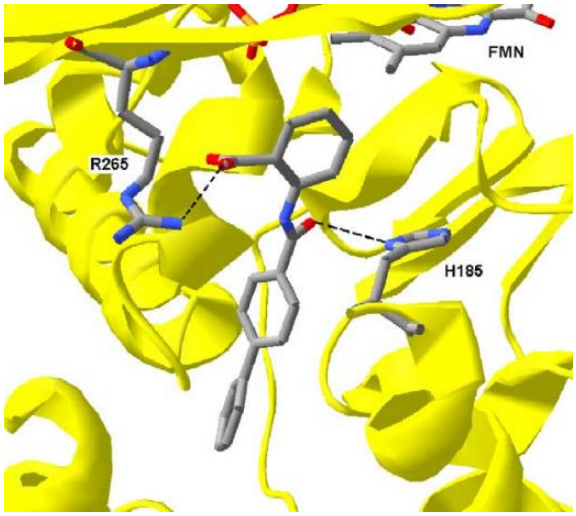


Figure 1.6 A SPROUT-designed molecule showing predicted hydrogen bonds in the ubiquinone binding site of PfDHODH reproduced from Heikkilä et al, 2006[74], with permission.

While these molecules, such as **22,** displayed only micromolar inhibition of the enzyme (Table 1.1), the best inhibitors found for the *Plasmodium* enzyme did not significantly inhibit human DHODH, demonstrating that selectivity is possible and providing a starting point for the development of future inhibitors.

Several other molecular scaffolds were also developed over the following years including a series containing a polar "head group" and hydrophobic aromatic region (e.g. **23**), predicted to make hydrogen bond contacts with the conserved amino acid residues His-185, Arg-265, and Tyr-528 and hydrophobic interactions with the hydrophobic cavity.[77]

A similar molecule to this series with polar and hydrophobic terminal portions is teriflunomide (**17**), and this has been computationally optimised to bind to PfDHODH, resulting in a series of analogues, including **24,** that were synthesised and tested for inhibition.[78]

Other such analogous molecules to be made were based on an *N*-arylaminomethylene malonate scaffold.[79] A high-throughput screen has identified an *N*-alkyl-5-(1H-benzimidazol-1-yl)thiophene-2-carboxamide[80] scaffold whose structure–activity relationships (SAR) have been investigated and the structure optimised to a lead compound (**25**) that proved efficacious in a mouse model of malaria with good selectivity over the human enzyme.[81] Further SAR and optimisation of the ADME properties resulted in a compound that was orally bioavailable and hence was chosen as a drug development candidate.[82] The structures of some of the most potent inhibitors discovered in each of these studies can be found in Table 1.1, along with their reported IC_{50} values for PfDHODH. Whilst it is not possible to directly compare these values, due to differences in the assays used to generate them, they provide

an idea of some of the PfDHODH inhibitors that have previously been made and their potency.

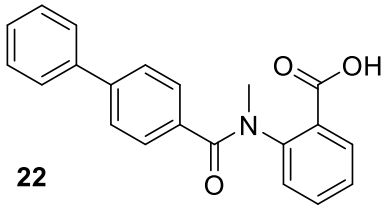
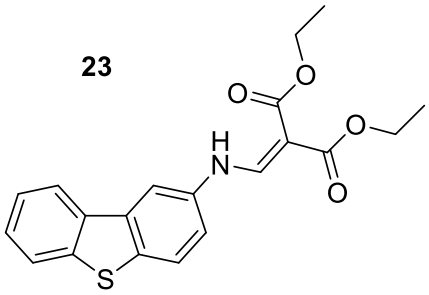
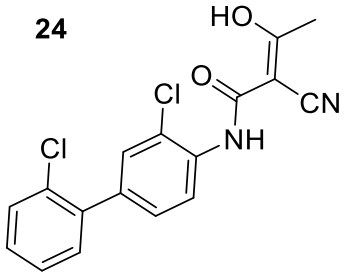
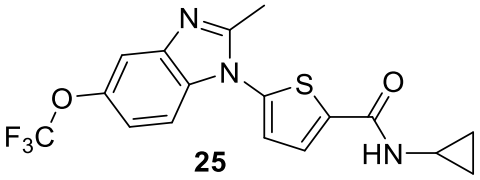
Molecule	IC_{50} for PfDHODH/µM
 **22**	42.6[74]
23 	0.16[77]
24 	4.0[78]
 **25**	0.022[81]

Table 1.1 The structures of some reported inhibitors of PfDHODH and their IC_{50} values for this enzyme

However, the most successful series of inhibitors yet discovered contains a triazolopyrimidine core and has led to the development of a molecule[83] (**26**) that is currently in clinical trials as an anti-malarial.[84] This series was discovered using a high-throughput screen of 220000 compounds resulting in a hit (**27**) that was found to be a good inhibitor of PfDHODH with an IC_{50} of 47 nM and over 4000 times selectivity over human DHODH.[85]

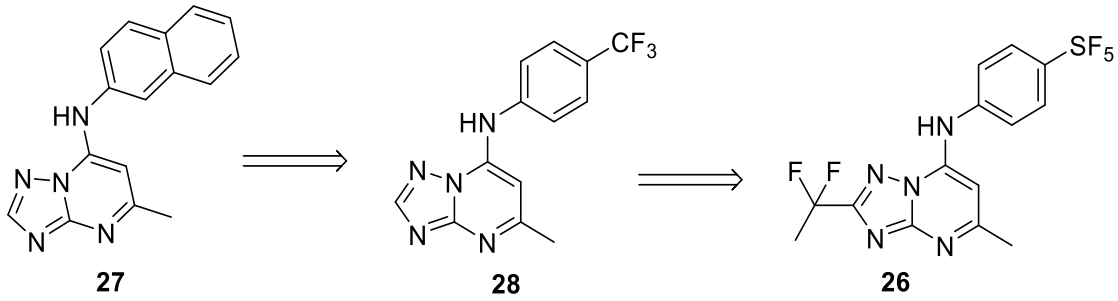


Figure 1.7 The original triazolopyrimidine hit (**27**) from a high-throughput screen and its development into a molecule with *in vivo* efficacy (**28**) and eventually to a clinical candidate (**26**)

Although simple SAR around this molecule produced good inhibitors of growth of the parasite *in vitro*, the efficacy in mice infected with *P. berghei* was poor. This was shown to be partly because of two altered amino acids in the *P. berghei* binding site compared to *P. falciparum* (Met-536 and Gly-181 in PfDHODH correspond to Val-536 and Ser-181 in PbDHODH) reducing the inhibitor binding and partly due to the maximum plasma concentration decreasing significantly on repeat dosing. Development of this series (Fig. 1.7) led to the synthesis of analogue **28** that is orally bioavailable and able to inhibit growth of *P. berghei* in mice (Fig. 1.8), the first time this had been achieved for *Plasmodium* DHODH.[86] Thus DHODH was fully validated as an anti-malarial target.

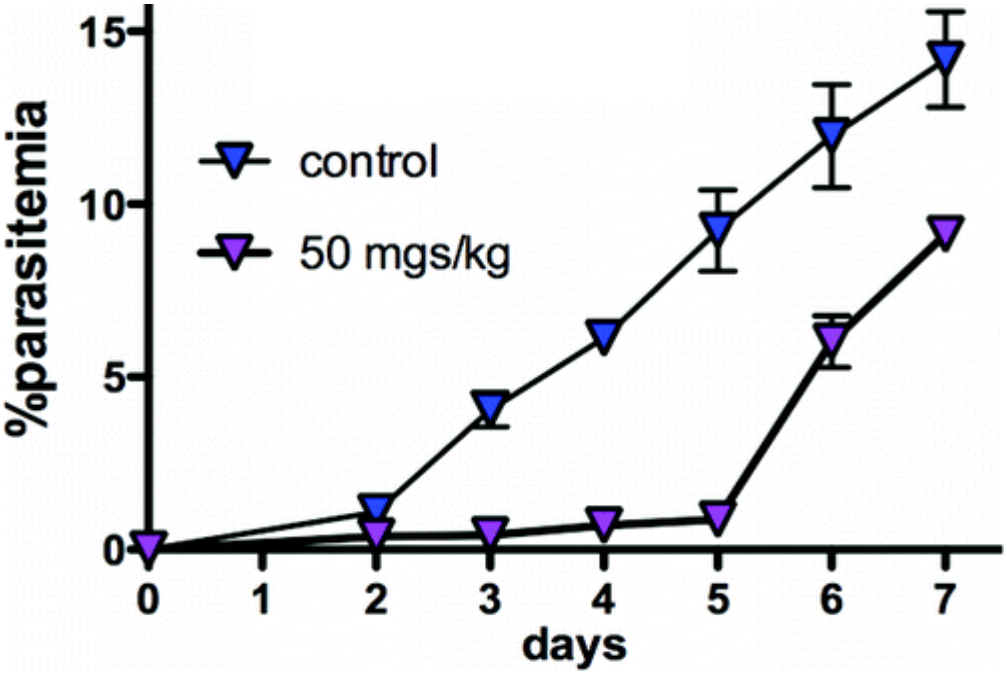
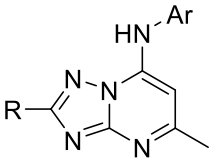


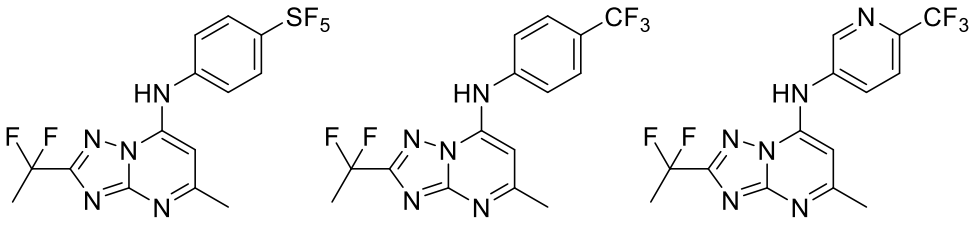
Figure 1.8 Graph showing efficacy of **28** in mice infected with parasites on day 0 with 2 doses per day of 50 mg/kg given on days 1–4. Reprinted with permission from Gujjar *et al.*,[86] copyright (2009) American Chemical Society.

Further work to optimise the compound focused on molecules with the general structure **29.** Changes to the aniline ring and, in particular, inclusion of the 4-SF_5 substituent was even better than the 4-CF_3 analogue as a compromise between activity and metabolic stability.



29

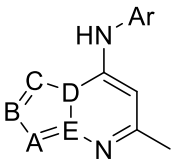
However, these compounds still had some loss of potency compared to **27** and thus substitution at the 2-position of the triazolo-ring was investigated in the hope of introducing interactions with a narrow hydrophobic channel in the protein. All alkyl substituents tested except *iso*-butyl were tolerated here with similar activity to the analogous compounds with just hydrogen at this position. The most potent molecules were obtained with unbranched haloalkyl R groups here, especially CF_2CH_3 which resulted in 4–40 times greater potency against PfDHODH and 25–240 times against the parasite with similar activity against *P. vivax* as well.[87] This was also good for metabolic stability, in conjunction with either the 4-CF_3 (**30**) or 4-SF_5 anilines (**26**) (Fig. 1.9), although aqueous solubility at pH 6.5 was low at 70–140 μM and 30–60 μM respectively.



26 **30** **31**

Figure 1.9 Structures of optimised triazolopyrimidines with improved activity and metabolic stability (**26** and **30**) and solubility (**31**)

Further SAR was carried out investigating changing the fused ring system to have either carbon or nitrogen at positions A–E of **32** but it was not possible to improve the compound in this way.



32

Changing any nitrogen to carbon except at position ‘C’ on structure **32** (above) significantly reduced potency, as did putting an amide linker before the

Ar-group, but the nitrogen at position C is important for metabolic stability and desirable pharmacokinetics.[88] Adding fluorine atoms into either or both aniline *meta*-positions led to a reduction in selectivity over mammalian DHODHs, from over 2500 times to only 100 times for human DHODH.[89] This is thought to be due to the increased hydrophobicity of the molecule interacting more strongly with the more hydrophobic binding pocket found in mammalian DHODH which helps counteract the additional hydrogen bond possible in PfDHODH, the origin of the previously seen selectivity.

The optimised compounds showed good oral bioavailability and efficacy in mice (Fig. 1.10) and were not cytotoxic and so **26** was chosen to be taken forward.

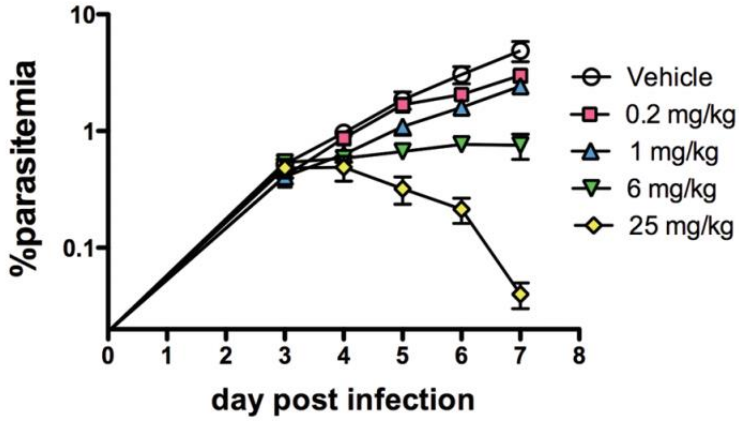


Figure 1.10 Graph showing efficacy of **26** in mice infected with parasites on day 0 with a single oral dose per day given on days 3–6. Reprinted with permission from Coteron *et al*.,[87] copyright (2011) American Chemical Society.

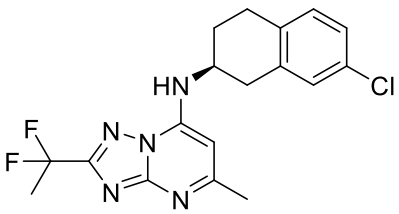
Compound **26** was effective against both the blood and liver forms of *P. falciparum*, including some drug-resistant strains. The pharmacokinetics are also good, allowing for high enough blood plasma concentrations for treatment of the disease to be sustained for more than eight days after one oral dose of 200–400 mg. Results of safety studies have also been encouraging: **26** was well tolerated in repeat-dose studies in mice and dogs and not mutagenic or active against any human enzymes or receptors.[83] This suggests a potential use as a single-dose treatment or chemopreventative taken once a week that would be better than current drugs, which must be dosed daily or are not effective against the liver form of *P. falciparum*.

In phase 1a human trials, **26** was safe at all doses tested (25–1200 mg). Headache was the most commonly observed side effect but there were no serious effects during the study. Pharmacokinetic parameters were promising, with a long half-life of 86–118 h depending on dose.[90]

At first **26** was given in a 150 mg dose 7 days after infection with *P. falciparum* parasites. This single dose resulted in parasite clearance initially but recrudescence occurred.[90] Increasing the dose to 400 mg resulted in complete clearance of asexual parasites with no serious side effects. However, gametocytes were not cleared even after an additional dose at day 23.[91] The compound has also been tested for use as a prophylactic, in a dose of 400 mg either 1 day or 7 days before volunteers were injected with *P. falciparum* sporozoites. Very good prophylactic activity was observed when taken 1 day before, with no parasitaemia at all, but efficacy was lower when taken 7 days prior to exposure.[92] Partial protection was observed when the drug was given 3 days before infection.[93]

In a phase 2a trial, a single 400 mg dose was given to patients with uncomplicated blood-stage malaria. For those infected by *P. falciparum*, parasitaemia was cleared and all had an adequate clinical response on day 14. However, none of those infected with *P. vivax* achieved this and even higher doses were only partially successful.[94] **26** has slightly lower activity against the *P. vivax* enzyme (IC_{50} 48 nM[83]) than the *P. falciparum* enzyme (IC_{50} 24 nM[83]) but this does not fully explain the difference in efficacy observed.

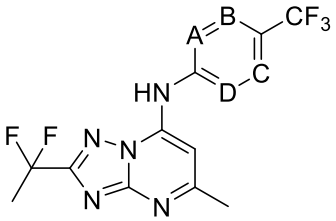
Despite the positive results so far obtained for **26**, back-up compounds have been designed in case of later failure. One problem with **26** is that although it does not inhibit human DHODH to a measurable extent, it is a low micromolar inhibitor of rodent DHODH which created problems with animal toxicity testing. Therefore a molecule was sought that would be inactive against all mammalian DHODHs, ideally with improved potency against PfDHODH so doses could be reduced. Replacing the Ar-group with a tetrahydro-2-naphthyl system could achieve such an increase in selectivity and, with a 7-Cl substituent (**33**), this also resulted in a slight increase in potency to give an IC_{50} value of 6.3 nM.[95]



33

However, the disadvantage of this compound was that the metabolic stability was lower, meaning that higher doses were needed for *in vivo* efficacy in a mouse model. Work was then done to find a compound that would have better

metabolic stability than this as well as greater solubility and selectivity than **26**. It was thought that reducing the hydrophobicity of the molecule would help with this and thus a series with the Ar-group replaced by nitrogen-containing heterocycles was synthesised with general structure **34**, where A, B, C and D are either carbon or nitrogen.



34

The pyridin-3-yl (B = N) compounds had higher solubility and potency than pyridin-2-yl (A = N) compounds. Analogues with two nitrogen atoms in this ring (A, D = N and B, C = N) were less potent, as were compounds synthesised with the CF_3 group switched to position B. Metabolic stability was good and human DHODH was not inhibited, even at a concentration of 100 µM.

Therefore, **31** was chosen as a backup compound with the best overall combination of these properties and only a small loss in potency (*P. falciparum* IC_{50} = 53 nM) compared to **26** and an X-ray crystal structure was obtained of this compound bound to the enzyme, in the channel leading to the active site (Fig. 1.11).[84]

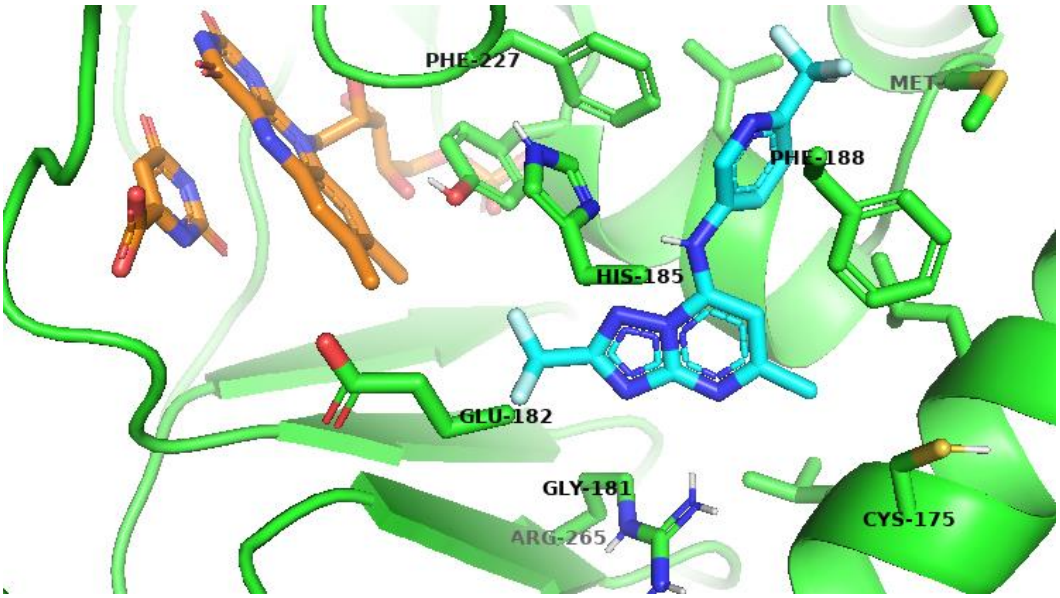
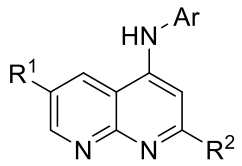


Figure 1.11 X-ray crystal structure showing **31** (blue) bound to PfDHODH, along with orotate and FMN (orange). Image created from structure 5TBO in the PDB.

Overall, despite the promising results thus far obtained for this series, there are some key disadvantages to these compounds, most notably their poor solubility, difficult formulation and inability to treat *P. vivax* malaria.

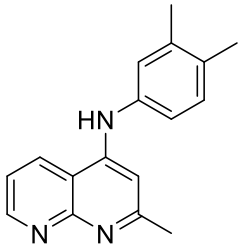
1.2.4 1,8-Naphthyridines as DHODH inhibitors

Beginning with *in silico* screening and computational design, work has been carried out in the Fishwick group to create novel DHODH inhibitors and investigate selectivity between species. Initially, efforts were focused on triazolopyrimidines and pyrimidones[96] but subsequently 1,8-naphthyridines (**35**) have been found to be much more promising for the creation of novel selective DHODH inhibitors as potential anti-malarials.



35

An X-ray crystal structure of an early 1,8-naphthyridine, **36**, bound to PfDHODH has been obtained (Fig. 1.12), showing this molecule bound in a similar mode to **31**.



36

A hydrophobic region, including residues Phe-188, Phe-227 and Leu-240, can be seen featuring π-stacking between the alanine group of the 1,8-naphthyridine and a phenylalanine residue. Two hydrogen bonds between the naphthyridine nitrogen atoms and an arginine residue are also observed, a key advantage of this molecular scaffold over the triazolopyrimidines which are observed to only make one hydrogen bond here (Fig. 1.13). This is likely to be due to the different orientation and much greater basicity of this nitrogen in the 1,8-naphthyridine compared to the corresponding atom in the triazolopyrimidine scaffold.

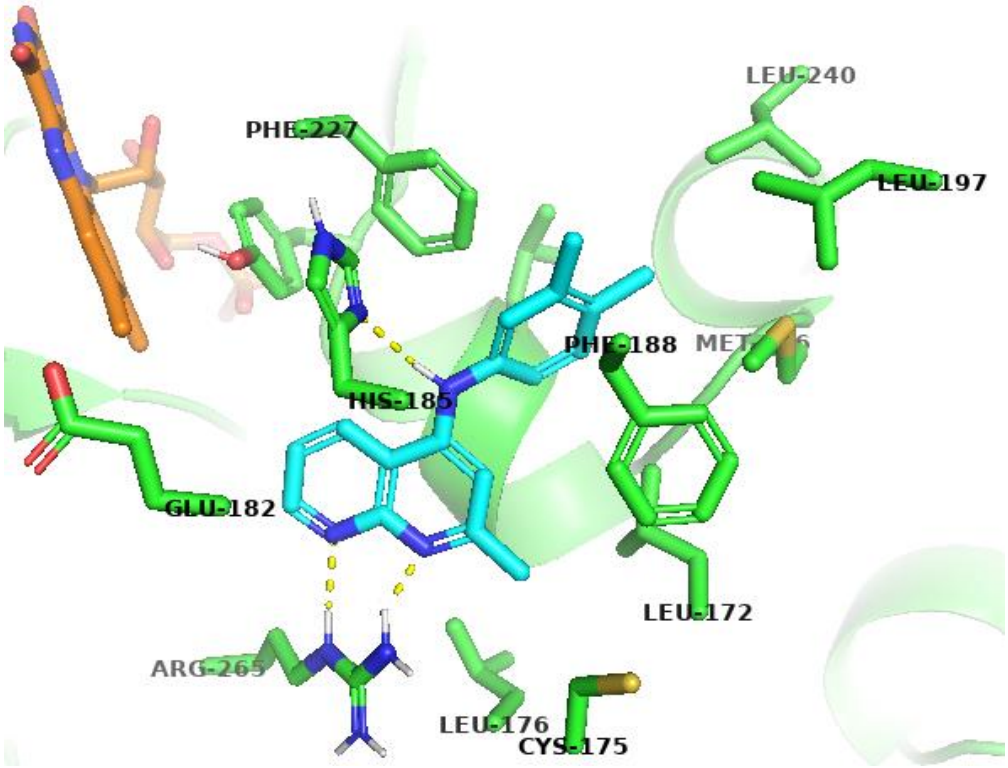


Figure 1.12 X-ray crystal structure of a 1,8-naphthyridine (**36,** blue) bound to *P. falciparum* DHODH, along with FMN (orange). Hydrogen bonds are shown by yellow dashed lines.

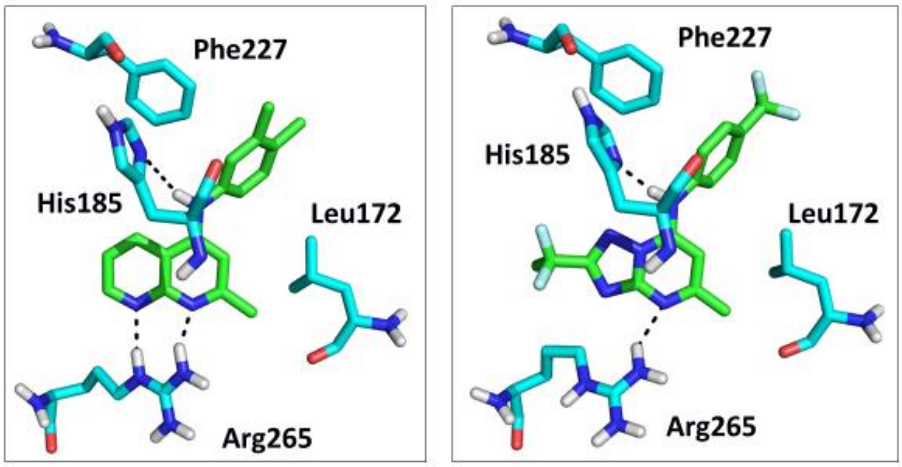


Figure 1.13 Comparison of the binding modes of the 1,8-naphthyridines (left) and triazolopyrimidines (right). Hydrogen bonds are shown by dashed lines.

1.2.4.1 Structure-activity relationships

Initial SAR has already been performed in the group, investigating different substituents at R^1, R^2 and Ar. Measurements of solubility in fasted-state simulated intestinal fluid (FaSSIF), metabolism (from ChemPartner, an external contract research organisation), enzyme inhibition and cellular inhibition (from Dr Glenn McConkey, University of Leeds) have been obtained on the synthesised compounds (Table 1.2).

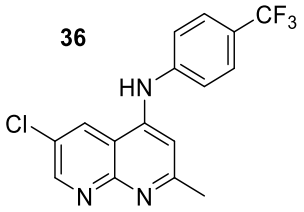
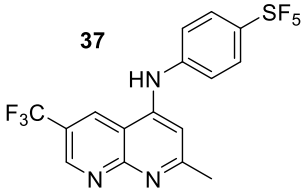
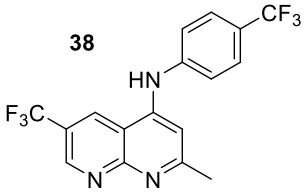
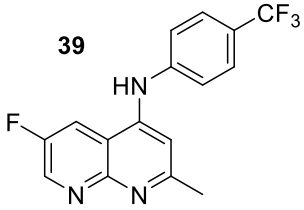
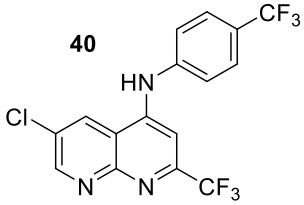
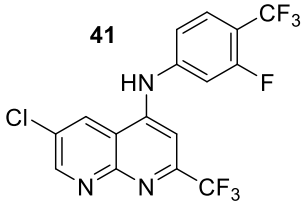
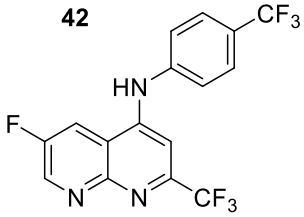
Compound	FaSSIF Solubility at pH 6.5/µM	Half-life in microsome /mins	Pf DHODH IC_{50}/nM	Hs DHODH IC_{50}/µM	*P. falciparum* cell EC_{50}/nM
36 	3.8	9.3	8	>10	1.3
37 	26	30	61	>10	6
38 	5.0	40	9	>100	5
39 	9.7	48	14	>10	6
40 	17	> 2000	198	>30	19
41 	101	319	118	>30	8
42 	3.6	1725	100	>100	83

Table 1.2 SAR around the 1,8-naphthyridine framework previously carried out in the Fishwick group, showing solubility in FaSSIF, half-life in mouse microsome and potency data.

To summarise this previously-established SAR, several compounds show good potency, both in enzyme and cellular assays and all show very good selectivity over human DHODH. SAR is summarised in Fig 1.14.

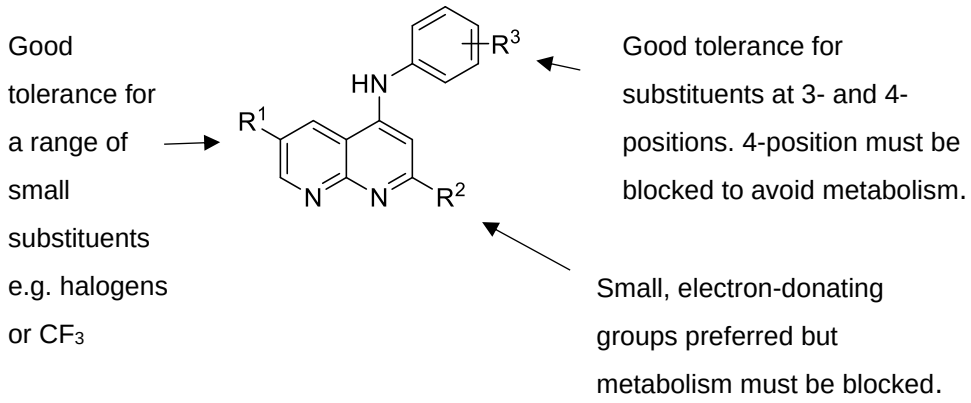


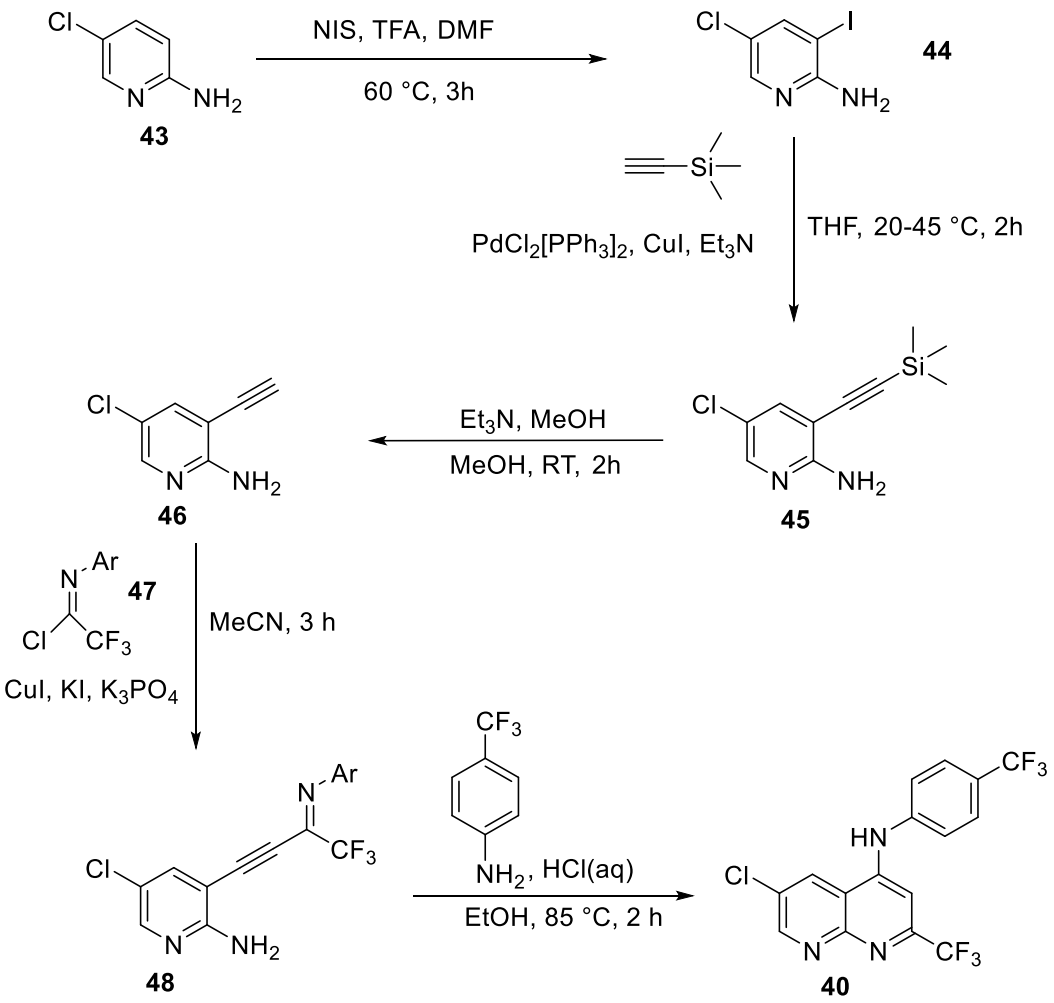
Figure 1.14 Structure-activity relationships discovered for the 1,8-naphthyridine series of inhibitors

Unlike in the triazolopyrimidine series,[83] compounds with Ar = 4-SF_5 aniline (**32**) are not very potent compared with having a CF_3 at this position (**33**). At the R^1 position, the order of potency is Cl>CF_3/F and, while replacement of the methyl group at R^2 with a trifluoromethyl resulted in a drop in potency in the enzyme assay, the potency in cells was only slightly lower than the methyl counterparts. This was necessary because those with R^2 = methyl show poor metabolic stability which increases dramatically on substitution with trifluoromethyl.

However, most of these compounds show very poor solubility, the one anomaly being compound **41** where the addition of a 3-fluoro group to the aniline seems to cause an increase of almost tenfold. This, along with the decrease in potency when changing the group at R^2 from methyl to trifluoromethyl to stop metabolism by cytochrome P450, shows that there is still much work to be done to find the best compromise of all these properties.

1.2.4.2 Synthetic routes to 1,8-naphthyridines

There are few known routes to 1,8-naphthyridines, none of which could be used to synthesise molecules with the desired substitution pattern (**35**). Hence a new synthetic route to such 1,8-naphthyridines has been developed at the University of Leeds by Professor Philip Kocienski (hereafter called the 'PK' synthesis, Scheme 1.3).



Scheme 1.3 The 'PK' synthesis. Synthetic route to **40** developed by Professor Philip Kocienski where Ar = trityl, 2-methylaniline or 2-aminobiphenyl. Other 1,8-naphthyridines (Table 1.2) were synthesised by an analogous route.

This has enabled the aforementioned SAR to be carried out. However, there are some issues with this route, notably the need to synthesise the imidoyl chloride **47** (2 steps) and the low yield of the second Sonogashira reaction. It is also possible that the displaced aniline in the final cyclisation could itself cyclise to give another undesired product, reducing yield and making purification difficult.

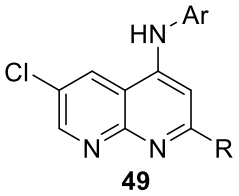
1.3 Project goals

The goals of this project were to:

- Develop a more efficient synthesis of 1,8-naphthyridines;
- Further explore SAR via synthesis of novel 1,8-naphthyridines as inhibitors of malarial DHODH
- Conduct lead optimisation of this series with the aim of producing good inhibitors with better ADME properties that may ultimately be taken further into clinical trials
- Design new inhibitor types for DHODH enzymes from other infective organisms such as *T. gondii*

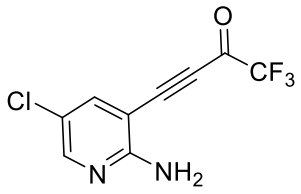
1.3.1 1,8-Naphthyridines as DHODH inhibitors for the treatment of malaria

The initial goal of the current project was to build on the work previously performed at Leeds on developing inhibitors of DHODH as anti-malarials. This involved lead optimisation with the aim of synthesising compounds which maintain good activity against the target and good selectivity over human DHODH while showing improved aqueous solubility over current lead compounds and ensuring good metabolic stability. As such, one aim was to develop a more efficient synthesis of 1,8-naphthyridines of general structure **49**, where R is either trifluoromethyl or difluoromethyl.



49

This enabled further investigation of the SAR of different Ar groups in the hope of improving the properties of the R = CF_3 series of compounds while maintaining their good metabolic stability. In order to do this, it was planned to investigate the possibility of going via an acetylenic ketone intermediate **50** to solve some of the problems with the previous route (Scheme 1.3) by removing the need to synthesise the imidoyl chloride and carry out the second Sonogashira reaction.



50

In this previous work, no molecules had yet been synthesised with R = CHF_2 so the aim was to be able to use this improved route to 1,8-naphthyridines to make and investigate SAR of these compounds; the smaller CHF_2 group may help to retain more of the potency and solubility seen in the R = CH_3 compounds while keeping the much improved metabolic stability of the CF_3 analogues.

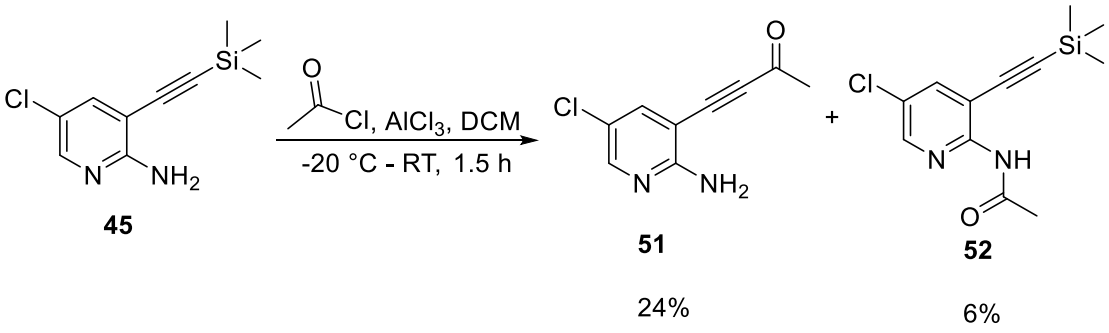
1.3.2 DHODH inhibitors as treatments for other infectious diseases

It was also planned to extend these studies to the design and synthesis of inhibitors of DHODH in other organisms such as *T. gondii*, in which this enzyme has recently become a validated drug target[68, 71, 72] but which has not yet been the focus of a medicinal chemistry programme to develop a potent inhibitor and hence a new drug for the treatment of toxoplasmosis. The existing 1, 8-naphthyridine series of compounds was tested for activity against this target in other species to determine whether it is a good starting point for drug development or whether a new molecular scaffold should be designed. Computational design was then utilised to develop potent inhibitors of DHODH from this species.

Chapter 2 Synthesis and Evaluation of 6-Chloro-1,8-naphthyridines

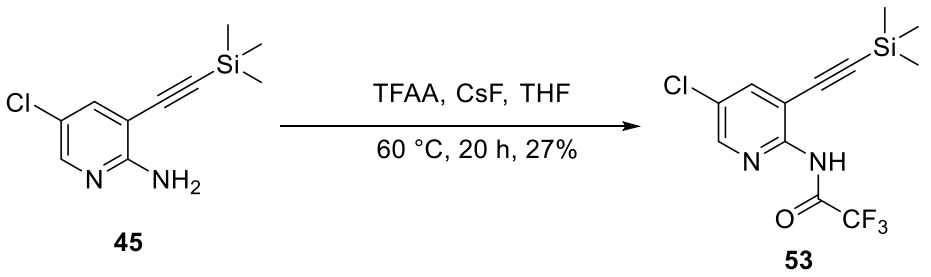
2.1 Development of a new synthetic route to 4-anilino-1,8-naphthyridines

In order to attempt to make the key intermediate in the proposed improved synthetic route to 1,8-naphthyridines, the first two steps of the PK synthesis (Scheme 1.3) were repeated: starting from **43** with an iodination giving **44** followed by Sonogashira coupling with ethynyltrimethylsilane[97] to make **45,** which was achieved in good yield. This compound was initially a grey colour, thought to be due to metal impurities, and could be produced in colourless form by washing with ammonium 1-pyrrolidinedithiocarbamate to remove residual metals.[98] From this molecule it was possible to make the methyl analogue of the ketone (**51**) directly in moderate yield (Scheme 2.1) by repeating a literature procedure with acetyl chloride mediated by aluminium trichloride.[99]



Scheme 2.1 Reaction of **45** with acetyl chloride in the presence of $AlCl_3$ to make **51**

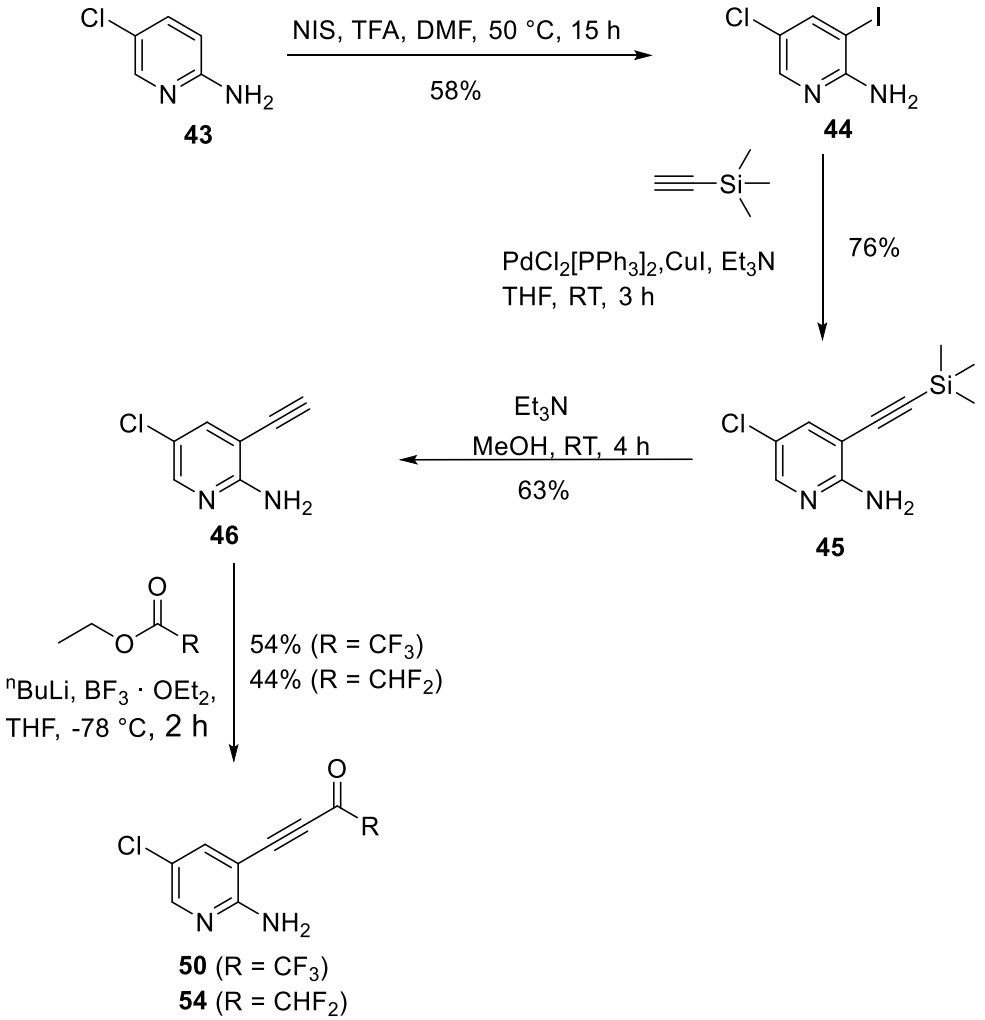
However, attempts to make the trifluoromethyl compound in an analogous reaction using trifluoroacetic anhydride instead of acetyl chloride were unsuccessful, producing only the desilylated alkyne **46**. Instead, microwave reactions using a range of Lewis acids in DMF were tried but this gave the same result. Changing the solvent to toluene resulted in the major product instead being the *N*-substituted amide **53** for each Lewis acid tried (palladium(II) chloride and zinc chloride) as well as in the absence of Lewis acid. The final set of conditions tried for this reaction used caesium fluoride in THF under reflux at 60 °C which had literature precedent using aldehydes or alkyl bromides as the electrophile.[100] In this case, using ethyl difluoroacetate as the electrophile resulted predominantly in desilylation to give **46,** while using trifluoroacetic anhydride gave **53** as the major product (Scheme 2.2).



45 **53**

Scheme 2.2 Attempted reaction with CsF that formed the amide **53**

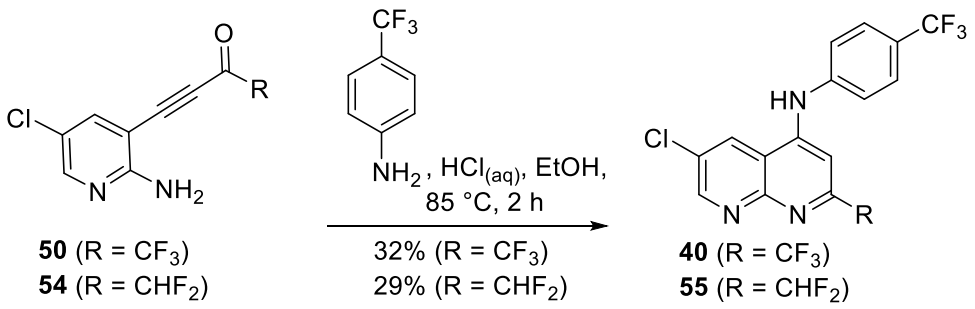
With none of these attempts to remove the trimethylsilyl group and add the trifluoroacetyl group simultaneously producing the desired product, the steps were tried separately. First a desilylation reaction developed by Professor Kocienski, University of Leeds, using triethylamine in methanol, proceeded to give **46** in good yield and using ^{n}butyllithium and boron trifluoride (to prevent double addition to the electrophile) to trifluoroacetylate terminal alkynes, adapted from a literature procedure using phenylacetylene,[101] was successful too, giving **50** in moderate yield (Scheme 2.3).



Scheme 2.3 Synthesis of acetylenic ketone intermediates

The disadvantage of using such a strong base was that this method might not prove suitable for the synthesis of the analogous CHF_2 compound due to the possibility of enolising the ester. However, it proved possible to make this compound in the same way as that for R = CF_3, albeit with a slightly lower yield (Scheme 2.3). This could be because the boron trifluoride forms a complex with the lithium acetylide,[102] making it less basic.

With the intermediate ketone in-hand, cyclisation reactions could now be undertaken to produce the final 1,8-naphthyridine compounds. The reaction of **50** was carried out first with 4-trifluoromethylaniline to make **40** (Scheme 2.4), a remake of a compound that had previously been synthesised in the group via a different route (Scheme 1.3), as a proof of concept for making 1,8-naphthyridines by this new route. This was successful, albeit in a yield of only 32%.



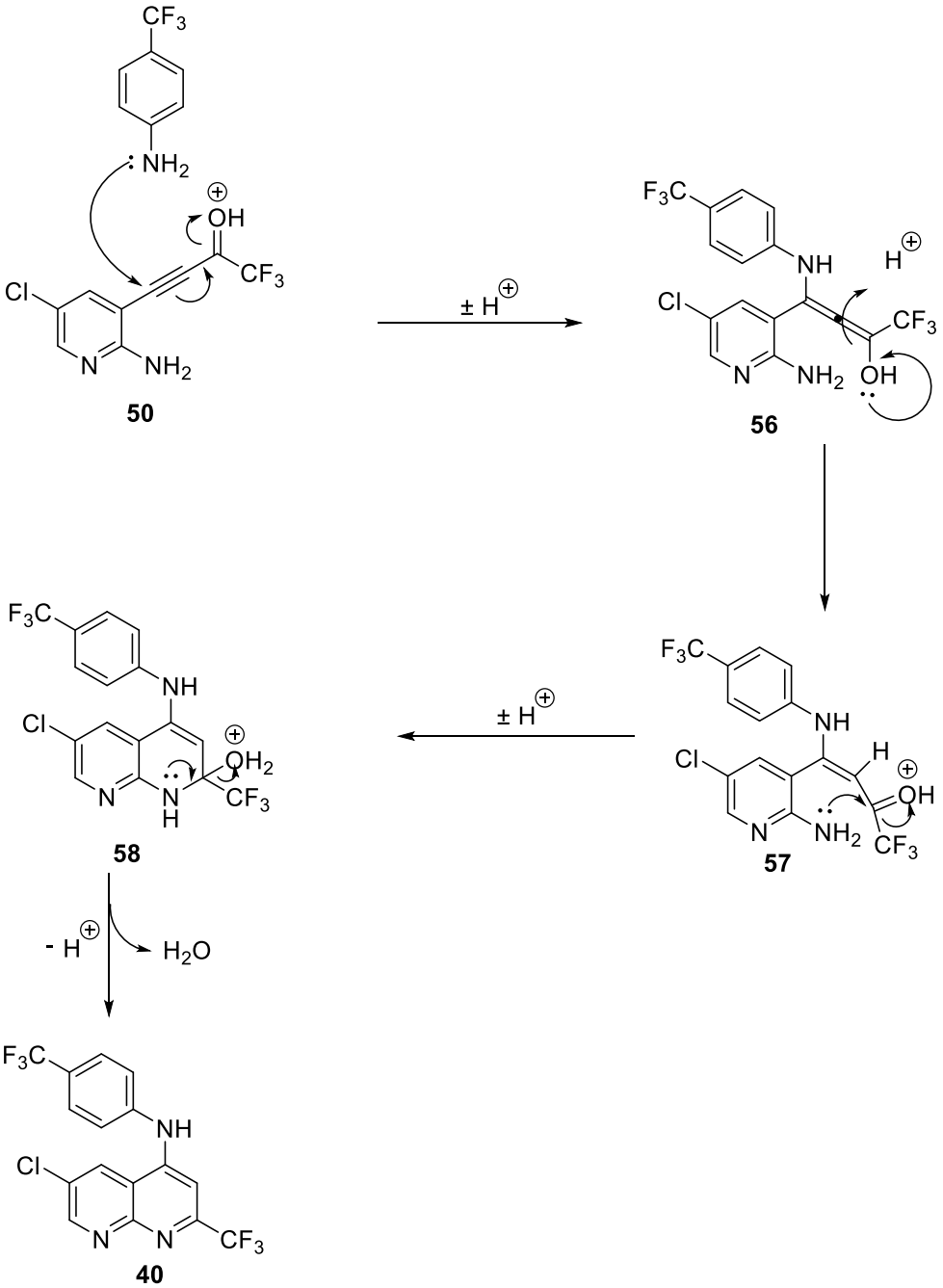
Scheme 2.4 Cyclisation of acetylenic ketone intermediates to 1,8-naphthyridines

The reaction to make **40** is proposed to proceed by the Michael addition of the aniline to give **56** which can then get protonated to give the enone **57**. This alkene intermediate could be either the *cis*- or *trans*- isomer, but the two are thought to be in equilibrium under the reaction conditions. Only the *cis*- isomer has the correct orientation to allow for an intermolecular cyclisation to **58** which can then aromatise through the loss of water, yielding the final product **40** (Scheme 2.5).

Cyclisation of **54** to make the analogous difluoromethyl compound **55** was similarly successful (Scheme 2.4). This is the first example of a novel 1,8-naphthyridine to be synthesised using this new, shorter route.

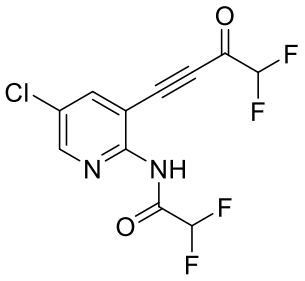
In order to synthesise a library of compounds with different aniline substituents, it was desirable to be able to make the ketone intermediate **54** in higher yield and on larger scale. Therefore investigations were performed, modifying the reaction conditions to try to improve yield. Changing the order of addition of boron trifluoride and ethyl difluoroacetate had no significant effect on yield but leaving out the boron trifluoride altogether resulted in a complex reaction mixture from which the desired product could be isolated in

low yield (10%). Scaling up the reaction also proved challenging, with significantly reduced yields observed when the reaction was performed above 3 mmol scale. One explanation for this could be that increased amounts of the doubly substituted product **59** were observed under these conditions.



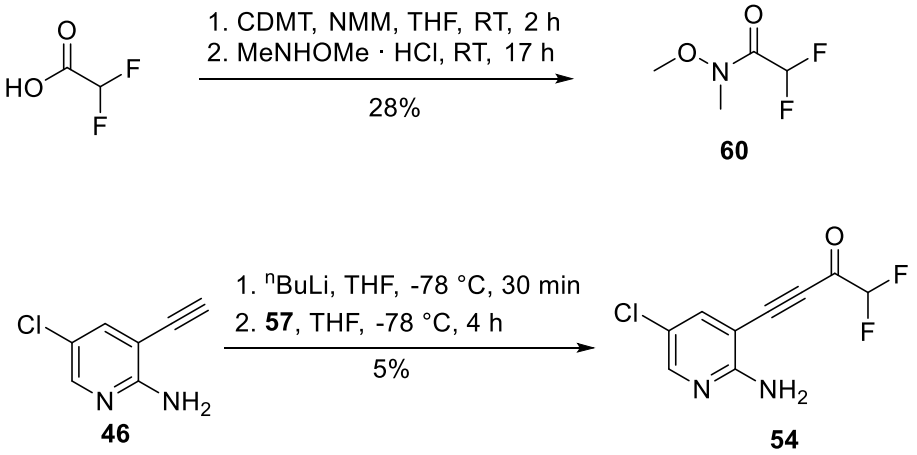
Scheme 2.5 Proposed mechanism for the cyclisation of acetylenic ketone intermediate **50** to the 1,8-naphthyridine **40**

Due to the difficulties encountered with attempting to increase the yield and scale of this step, other types of reaction were tried to make this key intermediate.



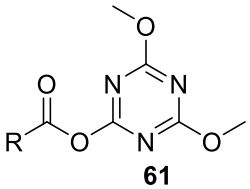
59

By first making the Weinreb amide **60** to alleviate the need to add boron trifluoride, it was hoped that these problems could be overcome. Initially this was attempted from ethyl difluoroacetate using a Grignard reagent as a base[103] but this did not yield any product. Therefore the reaction was performed using difluoroacetic acid, which was first activated using 2-chloro-4,6-dimethoxy-1,3,5-triazine with *N*-methylmorpholine with followed by displacement with *N*,*O*-dimethylhydroxylamine.[104] This gave the desired Weinreb amide **60** in low yield, enabling the synthesis of ketone **54** to be performed using this starting material (Scheme 2.6).



Scheme 2.6 Synthesis of acetylenic ketone intermediate using a Weinreb amide

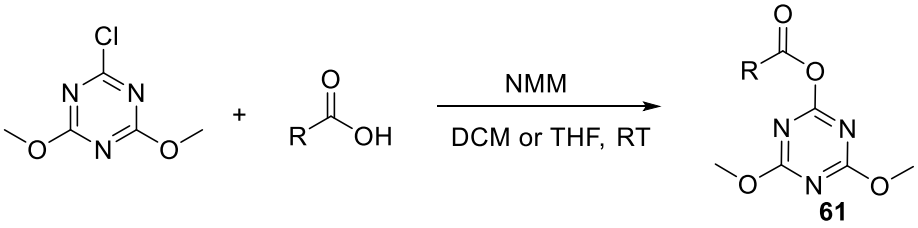
Given the very poor yields of ketone obtained using the Weinreb amide, a different approach was sought. It was reasoned that creating an activated ester **61** from the carboxylic acid might enable the desired ketone to be synthesised using a palladium catalysed approach, for which there was some literature precedent.[105]



61

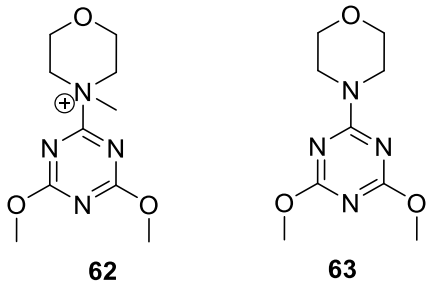
R = CF_3, CHF_2

The reaction was performed with either tri- or difluoroacetic acid according to the conditions reported by Yu *et al*.[105] or Rode, Son and Hong[106] for the activation of alkyl or aryl carboxylic acids (Scheme 2.7).



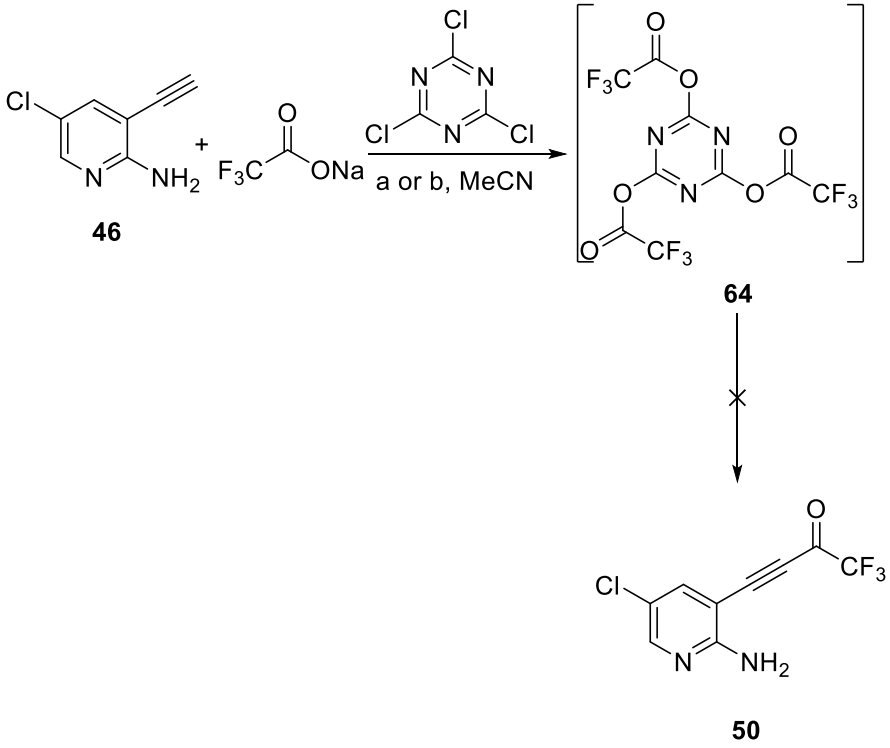
Scheme 2.7 Synthesis of activated ester intermediate, R = CF_3, CHF_2, CH_3

Monitoring this reaction using LCMS revealed that a peak was initially seen for a positive ion with mass 241, corresponding to attack by *N*-methylmorpholine to give **62**, which was slowly converted to a less polar molecule with mass 227, thought to correspond to loss of the *N*-methyl group to give **63**.



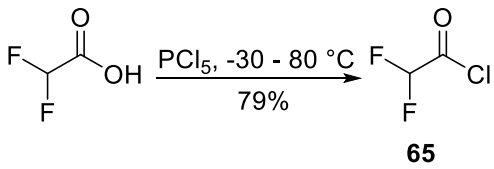
Even after the reaction was left overnight, no substitution was observed by either the tri- or difluoroacetic acid. This was thought to be due to the electron-withdrawing fluorine atoms greatly reducing the nucleophilicity of the carboxylate so the reaction was repeated using acetic acid instead but this gave the same result.

Also attempted was a 'one-pot' procedure, using cyanuric chloride and sodium trifluoroacetate in the hope of producing the activated ester **64** *in situ* without the need to use *N*-methylmorpholine, then coupling it directly to the alkyne **46** (Scheme 2.8). Mixing together first the sodium trifluoroacetate and cyanuric chloride for 10 minutes gave a white precipitate, indicating the production of NaCl and hence the successful substitution of the trifluoroacetate onto the triazine. Palladium acetate catalysis, reported to give high yields for the coupling of activated esters to phenylacetylene,[105] in this case gave only the amino substituted product. A similar literature procedure[107] for the synthesis of ynones using magnesium chloride also proved unsuccessful for the production of **50**.



Scheme 2.8 Attempted synthesis of ketone **50** by a one-pot coupling procedure: a = $Pd(OAc)_2$, 40 °C, b = $MgCl_2$, Et_3N, RT.

Another idea was to use difluoroacetyl chloride in an $AlCl_3$-catalysed reaction analogous to that used to make **51** (Scheme 2.1). Thus difluoroacetyl chloride (**65**) was made by the chlorination of difluoroacetic acid using PCl_5, with the product distilled off in good yield (Scheme 2.9).

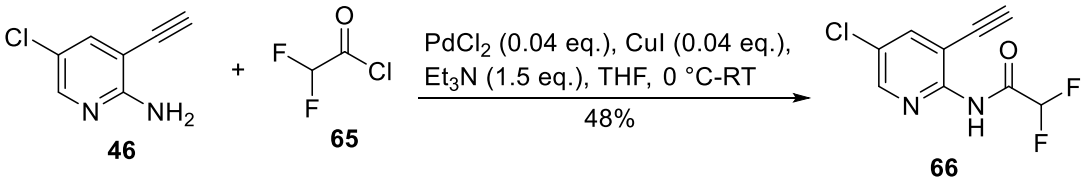


Scheme 2.9 Chlorination of difluoroacetic acid

However, the $AlCl_3$ catalysed reaction was unsuccessful in this case, giving only the product resulting from attack by the amino group. This is likely to be due to the increased electrophilicity of difluoroacetyl chloride over acetyl chloride greatly increasing the rate of this reaction and so what was the minor product in the case of acetyl chloride becomes the only isolated product.

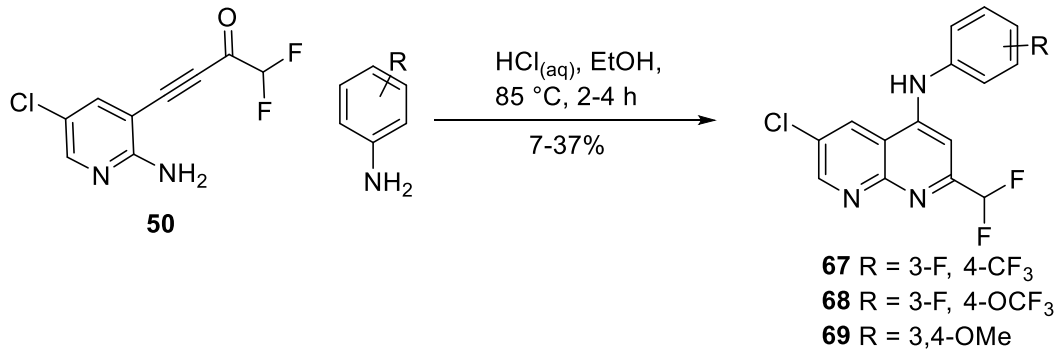
The coupling of acid chlorides to terminal alkynes under Sonogashira conditions has been reported[108] so this was attempted next. However, the

product of this reaction (**66**) was also the result of attack by the amine rather than the desired coupling (Scheme 2.10).



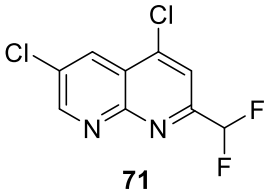
Scheme 2.10 Attempted Sonogashira reaction using difluoroacetyl chloride which yielded only the amide

With the lack of success achieved in trying to alter the method used to synthesise the ketone, it was decided to use the original conditions to make a small library of compounds with different anilines as Ar-groups to investigate SAR at this position. Hence, three further compounds were made by the same addition-cyclisation reaction as **55**, in low to moderate yields (Scheme 2.11).



Scheme 2.11 Addition-cyclisation reaction with varying anilines to create different 1,8-naphthyridines.

There is a clear trend in yields in these reactions, with those using the least nucleophilic anilines with electron withdrawing substituents to make **67** and **68** much lower than for the more nucleophilic aniline used in the synthesis of **69**. In the low yielding reactions, significant amounts of the chloro-substituted naphthyridine by-product **71** were obtained.



2.1.1 Biological and pharmacokinetic evaluation of 2-difluoromethyl-4-anilino-1,8-naphthyridines

The four novel 1,8-naphthyridines synthesised using this route were tested for efficacy against both the DHODH enzyme *in vitro* by collaborators at UT

Southwestern and in cellular assays against *Plasmodium falciparum* (isolate 3D7) by collaborators at Dundee (Table 2.1). Values obtained for **40** and the competitor compound **26** are included for comparison.

Both **55** and **67** show excellent inhibition of the parasite enzyme and cell growth, with nanomolar IC_{50} values obtained in *P. falciparum* and *P. vivax* enzyme assays and the *P. falciparum* cellular assay. They also showed outstanding selectivity for the parasite enzyme over human DHODH, for which no inhibition was observed even at a concentration of 100 μM.

Comparing **55** and **40**, changing the trifluoromethyl group to difluoromethyl has resulted in a significant improvement in activity against both the *P. falciparum* and *P. vivax* DHODH enzyme and the *P. falciparum cells*. This may be due to the less electron withdrawing nature of the difluoromethyl group resulting in an increase in the ability of the naphthyridine nitrogen atoms to act as hydrogen bond acceptors.

Not quite as potent were **68** and **69**: both these compounds had higher IC_{50} values in all three assays than **55** and **67**, with a small loss in potency against PfDHODH, and a larger decrease against PvDHODH for **67** in particular. **69** was also much less potent in the *P. falciparum* cellular assay.

The metabolic stability of these compounds was assessed using a liver microsome assay carried out by ChemPartner (Table 2.2). The difluoromethyl compounds tested showed a long half-life in both mouse and human models, with similar stability to the trifluoromethyl compound **40** and much better than the methyl analogue **36**. This demonstrates that the difluoromethyl group is metabolically stable and not oxidised by cytochrome P450s as is thought to be the case with a methyl group at this position. It was decided not to test **68** and **69** in this assay because they were not potent enough to be considered as lead compounds.

Solubility was measured by Dr Elisabeth Ruethlein in three different solutions: PBS buffer at pH 7.4, fasted-state simulated intestinal fluid (FaSSIF) at pH 6.5 and fasted-state simulated gastric fluid (FaSSGF) at pH 1.6 (Table 2.3). There is a general trend towards increased solubility in the FaSSIF solution compared to PBS, which can be rationalised by the proteins present in this solution helping to solubilise the relatively hydrophobic molecules. Most compounds were also more soluble at the lower pH of the FaSSGF medium compared to the more neutral FaSSIF. This suggests that they are being protonated under these acidic conditions.

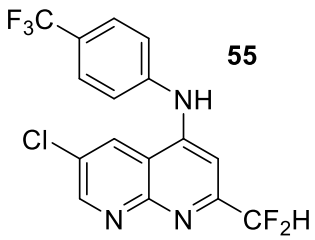
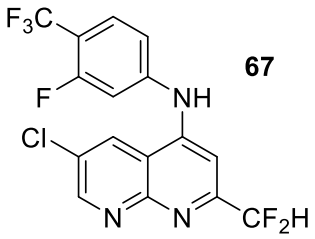
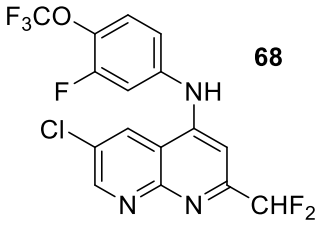
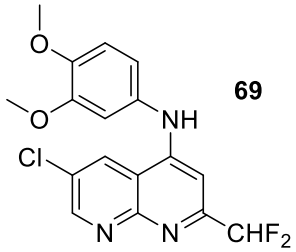
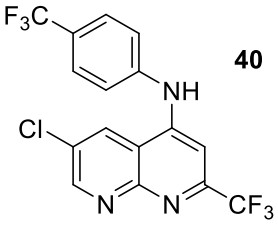
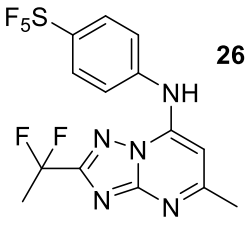
Structure	PfDHODH IC_{50}/nM	PvDHODH IC_{50}/nM	HsDHODH IC_{50}/µM	*P. falciparum* cell EC_{50}/nM
 **55**	14 ± 2	25 ± 3	> 100	7 ± 3
 **67**	31 ± 6	24 ± 5	> 100	5 ± 1
 **68**	41 ± 4	104 ± 6	> 100	27 ± 14
 **69**	48 ± 6	61 ± 7	> 100	315 ± 172
 **40**	26 ± 3	81 ± 5	> 100	43
 **26**	13 ± 4	26 ± 13	99[83]	11

Table 2.1 Data showing inhibition of DHODH enzyme from 3 different species; *P. falciparum*, *P. vivax* and human, *in vitro* and *P. falciparum* cells for the 2-difluoromethyl-substituted compounds compared to the trifluoromethyl compound **40** and the competitor compound **26**.

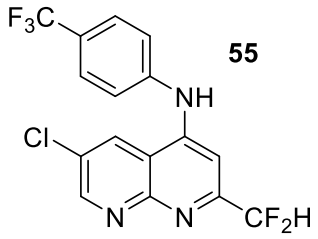
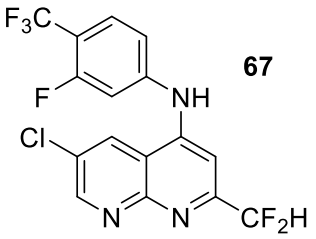
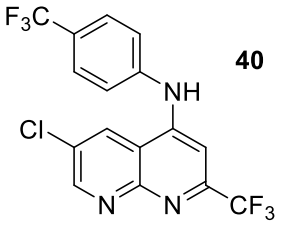
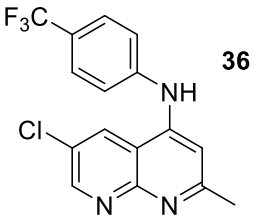
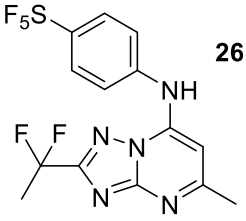
Structure	Human half-life/mins	Mouse half-life/mins
 **55**	575.2	381.1
 **67**	311.0	> 99% left in assay
 **40**	433.8	> 99% left in assay
 **36**	136.5	9.3
 **26**	519.2	124.6

Table 2.2 Liver microsome stability data.

Comparing **55** to **40**, having a more polar difluoromethyl group at the 2-position instead of trifluoromethyl results in solubility that is an order of magnitude greater across the three sets of conditions tested and also much better than that of the clinical candidate **26** under physiologically relevant conditions. It was proposed that adding oxygen atoms to the aniline part of the molecule could be a way of increasing solubility but this was not the case for the **69** in PBS and FaSSIF conditions; only under acidic FaSSGF conditions did this molecule show the desired improvement in solubility. The solubility of the trifluoromethoxy compound **68** was also disappointing, being lower than that of the corresponding trifluoromethyl compound **67**, and this compound

was also unstable under the assay conditions, with multiple peaks observed using HPLC.

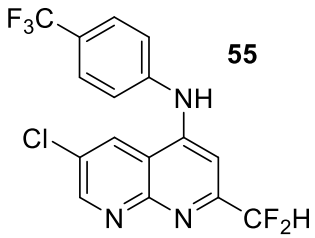
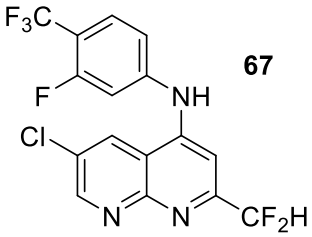
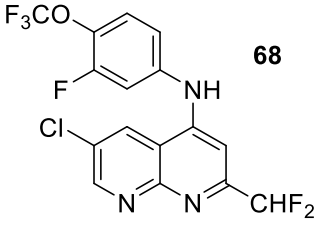
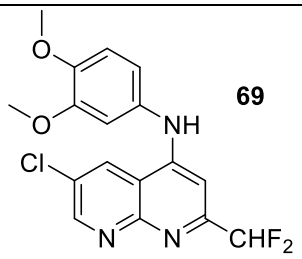
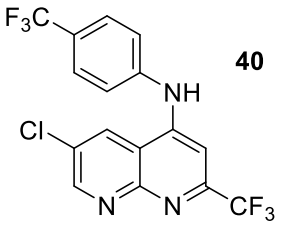
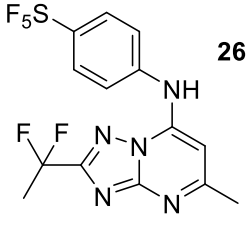
Structure	PBS (pH 7.4)/µM	FaSSIF (pH 6.5)/µM	FaSSGF (pH 1.6)/µM
 **55**	4.8	31	100
 **67**	6.4	52	67
 **68**	1.7*	13*	28*
 **69**	2.8	5.7	157
 **40**	0.6	6.3	1.0
 **26**	4.8	10	16

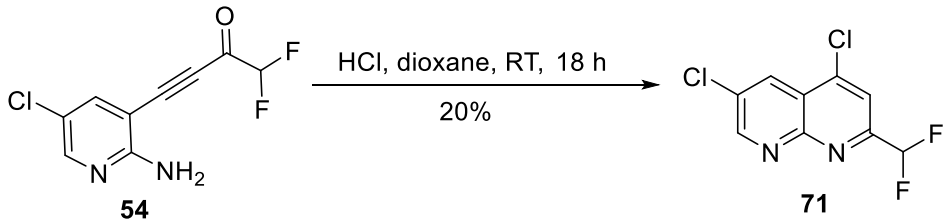
Table 2.3 Solubility of difluoromethyl compounds compared to **40** and **26** in 3 different media. * = some degradation observed – multiple peaks in HPLC.

In conclusion, the difluoromethyl group proved to be a good way of increasing solubility and activity over the trifluoromethyl analogues without compromising too much on metabolic stability. However, the solubility of these compounds was still not as good as is desired if they are to become orally taken drugs without the need for costly formulations like those required by **26**.

2.2 Investigating substituents other than anilines at the 4-position of the 1,8-naphthyridines

In order to further increase solubility of the 1,8-naphthyridines, it was decided to synthesise a series of analogues with heterocycles rather than anilines as the Ar-group. It was hypothesised that larger substituents at this position would be tolerated as long as π-stacking interactions were maintained, since there is a relatively large space at this site of the enzyme.

However, the synthesis used for the anilines did not work when the final step was tried with 2-aminobenzothiazole instead of the aniline. Therefore it was proposed that first cyclising the ketone (**50** or **54**) with HCl to make the corresponding 4-chloro- intermediate (**70** or **71**), previously isolated as a by-product in the cyclisation with anilines, could enable the desired heterocycle containing 1,8-naphthyridines to be made by a subsequent S_NAr reaction. This was achieved in low yield by adapting conditions reported for the synthesis of 4-chloroquinolines[109] (Scheme 2.12).



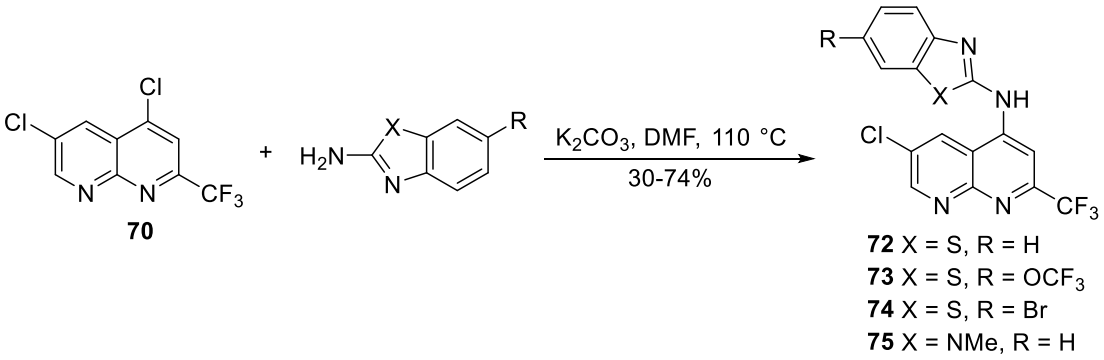
54 **71**

Scheme 2.12 Cyclisation of the ketone intermediate **54** with HCl

The poor yield of this reaction and the inability of the previous step to be performed on a large scale presented challenges for the synthesis of the small library of heterocycle containing compounds that was desired. Fortunately, Professor Philip Kocienski was able to make the trifluoromethyl analogue **70** on a large scale from imidoyl chloride intermediate **48**. Therefore, it was decided to carry out the desired SAR on the trifluoromethyl derivatives, easier and cheaper to make than the difluoromethyl ones, even though it was expected that difluoromethyl compounds would be more soluble and show

greater inhibition of the enzyme. The corresponding difluoromethyl analogues of any promising molecules could then be synthesised later if necessary.

Attempts to perform the S_NAr reaction using microwave radiation were unsuccessful but using a base instead allowed the desired products to be made in moderate to good yields (Scheme 2.13); attack occurred only at the chlorine resulting in the lowest energy intermediate, with the negative charge delocalised onto a nitrogen atom.



Scheme 2.13 S_NAr reaction of heterocycles using basic conditions

This reaction generally went to full conversion (by LCMS) in each case so the differing yields are likely to be due to the different degree to which each molecule precipitated out of solution.

The compounds were assessed for inhibition of DHODH enzyme by collaborators at UT Southwestern and in cellular assays against *Plasmodium falciparum* (isolate 3D7) by collaborators at Dundee (Table 2.4). All of the benzothiazole derivatives (**72**, **73** and **74**) were poor inhibitors of DHODH enzyme from both species of parasite and potency against *P. falciparum* cells was also much lower than for the anilino derivatives, with all three benzothiazole compounds having EC_{50} values above 1 μM. The benzimidazole compound **75** showed very good potency against the DHODH enzyme in both species; however, this compound was also poor at killing *P. falciparum* cells. This may be due to it failing to penetrate into the cell. All tested compounds showed no inhibition of human DHODH (IC_{50} > 100 μM).

These compounds have also been tested in solubility assays by Dr. Elisabeth Ruethlein (Table 2.5). The results for **72** showed PBS solubility of 157.3 μM and FaSSIF solubility of 88.7 μM, much better than any other 2-trifluoromethyl compounds that have been tested. However, some degradation was observed in these assays, with 4 peaks visible in the HPLC trace, casting some doubt on the validity of these numbers. Under acidic FaSSGF conditions all of the compound was degraded, thought to be due to the 2-aminobenzothiazole

being a good leaving group, especially when protonated, and hence being displaced by water. This could also explain why attempts to make this molecule under acidic conditions were unsuccessful. This instability to aqueous acid means that this molecule is not a suitable drug candidate regardless of its solubility.

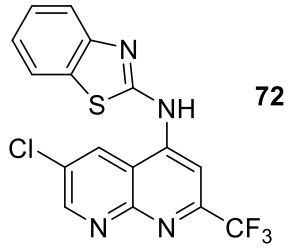
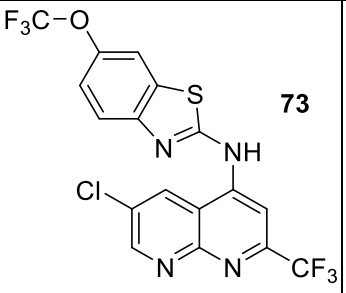
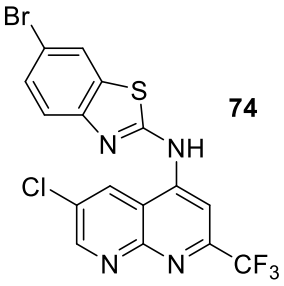
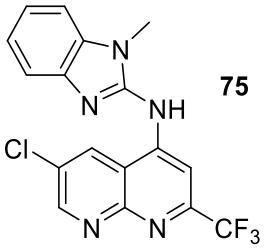
Structure	PfDHODH IC_{50}/nM	PvDHODH IC_{50}/nM	*P. falciparum* cell EC_{50}/nM
 **72**	15500 ± 7400	97000 ± 40000	2850 ± 1200
 **73**	9200 ± 3000	63000 ± 5000	1250 ± 830
 **74**	>100000	>100000	7350 ± 2650
 **75**	33 ± 5	21 ± 2	1820 ± 950

Table 2.5 Inhibition data on DHODH enzyme from 3 different species and *P. falciparum* cells for benzothiazole and benzimidazole compounds

Compound **73**, with a similar structure to **72**, showed similar problems of degradation under the assay conditions, but appeared to be less soluble anyway. The third benzothiazole-containing compound, **74**, was completely insoluble under all conditions tested; this molecule also has very low solubility in organic solvents. Pleasingly, replacement of the benzothiazole moiety with

a benzimidazole, resulted in a compound (**75**) that was stable under the assay conditions. However, this molecule was no more soluble than the anilino-derivatives.

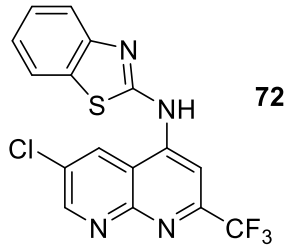
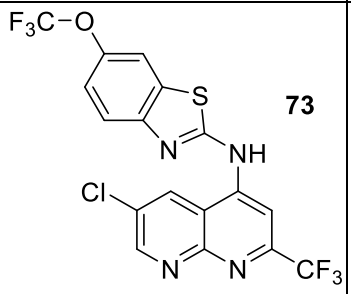
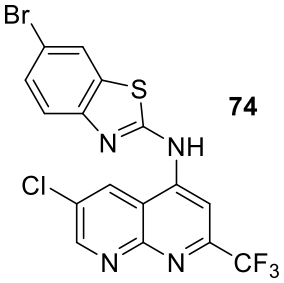
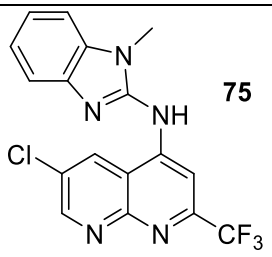
Structure	PBS (pH 7.4)/µM	FaSSIF (pH 6.5)/µM	FaSSGF (pH 1.6)/µM
 **72**	157*	89*	0.0*
 **73**	0.2*	2.2*	0.2*
 **74**	0.0	0.0	0.0
 **75**	0.2	1.0	9.9

Table 2.6 Solubility of 1, 8-naphthyridines with Ar = aminobenzothiazole or aminobenzimidazole groups. * = some degradation observed – multiple peaks in HPLC.

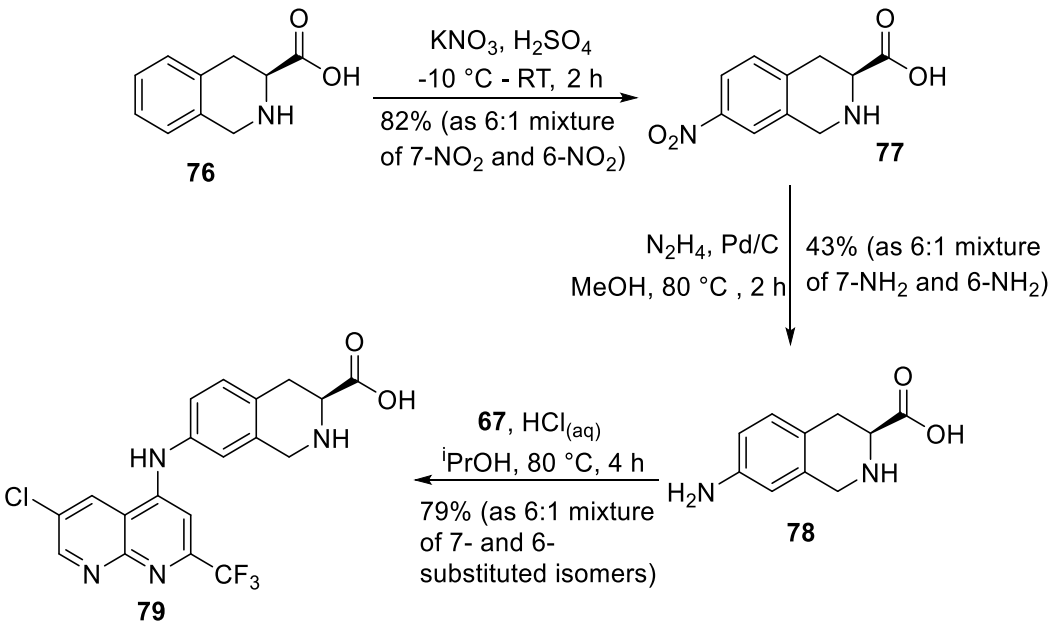
In conclusion, changing the aniline group to a benzothiazole resulted in a loss in potency against both enzyme and cells, and solubility was also very poor. The benzimidazole compound **75** retained potency against DHODH enzyme but was less potent against *P. falciparum* cells and was also less soluble in FaSSIF than the corresponding anilino derivatives. Therefore introducing a heterocycle in place of the aniline group did not achieve the aim of improving

the solubility of the 1,8-naphthyridine series while maintaining excellent potency.

2.3 Investigating more polar aniline substituents

Another way to potentially increase solubility would be to introduce an aniline with more polar groups on it. Including a zwitterionic amino acid moiety in the structure should ensure greater solubility in aqueous media and thus it was planned to synthesise **79**.

In order to make this by a similar S_NAr reaction, the aniline **78** must first be synthesised. This can be done by utilising a two-step literature procedure, starting with the nitration[110] of **76** to give **77** which can then be reduced[111] to **78** (Scheme 2.14).

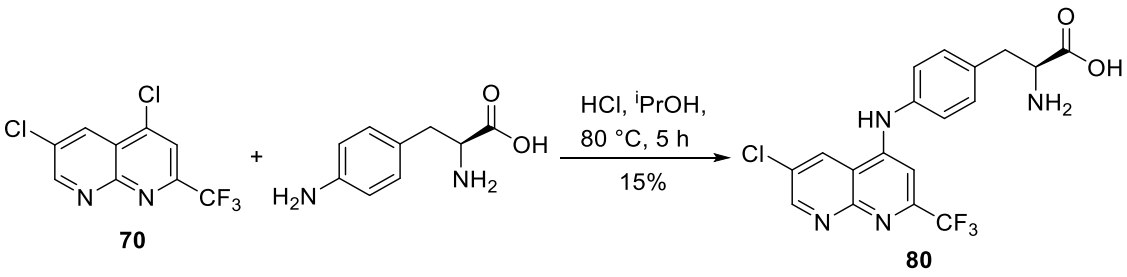


Scheme 2.14 Synthesis of amino acid containing 1,8-naphthyridine **79** which was obtained as an inseparable 6:1 mixture of 7- and 6-substituted isomers arising from a lack of selectivity in the nitration step

The nitration of **76** proceeded in good yield but gave a 6:1 mixture of 7-nitro and 6-nitro isomers; there is poor selectivity between the two positions *para* to the electron donating alkyl groups. The 7-position is thought to be the major product because it is *para* to the more electron-donating alkyl chain. This isomeric mixture was carried forward to the next step, a reduction to the amine **78**. Initially this was attempted using the literature procedure of hydrogen and

palladium on charcoal but this gave a yield of only 30% after 24 h, much worse than what had been reported (98% after 2 h[111]). Therefore the reaction was also attempted using hydrazine with the palladium catalyst to generate hydrogen *in situ*. This was observed using LCMS to go to completion within 2 h but gave only a moderate isolated yield, thought to be due to the product not being fully soluble in methanol and hence some being lost when the catalyst was filtered off. The mixture of isomers of **78** could not be separated so was used without further purification in the next step, the S_NAr reaction with **70**. Acid-catalysis was used in order to ensure that the secondary amine would be protonated and hence the aniline nitrogen would act as the nucleophile. This proceeded in good yield to give **79** as a mixture of 7- and 6- substituted isomers which also proved inseparable by both chromatography and crystallisation.

Due to the difficulties with purifying this compound, a further amino acid containing compound has been synthesised by an analogous S_NAr reaction (Scheme 2.15). By reacting the commercially available 4-amino-L-phenylalanine with **70**, the problem of separating isomers was avoided and **80** could be easily purified and tested to see whether or not the inclusion of such a polar group in the molecule would retain the potent enzyme inhibition observed with other 1,8-naphthyridines.



Scheme 2.15 Acid-catalysed S_NAr reaction used to make **78**

As expected, **80** did show a significant increase in solubility in all three media (Table 2.7). However, **79** and **80** were inactive against both the enzyme and parasite, with IC_{50} values greater than 10 μM.

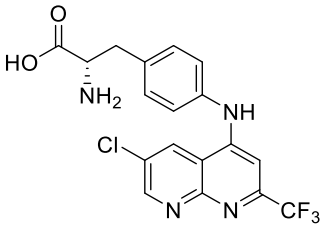
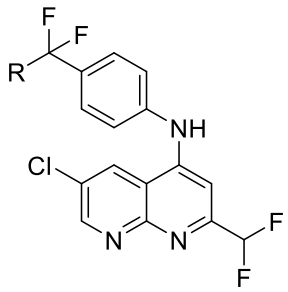
Structure	PBS (pH 7.4)/µM	FaSSIF (pH 6.5)/µM	FaSSGF (pH 1.6)/µM
 **80**	716	955	>2000

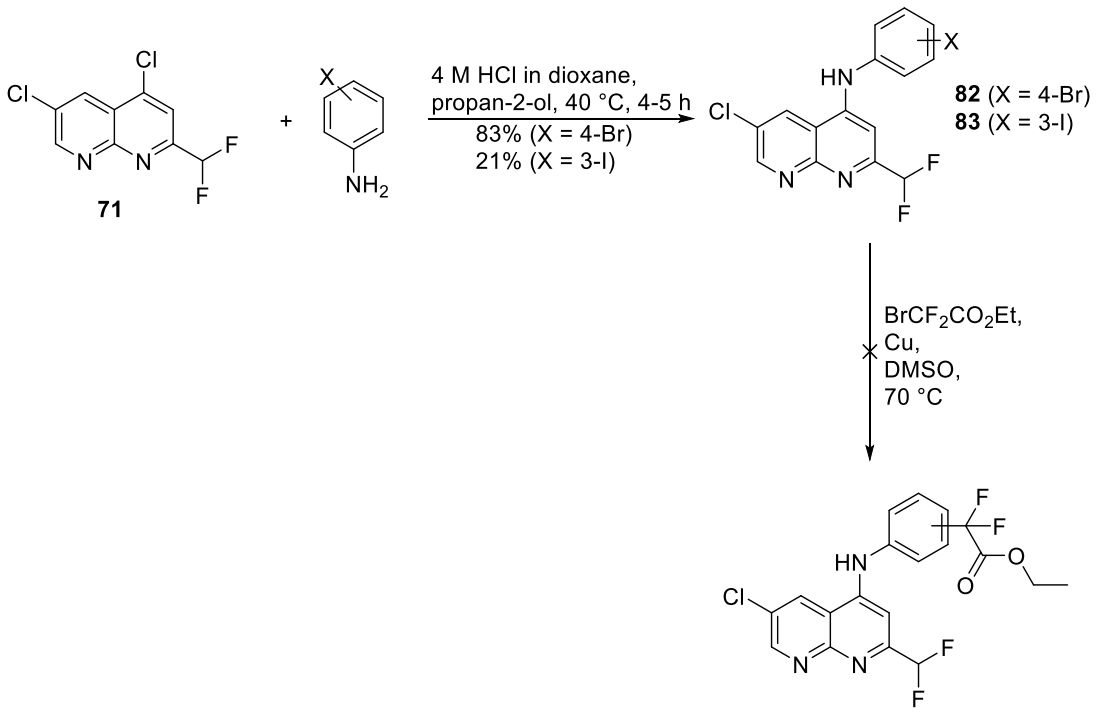
Table 2.7 Solubility of **80** in 3 different media

Drastic changes to either heterocycles or amino acid containing groups were not tolerated so smaller changes to anilines containing ester, alcohol or ether groups were investigated. It was also known that metabolism by cytochrome P450 was likely at any benzylic methylene groups and so molecules of general structure **81** were targeted.

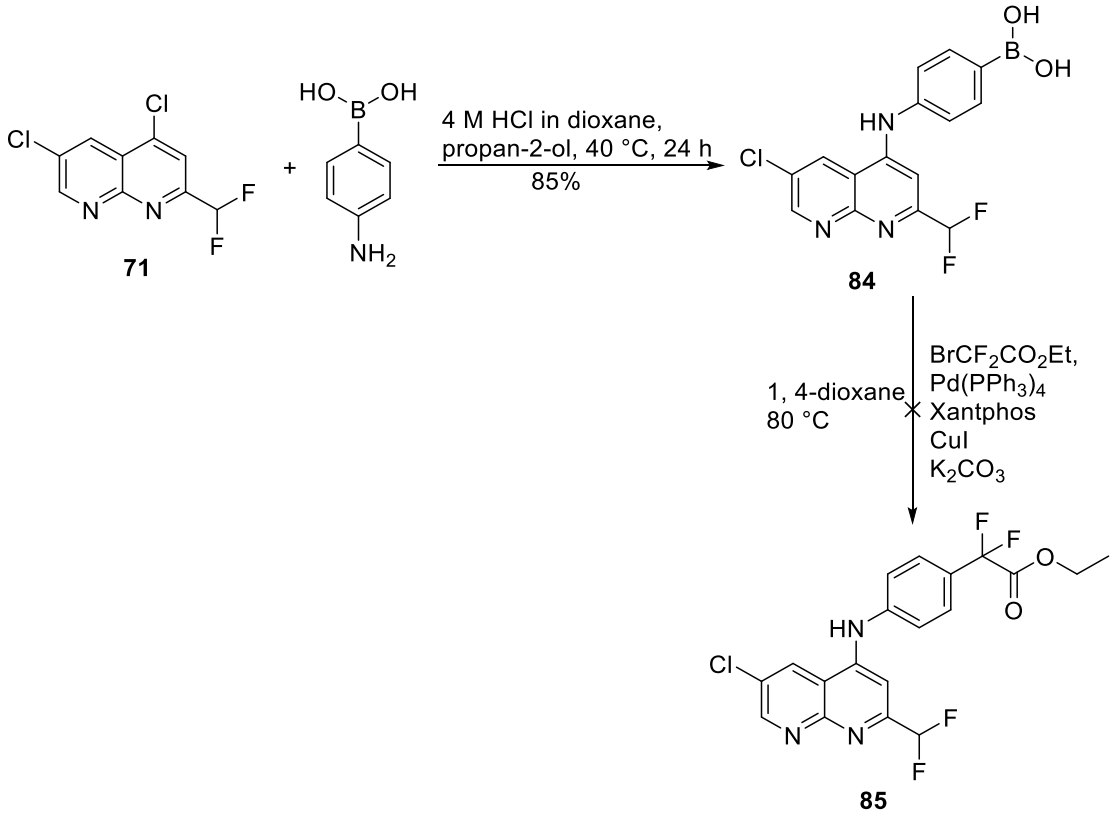

81

One way to make such a molecule could be a copper-mediated reaction between the bromo- or iodo-anilino derivatives and bromodifluoroacetate.[112] Therefore the necessary 1,8-naphthyridines were prepared by the S_NAr reaction of the aniline with **71**, and the copper-mediated coupling reaction was attempted (Scheme 2.16).

However, this was not successful, with starting material being recovered for both the 4-bromo- and 3-iodo-anilino compounds. Another option could be to make the boronic acid and attempt a palladium-catalysed cross-coupling reaction with ethyl bromodifluoroacetate.[113] The 1,8-naphthyridine containing a boronic acid unit was synthesised as before, but the palladium-catalysed reaction (Scheme 2.17) was unsuccessful, both on the 1,8-naphthyridine and the 4-aminobenzene boronic acid. There was some evidence (LCMS and NMR) for the boronic acid falling off but being replaced by a hydrogen rather than the desired group.

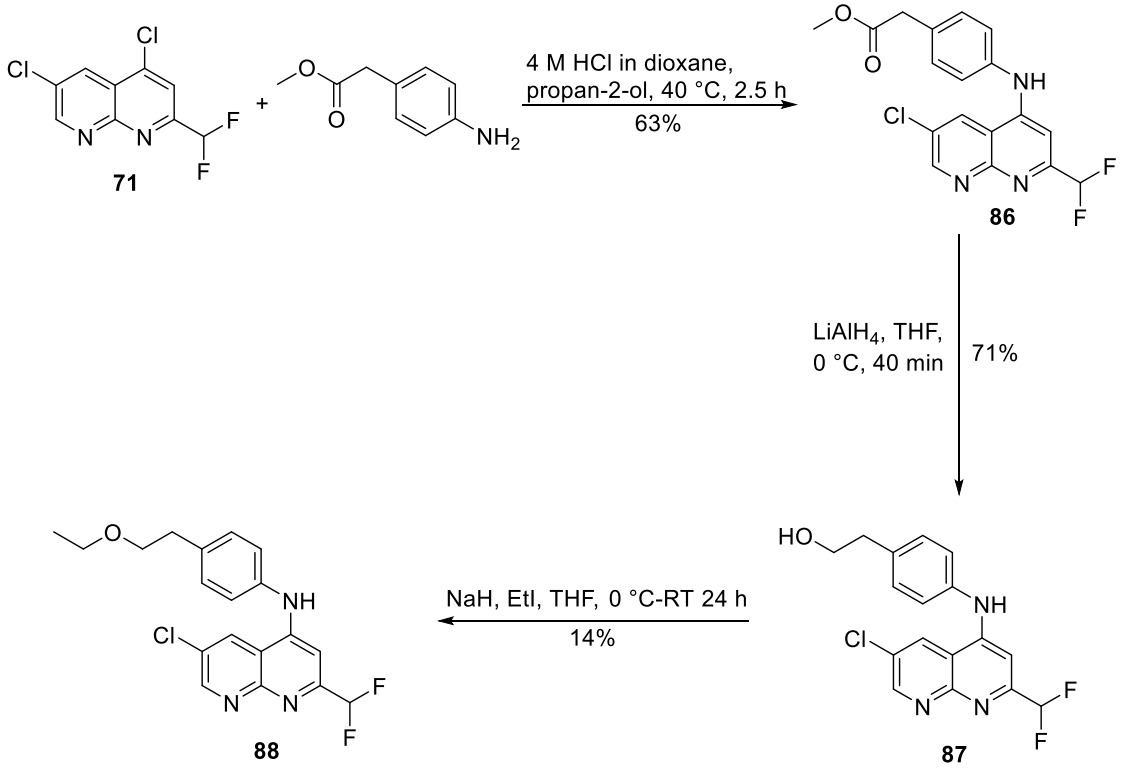


Scheme 2.16 Attempted synthesis of 1,8-naphthyridines containing an anilinodifluoroacetate unit using a copper-mediated reaction



Scheme 2.17 Attempted synthesis of 1,8-naphthyridines containing an anilinodifluoroacetate unit using a palladium-catalysed coupling reaction

With the difficulties encountered in the synthesis of this compound, it was decided to synthesise the analogue without fluorine atoms on the benzylic carbon as a model compound (Scheme 2.18).



Scheme 2.18 Synthesis of 1,8-naphthyridines with an ester, alcohol or ether group on the aniline

The S_NAr reaction with the commercially available methyl-(4-aminophenyl)acetate proceeded in good yield, as did the reduction of the ester **86** to the alcohol **87** using lithium aluminium hydride. The Williamson ether synthesis of **88**, however, had low yield of the desired product due to reaction at the N-H occurring as well as at the O-H. Synthesis of the ^{t}butyldimethylsilyl ether was also attempted but this was unsuccessful. Nevertheless, sufficient quantity of each compound was obtained for biological evaluation and the other newly synthesised 1,8-naphthyridines were also tested. Results of DHODH enzyme assays performed by collaborators at UT Southwestern and cell assays performed by collaborators at Dundee are shown in Table 2.8 and solubility assays in Table 2.9.

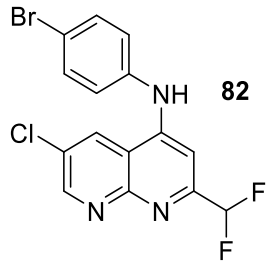
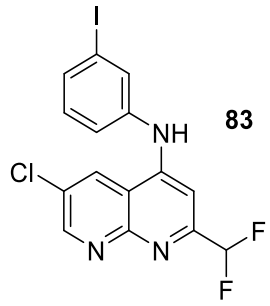
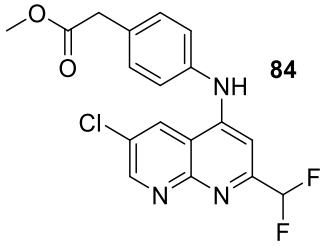
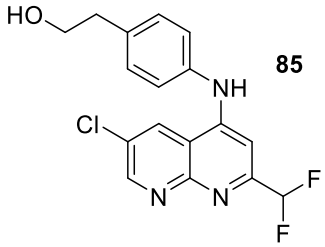
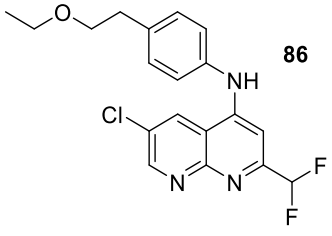
Compound structure	PfDHODH IC_{50}/nM	PvDHODH IC_{50}/nM	*P. falciparum* EC_{50}/µM
 **82**	31 ± 6	52 ± 4	38
 **83**	16 ± 5	15 ± 5	46 ± 3
 **84**	69 ± 10	350 ± 40	711 ± 205
 **85**	83 ± 11	290 ± 40	909 ± 182
 **86**	68 ± 7	380 ± 20	ND

Table 2.8 Inhibition values for compounds with different aniline groups against DHODH enzyme from *Plasmodium* parasites and *P. falciparum* 3D7 cells. ND = not determined.

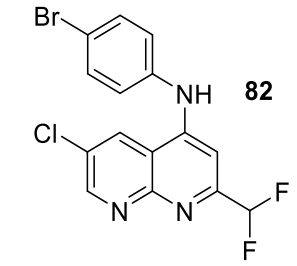
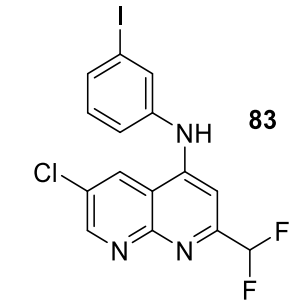
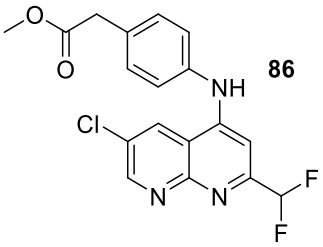
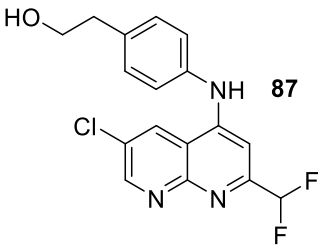
Compound structure	PBS (pH 7.4)/µM	FaSSIF (pH 6.5)/µM	FaSSGF (pH 1.6)/µM
 **82**	1.3	6.0	67
 **83**	1.1	11	61
 **86**	32	63	805
 **87**	20	57	>2000

Table 2.9 Solubility of compounds with different aniline substituents in 3 media.

Small changes in the aniline were tolerated well, with the 3-iodo- variant **83** in particular showing very good potency against DHODH enzyme from both *P. falciparum* and *P. vivax.* However, the ester, alcohol and ether variants all showed some loss in potency against PfDHODH and about tenfold decrease in potency against PvDHODH. All of these compounds retained a lack of inhibition of human enzyme. **82** and **83** also showed good potency against *P. falciparum* cells, albeit slightly lower than **55** but **86** and **87** were significantly less potent against *P. falciparum* cells than against the pure enzyme.

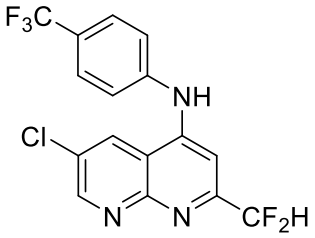
The compounds with a halogenated aniline showed poor solubility in PBS and FaSSIF and moderate solubility in FaSSGF. Those containing an ester or

alcohol group were much more soluble, with moderate values obtained in FaSSIF and high solubility in the acidic FaSSGF medium.

Overall, these 1,8-naphthyridines with more polar aniline groups were more soluble as expected but showed too great a loss in potency, especially against *P. falciparum* cells, to be useful lead compounds.

2.4 Full evaluation of front-runner compound 55

With excellent potency and selectivity, as well as moderate solubility, **55** was selected as a front-runner compound and was thus subjected to further assays to investigate whether it was suitable to be taken forward as a lead compound.



55

It was therefore sent to collaborators at UT Southwestern to test for inhibition of mammalian DHODH and at Monash to evaluate its metabolic stability, cytochrome P450 inhibition, permeability and *in vivo* pharmacokinetics.

2.4.1 Mammalian DHODH screening

It had already been discovered that these 1,8-naphthyridines have excellent selectivity for parasite DHODH over human but it was important to discover if they would inhibit any mammalian DHODH from a species that may be used in future toxicity studies. The competitor triazolopyrimidine compound **26,** currently in clinical trials, can inhibit rodent DHODH, which made animal toxicity testing more complicated.[95] Therefore, **55** was tested for inhibition of DHODH from six mammalian species: rat, mouse, dog, rabbit, monkey and minipig. Pleasingly, no inhibition was observed: IC_{50} values were greater than 100 μM for all species.

2.4.2 Metabolic stability

In order to test for metabolism by cytochrome P450, the compound was incubated in mammalian liver microsomes and its concentration was measured over time. Data were then fitted to an exponential decay function to determine the degradation rate constant (k) and hence calculate degradation

half-life ($t_{1/2}$), *in vitro* intrinsic clearance value (CL_{int}) and predicted *in vivo* CL_{int}, blood clearance values (CL_{blood}) and hepatic extraction ratio (E_H), taking into account the protein content, liver and body mass and hepatic blood flow values (Q) for each species according to equations 2.1–2.5. Values for these parameters are given in Table 2.10.

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \quad (2.1)$$

$$CL_{int,\ in\ vitro} = \frac{k}{mg\ protein\ mL^{-1}} \quad (2.2)$$

$$CL_{int} = CL_{int,\ in\ vitro} \times \frac{liver\ mass/g}{body\ mass/kg} \times \frac{mg\ protein}{g\ liver} \quad (2.3)$$

$$CL_{blood} = \frac{Q \times CL_{int}}{Q + CL_{int}} \quad (2.4)$$

$$E_H = \frac{CL_{blood}}{Q} \quad (2.5)$$

The compound showed a long half-life and low clearance in all 4 species so cytochrome P450 mediated metabolism is unlikely to be a problem *in vivo*.

Incubation in liver S9 fraction, containing aldehyde oxidase as well as cytochrome P450, was also carried out in the presence or absence of raloxifene, an aldehyde oxidase inhibitor, and NADPH, a cofactor required by cytochrome P450, and metabolic stability parameters calculated according to equations 2.1–2.5 (Table 2.11).

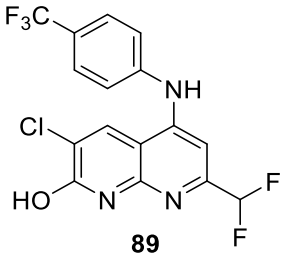
Species	Human	Rat	Mouse	Dog
Degradation half-life/min	240	>255	>255	>255
In vitro CL_{int}/µg min^{-1} mg protein^{-1}	7	<7	<7	<7
Predicted CL_{int}/mL min^{-1} kg^{-1}	6	<12	<18	<13
Predicted CL_{blood}/mL min^{-1} kg^{-1}	5	<10	<16	<10
Predicted E_H	0.22	<0.15	<0.13	<0.30

Table 2.10 Metabolic stability parameters for **55** in liver microsomes from 4 different species

Species	Human			Rat		
Conditions	+ NADPH	− NADPH	− NADPH, + raloxifene	+ NADPH	− NADPH	− NADPH, + raloxifene
Degradation half-life/min	62	105	>510	404	>510	>510
In vitro CL_{int}/ µg min^{-1} mg $protein^{-1}$	4	3	<1	1	<1	<1
Predicted CL_{int}/mL min^{-1} kg^{-1}	14	8	<2	3	<3	<3
Predicted CL_{blood}/mL min^{-1} kg^{-1}	8	6	<2	3	<3	<3
Predicted E_H	0.40	0.28	<0.08	0.05	<0.04	<0.04

Table 2.11 Metabolic stability parameters for **55** in liver S9 fraction from human and rat

In human S9 fraction, **55** displayed an intermediate degradation rate in the presence of NADPH which was only slightly reduced in the absence of NADPH but a low rate in the absence of NADPH unless raloxifene was also added. This suggests that aldehyde oxidase mediated metabolism is the major degradation pathway for this compound and a degradation product with mass increased by 16 was observed. This product is thought to be **89**: it is known that aldehyde oxidase can oxidise at the 2-position of pyridine-like heterocycles.[114] The degradation rates without raloxifene in the rat S9 fraction are much lower than for human because rats have much lower aldehyde oxidase activity. This metabolic susceptibility must be addressed in order for the compound to have a long enough lifetime to be efficacious in humans.



89

2.4.3 Cytochrome P450 inhibition

The compound was screened for inhibition of several isoforms of cytochrome P450 to check the potential to inhibit cytochrome P450-mediated metabolism of concomitantly administered drugs (Table 2.12).

Isoform	IC_{50}/μM
CYP1A2	4.6
CYP2C9	20.7
CYP2C19	12.7
CYP2D6	6.0
CYP3A4	>20

Table 2.12 IC_{50} values for the inhibition of cytochrome P450 isoforms by **55**

Overall, low to moderate cytochrome P450 inhibition was observed.

2.4.4 Permeability

The permeability across Caco-2 cell monolayers was measured to give an indication of uptake of the molecule in the body. Permeability was assessed in the apical to basolateral (A–B) and basolateral to apical (B–A) directions in order to also assess the potential for efflux. The mass balance was calculated by measuring the mass of the donor and acceptor solutions initially and after 4 h (equation 2.6). The apparent permeability coefficient (P_{app}) was determined based on the rate of compound appearance in acceptor buffer at steady state using equation 2.7, where dQ/dt is the apparent steady-state transport rate/μmol s^{-1}, A is the surface area of the Caco-2 monolayer (0.3 cm^2) and the concentration in the donor solution at the start/μmol cm^{-3} is $C_{Donor}^{initial}$.

$$Mass\ balance\ (\%) = \frac{Mass_{Donor}^{final} + Mass_{Acceptor}^{final}}{Mass_{Donor}^{initial}} \times 100 \quad (2.6)$$

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_{Donor}^{initial} \times A} \quad (2.7)$$

By calculating P_{app} for both A–B and B–A directions, the apparent efflux ratio (E_R) can be obtained by using equation 2.8.

$$E_R = \frac{P_{app,\ B-A}}{P_{app,\ A-B}} \quad (2.8)$$

The values obtained for these parameters are shown in Table 2.13.

Mass balance/%		$P_{app} \times 10^{-6}$/cm s^{-1}		E_R
A–B	B–A	A–B	B–A	
53 ± 6	73 ± 8	20 ± 3	13 ± 2	0.7 ± 0.1

Table 2.13 Mass balance and P_{app} data for **55** across Caco-2 cell monolayers. Values are presented as the mean ± SD of 3 replicates.

The mass balance was relatively high in the B–A direction but lower for A–B, which may be due to this lipophilic compound (cLog $D_{7.4}$ = 4.7) having high membrane retention and low levels of desorption into the acceptor medium. The A–B P_{app} value indicates moderate permeability, although this may be underestimated due to the relatively low mass balance. The B–A P_{app} value is slightly lower than A–B but not too dissimilar, so there is unlikely to be polarised transport across the membrane for this compound.

2.4.5 *In vivo* pharmacokinetics

The compound was introduced into mice and rats, either orally or by intravenous administration, to study the plasma exposure and pharmacokinetics.

The plasma concentration was monitored over 24 h after oral administration of 20 mg kg^{-1} in mice (Fig. 2.1) and the following exposure parameters were determined: area under the plasma concentration versus time curve from time zero to the last time point with measurable concentration ($AUC_{0–last}$), maximum plasma concentration (C_{max}) and the time to achieve C_{max} (T_{max}) (Table 2.14). The apparent half-life could not be calculated due to the very high plasma concentration still remaining at the end of the 24 h sampling time but is evidently very long, as predicted from the low CL_{blood} value obtained from the mouse liver microsome experiment.

Metabolites observed (by LCMS) were a mono-oxygenated product and the result of both oxygenation and cleavage of the aniline group.

The mice were not observed to have any side effects or adverse reaction to the compound, so toxicity appears to be low based on this study.

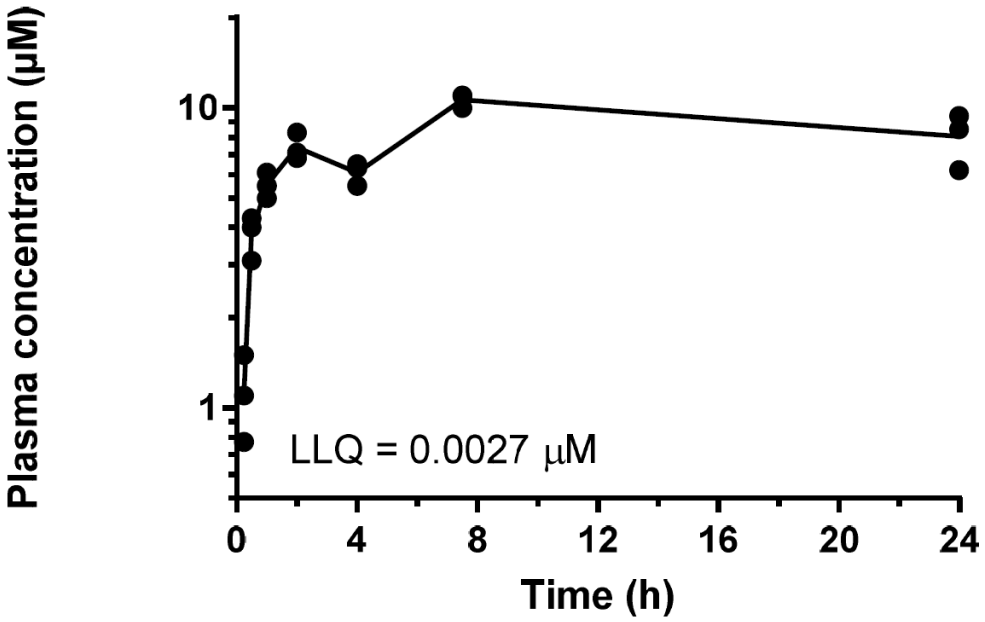


Figure 2.1 Plasma concentration of **55** in male Swiss outbred mice after oral administration at 20 mg/kg.

C_{max}/μM	11
T_{max}/h	7.5
$AUC_{0–last}$/h μM	205

Table 2.14 Exposure parameters for **55** in male Swiss outbred mice following oral administration at 20 mg/kg.

The compound was given to rats by both intravenous administration and orally and the plasma concentration was measured over time (Fig. 2.2), as was the concentration present in urine. This allowed the determination of several pharmacokinetic parameters: apparent half-life ($t_{1/2}$), plasma clearance (CL), apparent volume of distribution at steady state (V_{ss}), area under the concentration versus time curve from time zero to infinity ($AUC_{0–inf}$), $AUC_{0–last}$ and oral bioavailability (BA). These were calculated using equations 2.9–2.12.

$$t_{\frac{1}{2}} = \frac{\ln 2}{\lambda_z} \tag{2.9}$$

$$plasma\ CL = \frac{dose_{IV}}{AUC_{IV}} \tag{2.10}$$

$$plasma\ V_{ss} = \frac{AUMC_{IV}}{AUC_{IV}} \times plasma\ CL \tag{2.11}$$

$$BA = \frac{AUC_{oral} \times dose_{IV}}{AUC_{IV} \times dose_{oral}} \tag{2.12}$$

where AUC_{IV} is the $AUC_{0–inf}$ after IV administration, λ_z is the terminal elimination rate constant after IV administration and $AUMC_{IV}$ is the area under

the first moment of the plasma concentration versus time curve from time zero to infinity after IV administration.

Pharmacokinetic parameters for IV administration are given in Table 2.15, and oral administration in Table 2.16.

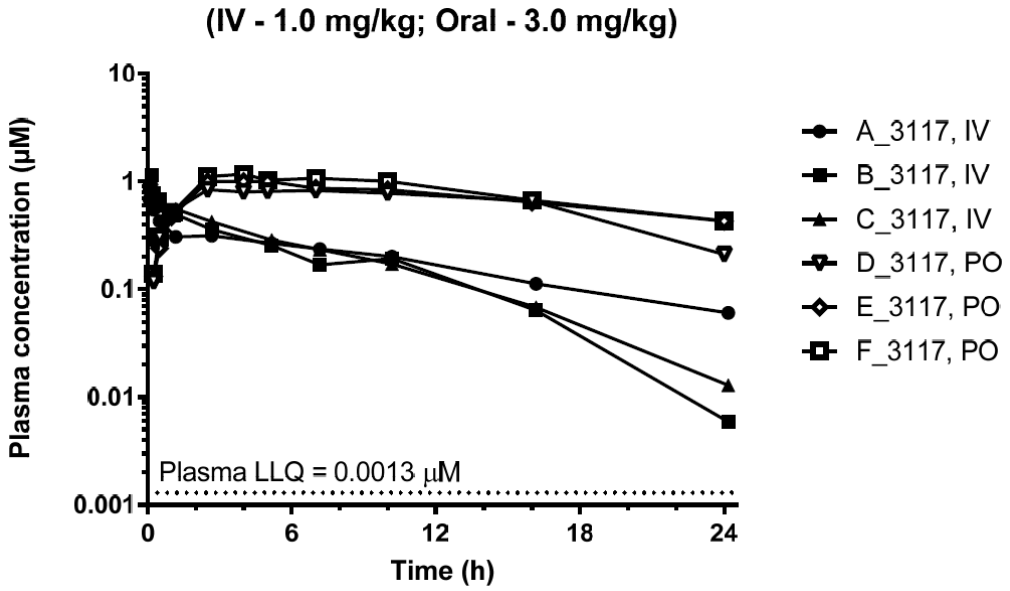


Figure 2.2 Plasma concentrations of **55** in 6 male Sprague–Dawley rats following IV administration at 1.0 mg/kg or oral administration (PO) at 3.0 mg/kg

Apparent $t_{1/2}$/h	4.9 ± 2.9
Plasma CL/mL min^{-1} kg^{-1}	9.8 ± 1.1
Plasma V_{ss}/L kg^{-1}	4.7 ± 1.4
Plasma $AUC_{0–last}$/h µM	4.3 ± 0.2
Plasma $AUC_{0–inf}$/h µM	4.6 ± 0.6
Dose in urine/%	<0.10

Table 2.15 Plasma pharmacokinetic parameters for **55** in male Sprague–Dawley rats following IV administration at 1.0 mg/kg

Apparent $t_{1/2}$/h	11 ± 3.6
Plasma C_{max}/μM	1.0 ± 0.2
T_{max}/h	3.8 ± 1.3
Plasma $AUC_{0–last}$/h μM	17 ± 2
Plasma $AUC_{0–inf}$/h μM	23 ± 5
Dose in urine/%	<0.05

Table 2.16 Plasma pharmacokinetic parameters for **55** in male Sprague–Dawley rats following oral administration at 3.0 mg/kg

Fig. 2.1 shows that **55** was slowly absorbed after oral administration, with mean maximum plasma concentrations of around 1 μM occurring after around 2.5–5.0 h, with a long half-life (11 h), much longer than that observed after IV administration (4.9 h). This could be due to saturation of the clearance mechanism. BA was calculated (equation 2.12) to be 123 ± 14%; this value of above 100% could also be explained by saturation. With both types of administration, the amount of unreacted **55** excreted in the urine was below the detection limit, suggesting that metabolism rather than direct urinary excretion is the major pathway for *in vivo* elimination of the compound; the same two metabolites were detected in the rat as in the mouse study.

The blood to plasma partitioning ratio (B/P) was measured to be 5.2 and this allowed for the calculation of blood pharmacokinetic parameters according to equations 2.13–2.15.

$$blood\ CL = \frac{plasma\ CL}{B/P} \tag{2.13}$$

$$blood\ V_{ss} = \frac{plasma\ V_{ss}}{B/P} \tag{2.14}$$

$$blood\ AUC = plasma\ AUC\ \times B/P \tag{2.15}$$

Blood pharmacokinetic parameters for IV administration are given in Table 2.17, and oral administration in Table 2.18.

Blood CL/mL min^{-1} kg^{-1}	1.9 ± 0.2
Blood V_{ss}/L kg^{-1}	0.9 ± 0.3
Blood $AUC_{0–inf}$/h μM	24 ± 3

Table 2.17 Blood pharmacokinetic parameters for **55** in male Sprague–Dawley rats following IV administration at 1.0 mg/kg

Blood C_{max}/μM	5.2 ± 0.8
Blood $AUC_{0–inf}$/h μM	119 ± 27

Table 2.18 Blood pharmacokinetic parameters for **55** in male Sprague–Dawley rats following oral administration at 3.0 mg/kg

The B/P ratio of 5.2 is quite high, indicating that **55** is largely distributed into erythrocytes. Blood clearance is low, as predicted in the liver microsome experiment.

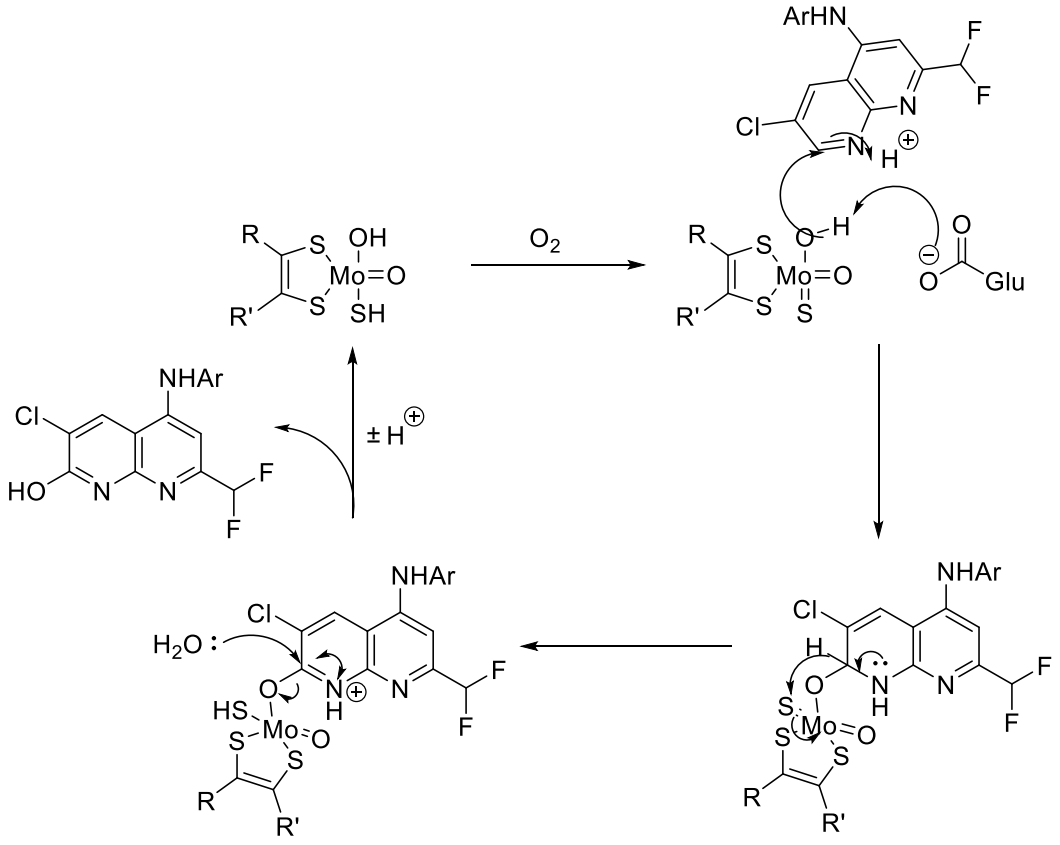
Overall, the oral bioavailability of this compound in rats is very good. This is likely to be because of reasonable absorption (moderate cell permeability was observed in the Caco-2 cell monolayer experiment) and low first pass elimination (very low predicted hepatic extraction ratio in liver microsomes). However, it is unknown how well this would translate into humans, where the higher aldehyde oxidase activity levels could result in faster metabolism. Therefore, similar compounds to **55** which are not metabolised so quickly by aldehyde oxidase would be highly desirable.

Chapter 3 Investigating Aldehyde Oxidase Metabolism

3.1 Aldehyde oxidase

Aldehyde oxidase (AO) is a molybdoflavoprotein consisting of two subunits.[115] It is located in the cytosol of cells in many tissues, especially the liver.[116] AO can catalyse the oxidation of substrates including: aldehydes to carboxylic acids, iminium ions to lactams, and, most importantly for medicinal chemists, aromatic azaheterocycles.[117] Molecules containing pyridine, pyrimidine or pyrazine motifs are known to be potential substrates for this enzyme.[118]

The oxidation of heterocycles by AO has been proposed to occur via nucleophilic attack at the carbon next to a pyridine-like nitrogen, followed by re-aromatisation via hydride shift and finally attack by water to release the enzyme again[119] (Scheme 3.1). There is also some evidence that these steps may be concerted with a tetrahedral transition state rather than stepwise with a tetrahedral intermediate.[119]



Scheme 3.1 Proposed mechanism for the oxidation of 1,8-naphthyridines by AO

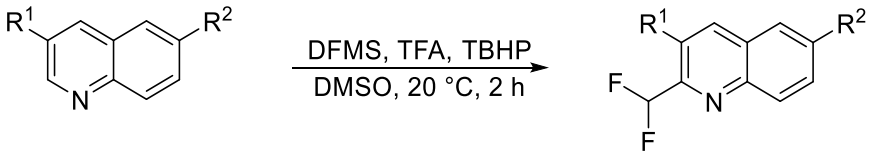
The most obvious way to prevent oxidation of the 1,8-naphthyridines by AO would be to introduce a substituent into the 7-position of the 1,8-naphthyridine where the oxidation is thought to happen, but there is also a precedent for

stopping metabolism by AO by altering substituents at other positions.[120] Changing the sterics of the molecule could block the enzyme from binding and hence reduce metabolism. Changing the electronics of the aromatic ring could also affect the rate of oxidation by AO. Making a molecule more electron rich should reduce AO metabolism because the nucleophilic attack would be slowed down.[121] For example, in one study, a series of pyrimidines was found to be a substrate for AO when they contained an electron-withdrawing group but not an electron-donating group.[122] However, in a series of isoquinolines, molecules with an electron-donating substituent were more susceptible to AO metabolism than those containing an electron-withdrawing group.[122]

Recently, a lot of work has been done to try to predict AO metabolism with some success, but there is still no *in silico* method capable of reliably predicting whether or not a molecule will be a good substrate for AO[123] and so experimental data are very important. A chemical 'litmus test' (Baran test) has been developed, using bis(((difluoromethyl)sulfinyl)oxy)zinc (DFMS) to introduce a difluoromethyl group. This reaction goes via a difluoromethyl radical and is thought to mimic the reactivity of AO and hence predict whether a molecule may be a good substrate for this enzyme.[124] However, this test is not able to predict if a molecule is the incorrect shape for binding to the active site. Therefore, it is useful for indicating the potential of a molecule to be metabolised by AO but can give some false positives.

3.2 Investigating AO susceptibility of a quinoline series using the 'litmus test'

In order to understand how altering remote substituents could affect AO susceptibility, a series of quinolines with different substituents at the 3- and 6-positions was treated with the DFMS reagent (Scheme 3.2).



Scheme 3.2 Reaction of 6-substituted quinolines under the Baran 'litmus test' to evaluate susceptibility to metabolism by AO

In each case, the reaction mixture was stirred at 20 °C for two hours then analysed by LCMS to determine the relative sizes of the starting material and

product (M+50) peaks and hence the extent of conversion of the reaction. For each substrate, the reaction was run in duplicate (Table 3.1).

R^1	R^2	Run 1 conversion/%	Run 2 conversion/%	Mean conversion/%
H	H	100	70	85
H	F	21	34	28
H	Cl	80	74	77
H (+ 2-Me)	Cl	72	79	75
H	Br	73	74	74
H	CF_3	68	81	74
H	OMe	76	65	70
H	COOMe	74	67	71
F	H	29	24	26
Cl	H	59	60	60
Br	H	73	48	60
CF_3	H	43	32	38
OMe	H	74	67	70
COOEt	H	87	83	85

Table 3.1 Results of the Baran 'litmus test' for predicting AO metabolism on a series of quinolines. % conversion was determined by LCMS.

The results were mostly reproducible with little variation in the conversion observed in each run; the largest difference was for quinoline ($R^1 = R^2 = H$) where the low mass of this molecule made detection in the LCMS more difficult. Two series of quinolines were tested with substituents at either the 3-position or the 6-position. The 3-position of the quinoline is adjacent to the likely site of difluoromethylation (the 2-position) so any electronic effects would be strongly felt and there may also be some steric hindrance which could affect the rate of reaction. The 6-position, conjugated to the 3-position, is more remote, so steric effects would be reduced but electronic effects could still be observed.

Comparing the data for compounds with R^1 = H, 6-fluoroquinoline showed by far the lowest conversion (28%) with all the other substituents giving similar conversion levels of 70–80%. Surprisingly, the compound with a methyl group blocking the 2-position gave similar conversion to the analogous compound without the methyl there. It would be expected that blocking this position would block AO metabolism but it is known that the DFMS reagent can also react at other positions such as the 4-position, whereas the actual enzyme is usually selective for only one position.[124]

Molecules with R^2 = H and different R^1 substituents showed more variation. Again, it was the fluoro- group that resulted in the lowest percentage conversion (26%). Conversion was quite low (38%) for R^1 = CF_3, another σ-withdrawing group. Higher conversion levels were observed for π-donor substituents such as methoxy, but the highest of all (85%) was the π-withdrawing ester group. This suggests that σ-withdrawing groups may be able to reduce the rate of oxidation by AO, although there may also be steric factors.

A pyrimidine series was also tested in this way but all of these molecules proved to be very good substrates, with a mixture of mono- and di-substituted products seen in each case, reflecting the 1.5 equivalents of DFMS used in the reaction.

3.3 *In vitro* metabolism of the quinoline series

Some of these quinolines were sent to collaborators at Monash University to assess their metabolism by cytochrome P450 and AO in order to validate the results of the ‘litmus test’. Compounds were incubated in liver S9 fraction, containing AO as well as cytochrome P450, in the presence or absence of raloxifene, an AO inhibitor, and NADPH, a cofactor required by cytochrome P450. Half-life and *in vitro* intrinsic clearance value ($CL_{int,\ in\ vitro}$) were calculated according to equations 2.1 and 2.2 and the area of the M+16 LCMS peak after 120 min was recorded (Table 3.2). No data could be obtained for the 6-COOMe analogue due to complete hydrolysis of the ester.

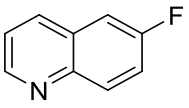
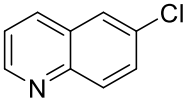
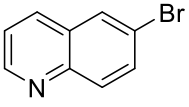
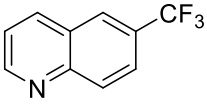
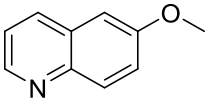
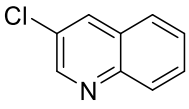
Compound	Conditions	Degradation half-life/min	*In vitro* CL_{int}/ µg min^{-1} mg $protein^{-1}$	M+16 peak area after 120 min
 **90**	+ NADPH	28	10	ND
	− NADPH	60	5	ND
	− NADPH, + raloxifene	70	4	ND
 **91**	+ NADPH	22	13	ND
	− NADPH	90	3	10
	− NADPH, + raloxifene	93	3	7
 **92**	+ NADPH	20	14	ND
	− NADPH	92	3	14
	− NADPH, + raloxifene	93	3	ND
 **93**	+ NADPH	19	15	49
	− NADPH	22	13	59
	− NADPH, + raloxifene	24	12	11
 **94**	+ NADPH	15	18	ND
	− NADPH	>255	<2	ND
	− NADPH, + raloxifene	>255	<2	ND
 **95**	+ NADPH	ND	ND	28
	− NADPH	ND	ND	46
	− NADPH, + raloxifene	ND	ND	15

Table 3.2 Metabolic stability parameters and oxygenated product formation in human liver S9 fraction for a series of quinolines. ND = not determined

The 6-haloquinolines **90**, **91** and **92** all had a short half-life and correspondingly high clearance with NADPH present but much longer half-life without NADPH, indicating metabolism by cytochrome P450. Adding

raloxifene slightly increased the half-life further, showing that these compounds are only metabolised to a low degree by AO. However, even with no NADPH and raloxifene present, the half-life is still less than 100 minutes for these compounds, which suggests that there is some clearance occurring that is not caused by either AO or cytochrome P450.

This was observed to an even greater degree with 6-(trifluoromethyl)quinoline (**93**), which has a half-life of only 24 minutes when metabolism from both cytochrome P450 and AO is inhibited and a similar half-life across all three sets of conditions. However, the area of the LCMS peak for the oxygenated metabolite is much smaller in the presence of raloxifene which would be indicative of this compound being a substrate for AO.

6-Methoxyquinoline (**94**) had a short half-life in the presence of NADPH but was very stable in the absence of NADPH, whether or not raloxifene was added. This means that it is metabolised only by cytochrome P450. It is hypothesised that the strongly electron-donating 6-methoxy group is suppressing oxygenation of this quinoline by AO.

Limited data were obtained for 3-chloroquinoline due to poor detection of this compound by LCMS but there appears to be some metabolism by AO because the M+16 peak was smaller in the presence of raloxifene.

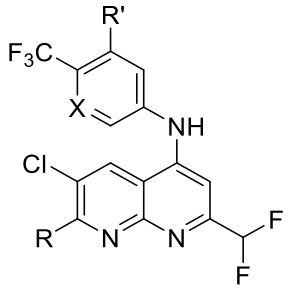
3.4 Quinoline series conclusions

The results of incubating with DFMS in the ‘litmus test’ predicted that electron-withdrawing substituents such as fluoro- or trifluoromethyl- groups would reduce the susceptibility of quinolines to metabolism by AO. When these compounds were incubated in liver S9 fraction, some AO oxidation was observed at a level which is believed to be similar to the other halogenated quinolines, although the high level of clearance by other pathways made it difficult to draw firm conclusions. Surprisingly, it was the 6-methoxyquinoline (**95**) that was most resistant to metabolism by AO, despite the relatively high conversion observed in the ‘litmus test’.

Overall, it is definitely possible for remote substituents to affect metabolism of a compound by AO. Extrapolating these results to the 1,8-naphthyridine series shows the potential for reducing the rate of metabolism of these compounds by changing the molecule at positions other than the site of metabolism, but it remains unclear which group would be best to achieve this.

Chapter 4 1,8-Naphthyridines with a Substituent at the 7-Position

One way to overcome the liability of the 1,8-naphthyridines to metabolism by AO is to introduce a substituent at the 7-position where this oxidation is understood to take place. Therefore it was decided to synthesise a library of compounds of general structure **96**.



96

4.1 7-Amino- derivatives

It was hypothesised that having R = NH_2 might not only block this site of metabolism but also improve solubility and potency, since it would make the naphthyridine nitrogen a stronger hydrogen bond acceptor.

First, *in silico* docking experiments were performed using Glide to check what effect the introduction of a 7-amino group might have on binding affinity. The results suggested that there would be very little change, with a docking score of −11.267 for **104** compared to −11.318 for the analogous molecule without the additional 7-amino group. The predicted binding pose (Fig. 4.1) was also very similar to the X-ray crystal structure previously obtained for a 1,8-naphthyridine bound to PfDHODH (Fig. 1.12).

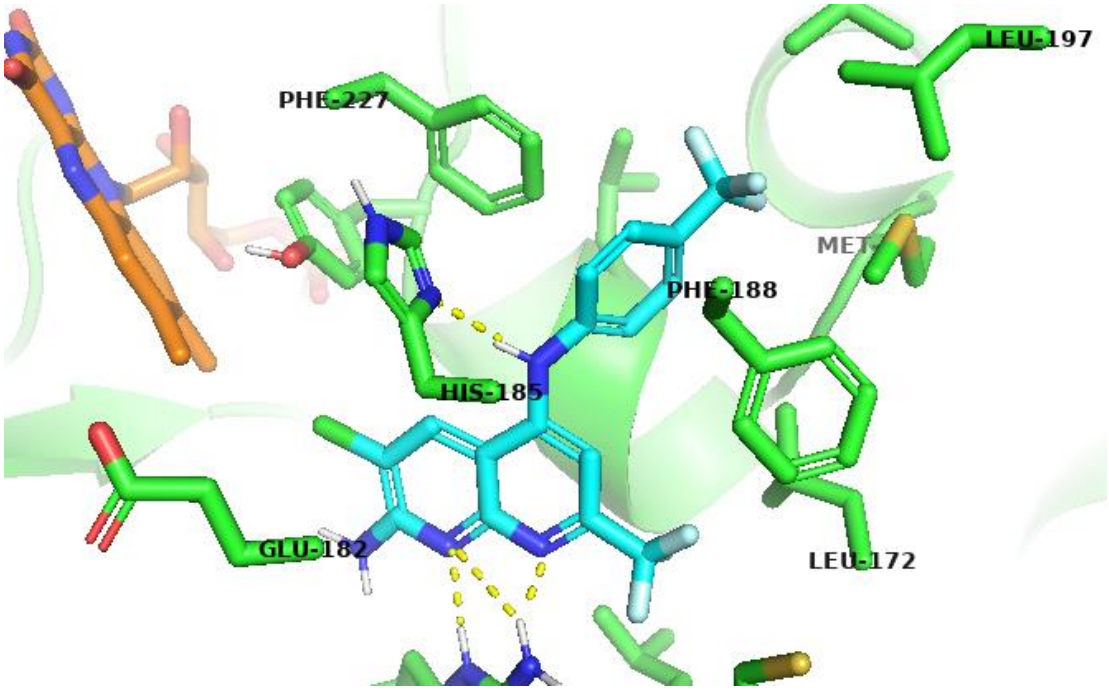
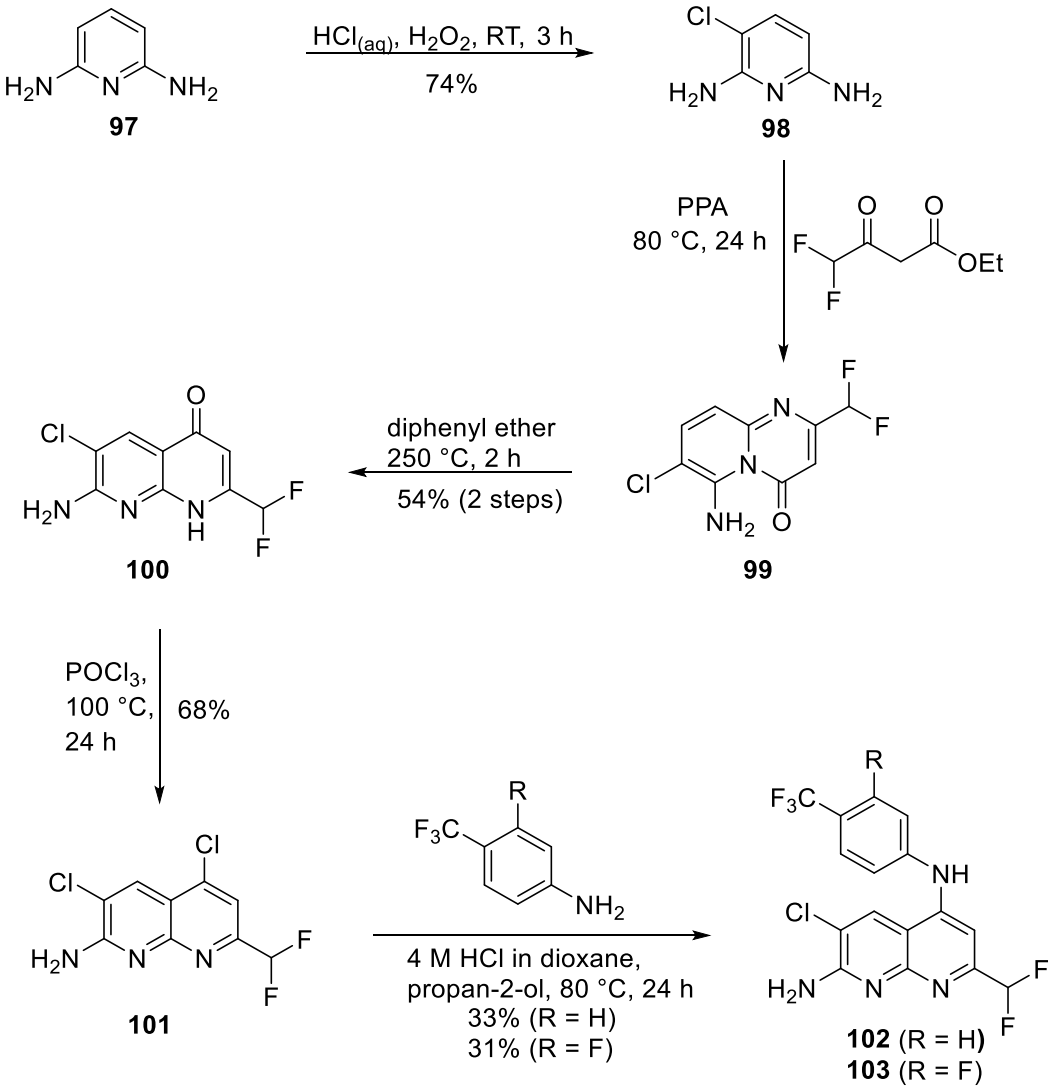


Figure 4.1 Predicted binding mode of a 7-amino-1,8-naphthyridine (**104,** blue) bound to *P. falciparum* DHODH (green), along with FMN (orange). Hydrogen bonds to histidine and arginine residues are shown by yellow dashed lines.

4.1.1 Synthesis

In order to make such analogues a new synthetic route was used, inspired by the synthesis of 1,8-naphthyridines by Ferrarini et al.[125, 126] based on the Conrad-Limpach quinoline synthesis (Scheme 4.1). The first step was the chlorination of 2,6-diaminopyridine using hydrochloric acid and hydrogen peroxide[127] which proceeded in good yield. The polyphosphoric acid (PPA) catalysed cyclisation of **98** with a β-ketoester[125] resulted in the formation of pyrido[1,2-a]pyrimidin-4-one **99** which rearranged to **100** upon refluxing in diphenyl ether[126] for 2 h. This was chlorinated using $POCl_3$, then the desired final compounds were synthesised by the S_NAr reaction of **101** with two different anilines. This required heating to 80 °C overnight to obtain moderate yields of each compound, sufficient for biological evaluation. Some 2-(trifluoromethyl)- analogues of this series (**104**, **105** and **106**) were also synthesised in the same way, by MChem student Kyle Orritt. A similar route was attempted to make the analogous compounds with fluoro- or methyl groups at the 7-position but the rearrangement of the pyrido[1,2-a]pyrimidin-4-one intermediate did not occur in the absence of the electron-donating amino group.



Scheme 4.1 Synthetic route to make 1,8-naphthyridine analogues with an amino- group at the 7-position

4.1.2 Biological evaluation of 7-amino-1,8,naphthyridines

The five 1,8-naphthyridines synthesised using this route were tested for efficacy against both the DHODH enzyme *in vitro* by collaborators at UT Southwestern and in cellular assays against *Plasmodium falciparum* (isolate 3D7) by collaborators at Dundee (Table 4.1).

None of these compounds inhibited human DHODH but unfortunately they were also not very potent against either the parasite enzyme or cells. The best of these compounds, **103**, had an IC_{50} value of 1.2 μM for *P. falciparum* DHODH enzyme, 0.98 μM for *P. vivax* enzyme and 0.52 μM for *P. falciparum* cells, a decrease of more than tenfold on the previous compounds without an amino group at the 7-position. This loss in potency may be due to the unfavourable introduction of a polar amino group into a region of the enzyme that is quite hydrophobic.

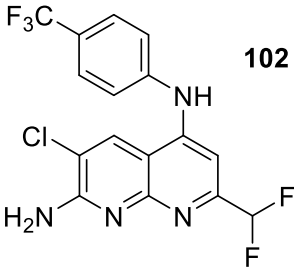
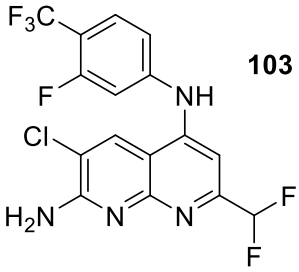
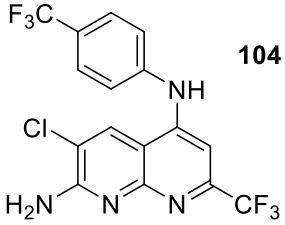
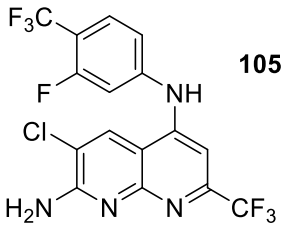
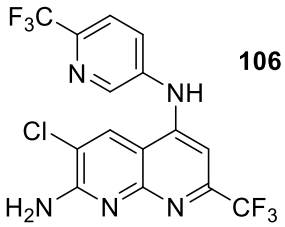
Structure	PfDHODH IC_{50}/nM	PvDHODH IC_{50}/nM	*P. falciparum* cell EC_{50}/nM
 **102**	1700 ± 200	1700 ± 400	ND
 **103**	1200 ± 100	980 ± 120	518 ± 65
 **104**	2300 ± 200	4300 ± 500	2570 ± 170
 **105**	1400 ± 200	1900 ± 100	542 ± 133
 **106**	5700 ± 600	4600 ± 800	2785 ± 130

Table 4.1 Inhibition data for DHODH enzyme from two different *Plasmodium* species and *P. falciparum* cells for 7-amino-1,8-naphthyridines. ND = not determined

As with the other series, 2-difluoromethyl compounds are more potent than the 2-trifluoromethyl analogues. A trend can also be observed with the aniline groups: the most potent compounds are those with 3-fluoro-4-(trifluoromethyl)aniline, followed by 4-(trifluoromethyl)aniline with the more polar aminopyridine-containing molecule **106** the least potent.

The solubility of these compounds (except **102**, where insufficient material was available) was measured in three different solutions: PBS buffer at pH 7.4, FaSSIF at pH 6.5 FaSSGF at pH 1.6 (Table 4.2).

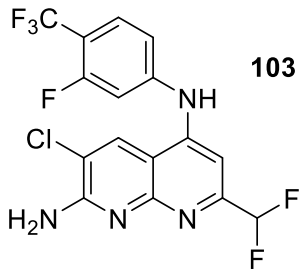
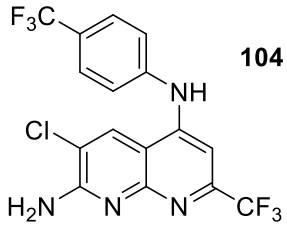
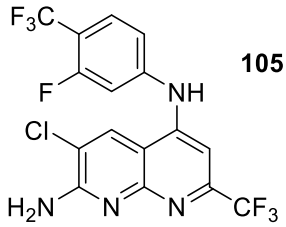
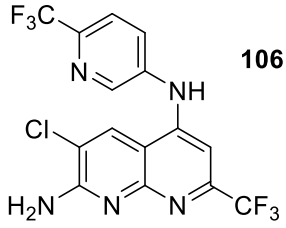
Structure	PBS (pH 7.4)/µM	FaSSIF (pH 6.5)/µM	FaSSGF (pH 1.6)/µM
 **103**	6.6	99	112
 **104**	0.0	0.0	16
 **105**	0.0	0.6	12
 **106**	2.2	9.4	58

Table 4.2 Solubility of 7-amino-1,8-naphthyridines in three media

The 2-difluoromethyl compound **103** showed decent solubility, up to 99 µM in FaSSIF. The 2-trifluoromethyl compounds, however, had very low solubility; only **106** with the more polar aminopyridine group dissolved at all in the neutral media.

4.2 7-Methylamino- and dimethylamino derivatives

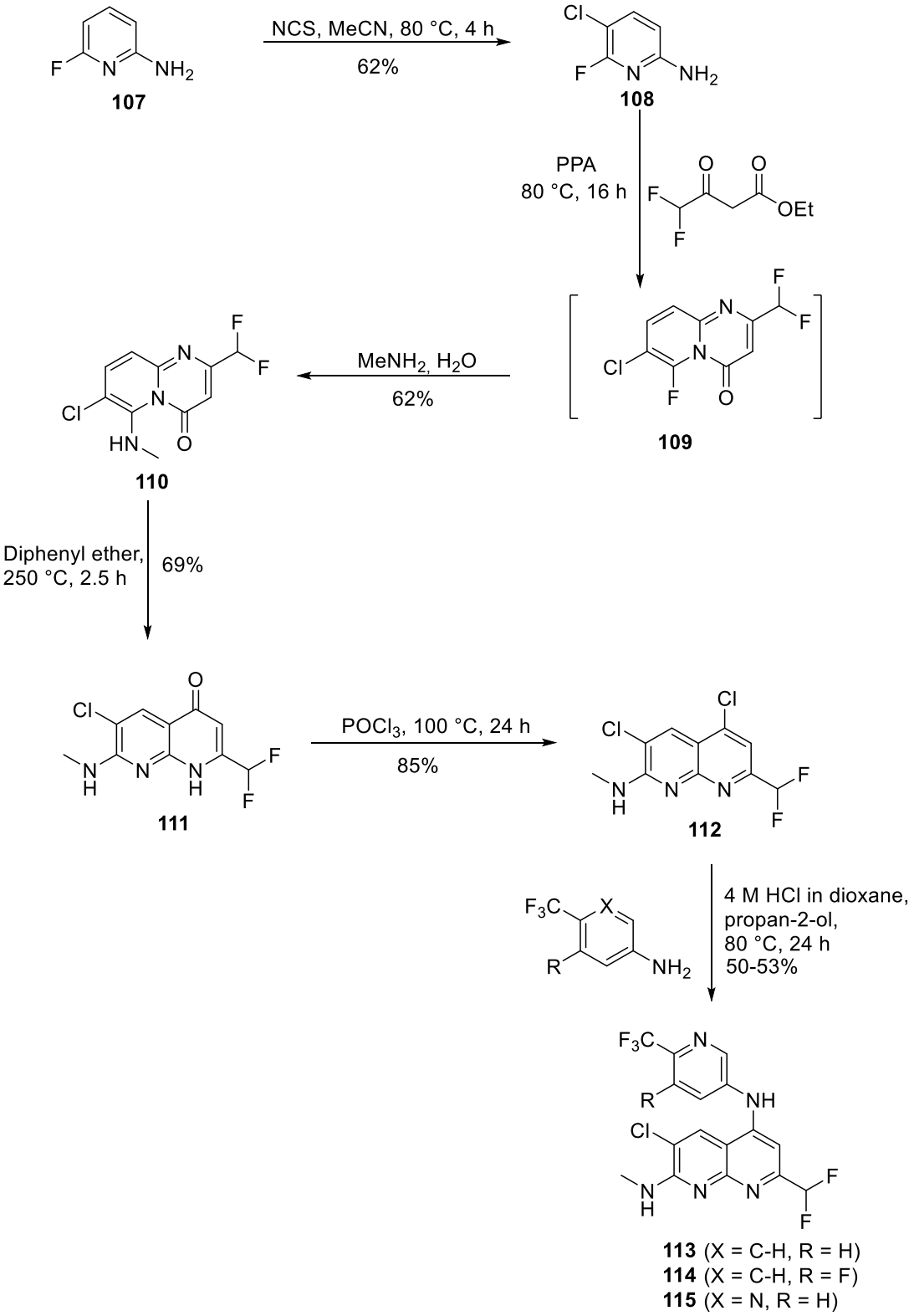
It was decided to synthesise 7-methylamino and dimethylamino derivatives; these less polar groups may restore some potency.

4.2.1 Synthesis

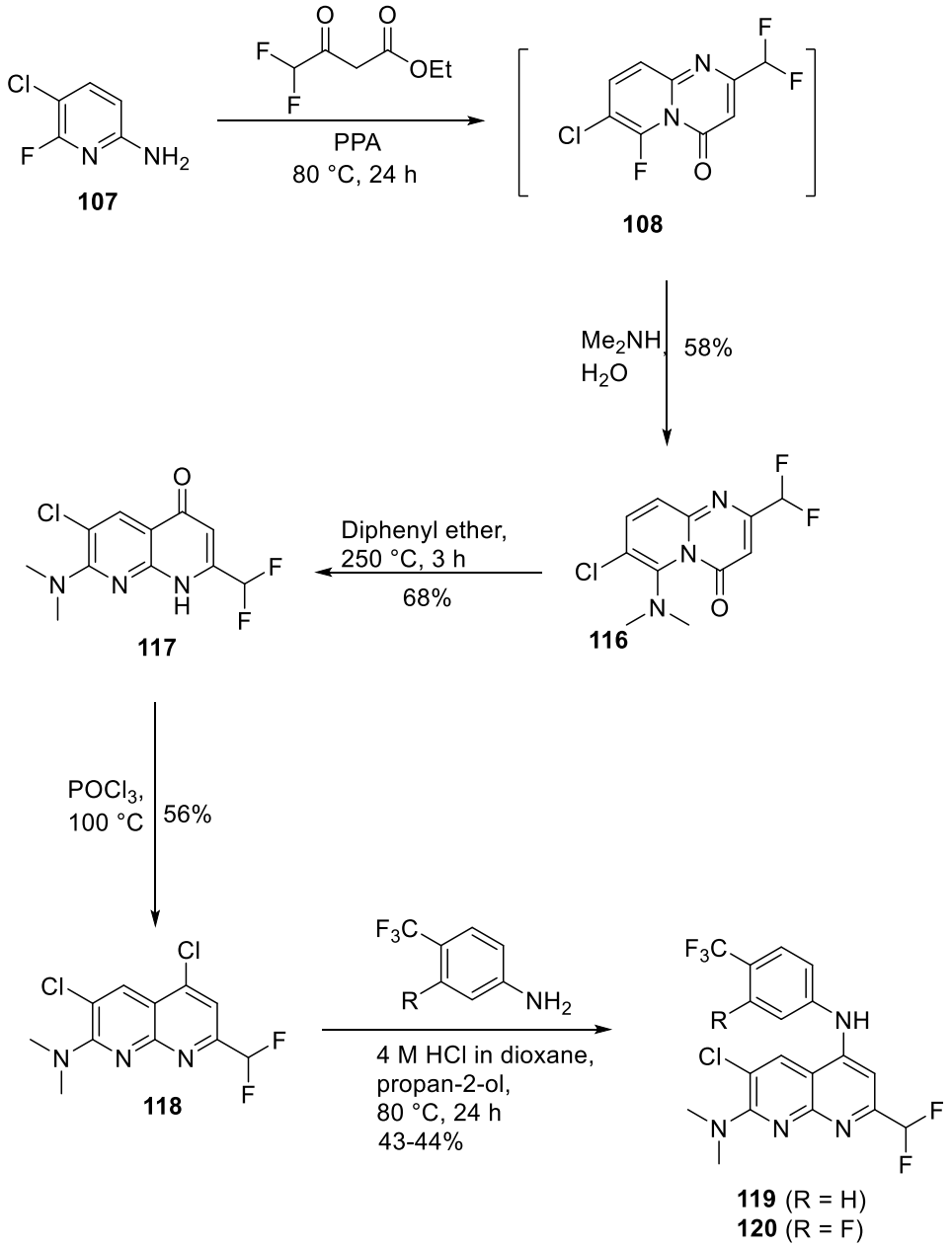
Attempts to make these compounds from the 7-amino derivatives by reductive amination, using either formaldehyde and sodium cyanoborohydride or formaldehyde and formic acid, were unsuccessful. This is probably due to the lack of reactivity of this conjugated amine. Instead, the 7-methylamino compounds were able to be made by starting with a fluoride at the corresponding position which could later be substituted for methylamine (Scheme 4.2).

Starting with the unsymmetrical pyridine **107**, the chlorination could conceivably occur either ortho or para to the amino group. The major product was the desired one, **108**, with the chloride para to the amino group, but the other isomer was also made as a minor product. These were separable by chromatography, allowing the desired product to be obtained in moderate yield. In this case, the following cyclisation step was neutralised using methylamine and an *in situ* substitution reaction occurred immediately to generate intermediate **110** in moderate yield. This was followed by rearrangement and chlorination to give the final intermediate **112** in good yield. This could then be used for an S_NAr reaction with two different anilines or an aminopyridine; this more polar substituent was chosen in the hope of improving solubility.

7-Dimethylamino variants were synthesised using an analogous method, quenching the cyclisation step with dimethylamine (Scheme 4.3).



Scheme 4.2 Synthetic route to make 1,8-naphthyridine analogues with a methylamino group at the 7-position



Scheme 4.3 Synthetic route to make 1,8-naphthyridine analogues with a dimethylamino group at the 7-position

4.2.2 Biological evaluation

These compounds were tested for efficacy against the DHODH enzyme by collaborators at UT Southwestern and in cellular assays against *Plasmodium falciparum* (isolate 3D7) by collaborators at Dundee (Table 4.3).

As with all previously tested 1,8-naphthyridines, there was no observed inhibition of human DHODH (IC_{50} > 100 μM).

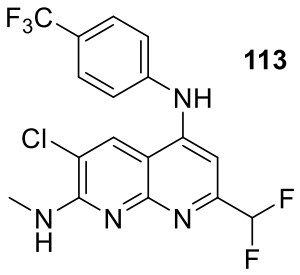
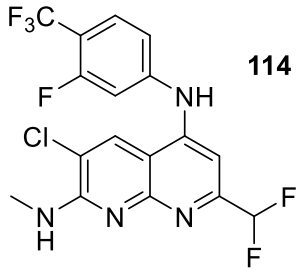
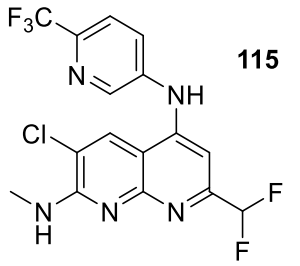
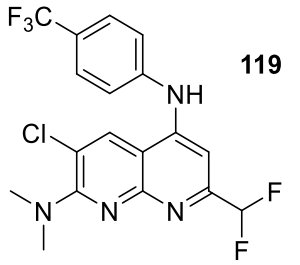
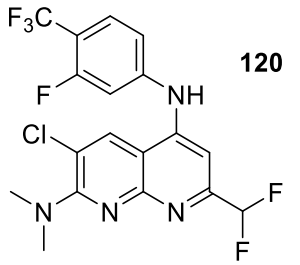
Structure	PfDHODH IC_{50}/nM	PvDHODH IC_{50}/nM	*P. falciparum* cell EC_{50}/nM
 **113**	2400 ± 400	3800 ± 200	1780 ± 540
 **114**	2300 ± 600	1800 ± 100	546 ± 230
 **115**	3400 ± 300	3100 ± 500	2200 ± 450
 **119**	>100000	>100000	9660 ± 830
 **120**	>100000	>100000	6090 ± 130

Table 4.3 Inhibition data against DHODH enzyme from two different *Plasmodium* species and *P. falciparum* cells for 7-methylamino- and 7-dimethylamino- analogues

Disappointingly, the 7-methylamino-1,8-naphthyridines were no more active against either the DHODH enzyme or *P. falciparum* cells than the 7-amino analogues. The most potent of these compounds was again the one containing a 3-fluoro-4-(trifluoromethyl)aniline group but even this molecule

had IC_{50} values of only 2.3 μM (*P. falciparum*) and 1.8 μM (*P. vivax*), and an EC_{50} of 0.55 μM against *P. falciparum* cells. The 7-dimethylamino variants did not inhibit the DHODH enzyme at all, with IC_{50} values above 100 μM for both species. They were also only very weakly active against *P. falciparum* cells. This may be because the dimethylamino group is too large to allow these molecules to bind to the enzyme.

Solubility of these compounds was measured in three different solutions: PBS buffer at pH 7.4, FaSSIF at pH 6.5 and FaSSGF at pH 1.6 (Table 4.4). Both of the 7-dimethylamino compounds (**119** and **120**) and the 7-methylamino derivative **113** were not very soluble (<20 μM) in FaSSIF. However, 7-methylamino analogues with either an additional 3-fluoro group on the aniline (**114**) or with a nitrogen at this position (**115**) were moderately soluble in FaSSIF, up to 53 μM and 44 μM respectively. All these molecules were much more soluble in the acidic FaSSGF medium, likely reflecting protonation under these conditions.

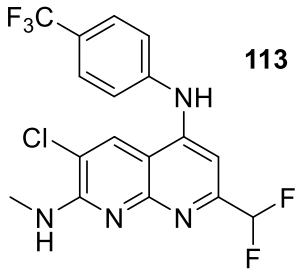
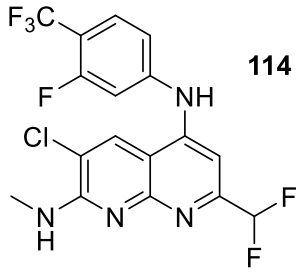
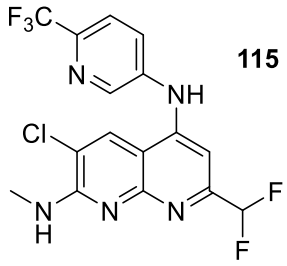
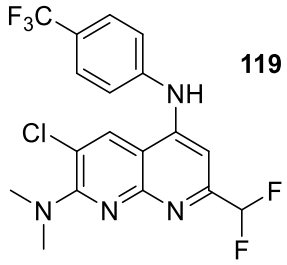
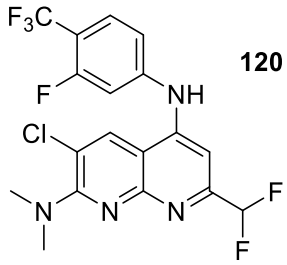
Structure	PBS (pH 7.4)/µM	FaSSIF (pH 6.5)/µM	FaSSGF (pH 1.6)/µM
 **113**	0.4	17	302
 **114**	2.1	53	629
 **115**	5.8	44	617
 **119**	0.9	19	400
 **120**	0.2	8.3	162

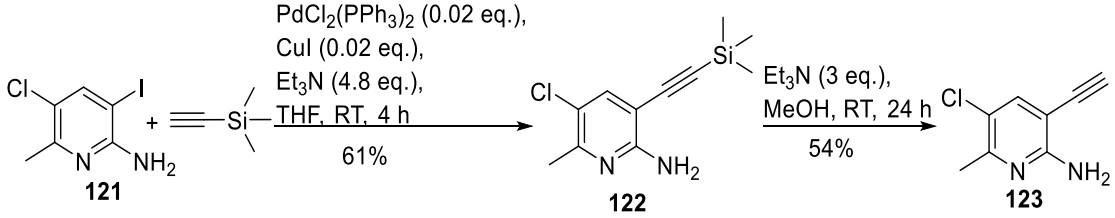
Table 4.4 Solubility measurements in three different media

4.3 7-Methyl-1,8-napthyridines

Next, it was decided to try a smaller, non-polar group such as methyl at the 7-position to investigate whether this would be tolerated better than the amino, methylamino or dimethylamino groups.

4.3.1 Attempted synthesis of 7-methyl-1,8-naphthyridines

A similar synthesis to the ketone intermediate was employed, starting from the iodide **121** (provided by Dr. Martin McPhillie) which then underwent a Sonogashira reaction to give **122** followed by desilylation to **123** (Scheme 4.3).



Scheme 4.3 Sonogashira and desilylation reactions used to make 7-methyl naphthyridine derivatives

These steps proceeded in good yield but the subsequent reaction, using ^{n}butyllithium to deprotonate the alkyne so that it can attack the ethyl difluoroacetate, was unsuccessful. This is likely to be due to the strong base removing a proton from the methyl group rather than from the alkyne. Hence it would be necessary to employ a different route in order to synthesise the desired 7-methyl-1,8-naphthyridines.

It is likely that the methyl group would be metabolised by cytochrome P450, as has been observed for 1,8-naphthyridines with a methyl group at the 2-position, and therefore 7-methyl-1,8-naphthyridines would be predicted to have high clearance *in vivo*. Hence, it was decided that it was not worth trying alternative methods to make such compounds.

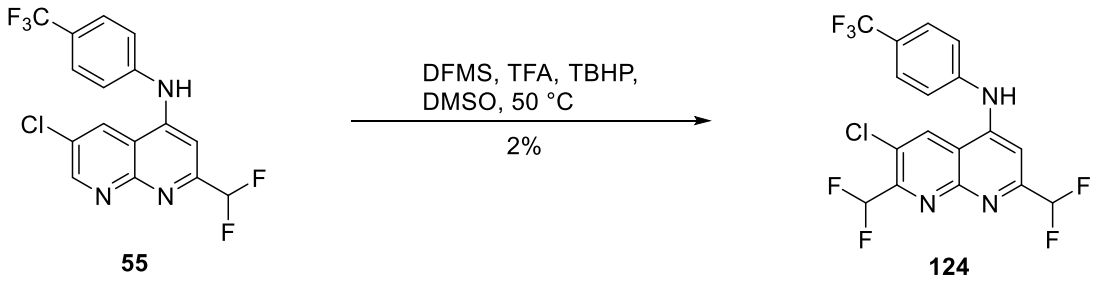
4.4 7-Difluoromethyl-1,8-napthyridines

A metabolically stable, small, non-polar substituent was desired for the 7-position of the 1,8-naphthyridines. Therefore, a difluoromethyl group at this position may be tolerated here.

4.4.1 Synthesis

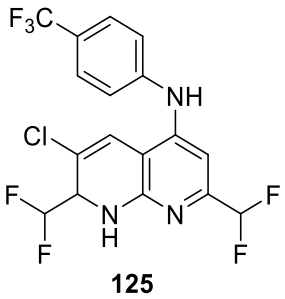
A difluoromethyl group could be introduced at the 7-position of the 1,8-naphthyridine core using DFMS in a preparative scale version of the Baran

'litmus test'[124] on **55.** The same conditions were used, but with two equivalents of DFMS and a higher temperature to increase the yield (Scheme 4.4).



Scheme 4.4 Synthesis of 7-(difluoromethyl)- derivative using DFMS

However, the yield of desired product was poor, although sufficient quantity was obtained for enzyme assays. The major LCMS peak was two mass units higher than would be expected, and the major product of this reaction was confirmed by NMR to be the dihydro molecule **125**.



125

Several oxidising agents, TBHP, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and palladium acetate, were tried in order to convert this to the desired product **124** but all were unsuccessful, demonstrating the unusually high stability of this dihydronaphthyridine.

Synthesis of this molecule was also attempted by reacting DFMS with 5-chloro-pyridin-2-amine (**43**) to introduce the difluoromethyl group in the first step and subsequently take it through the whole synthetic route. However, this was unsuccessful, with only unreacted starting material obtained.

124 was tested against DHODH enzyme by collaborators at UT Southwestern. It was less potent than **55**, the addition of the extra difluoromethyl group resulting in an IC_{50} value of 77 ± 10 nM against PfDHODH and 84 ± 14 nM against PvDHODH. This small loss in potency may be due to the additional electron-withdrawing group reducing the ability of the naphthyridine nitrogen to act as a hydrogen bond acceptor. Unlike any of the other 1,8-naphthyridines previously tested, it also showed some inhibition of human DHODH, with an IC_{50} of 19 ± 5 μM. Therefore, work on this compound was

discontinued due to these disappointing assay results as well as the difficulties with synthesis.

4.5 Summary of 7-substituted 1,8-naphthyridines

Adding a substituent at the 7-position to the proposed site of oxidation by AO was the most obvious way to try to avoid this metabolic liability. Several different groups were tried in this position but all resulted in some loss in potency, ranging from a small loss with the difluoromethyl group to a complete loss of activity in the case of the dimethylamino group. There were also some solubility issues, difficulties with synthesis and, in the case of the difluoromethyl group, some inhibition of human DHODH. Therefore none of the 7-substituted 1,8-naphthyridines that were made had a good enough combination of properties to be considered as lead compounds and a different approach was needed.

Chapter 5 1,8-Naphthyridines with Different Substituents at the 6-Position

It appeared from both the 'litmus test' experiments and the aldehyde oxidase assay results (Chapter 3) that altering the substituent at the 6-position could significantly reduce the amount of oxidation by AO observed at the 7-position. Therefore, it was decided to synthesise analogues of **55** with either a fluoro- or trifluoromethyl- substituent instead of chloro-, along with the preferred difluoromethyl group at the 2-position and three different anilines or aminopyridines in each case.

The desired compounds were docked into the PfDHODH enzyme using Glide, and were predicted to bind with similar affinity to **55**. Both the 6-fluoro- and 6-(trifluoromethyl)-1,8-naphthridines had a predicted binding mode (Fig. 5.1) that was consistent with the X-ray crystal structure previously obtained for a 1,8-naphthyridine in PfDHODH (Fig. 1.12).

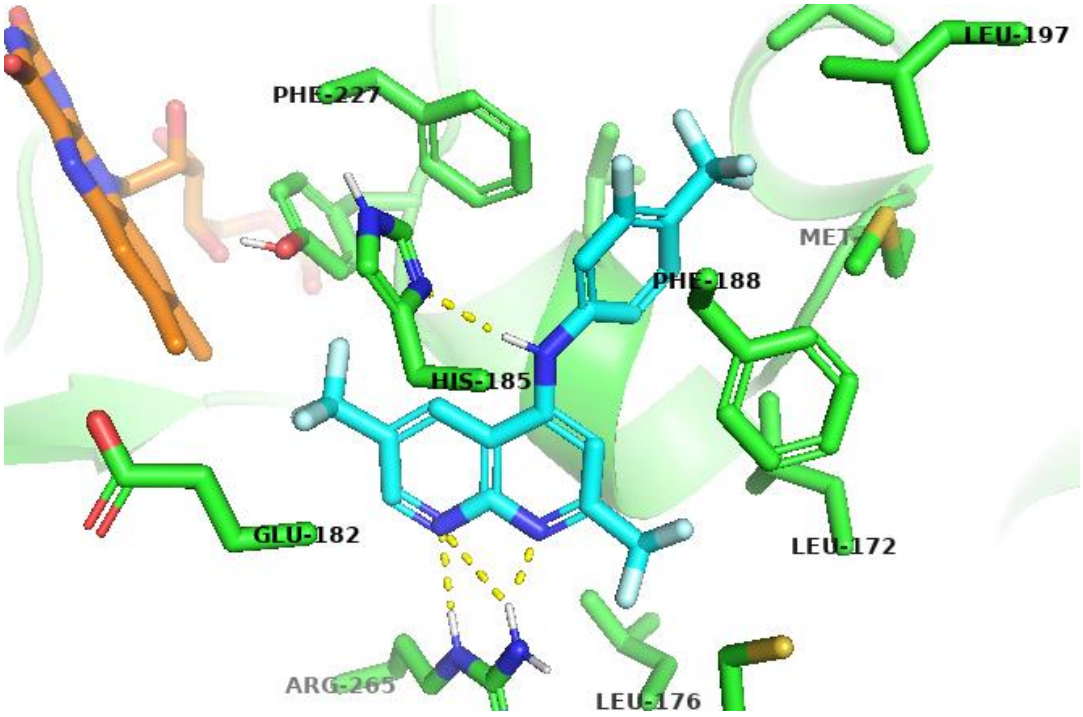
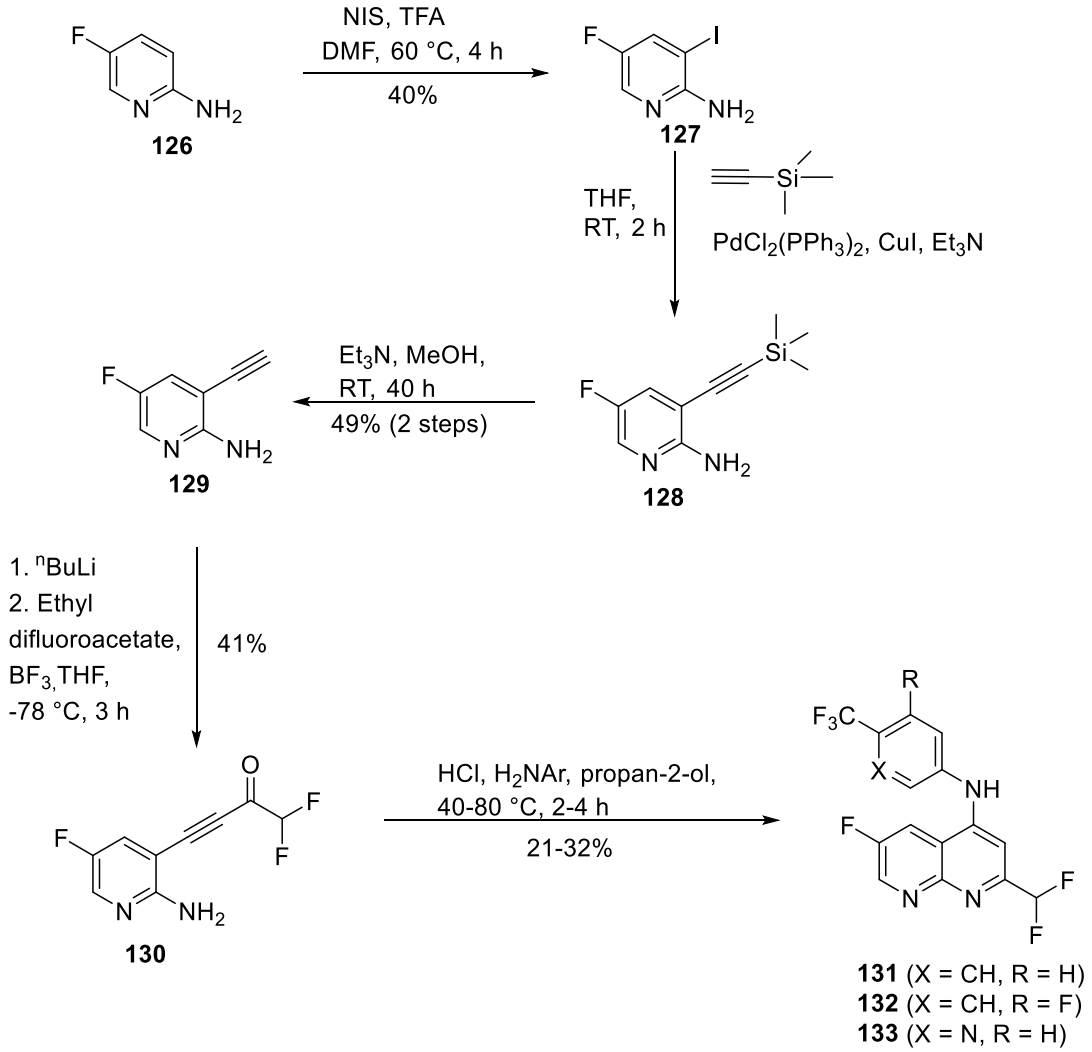


Figure 5.1 Predicted binding mode of a 6-(trifluoromethyl)-1,8-naphthyridine (**138,** blue) bound to *P. falciparum* DHODH (green), along with FMN (orange). Hydrogen bonds are shown by yellow dashed lines.

5.1 6-Fluoro- and 6-(trifluoromethyl)-1,8-naphthyridines

5.1.1 Synthesis

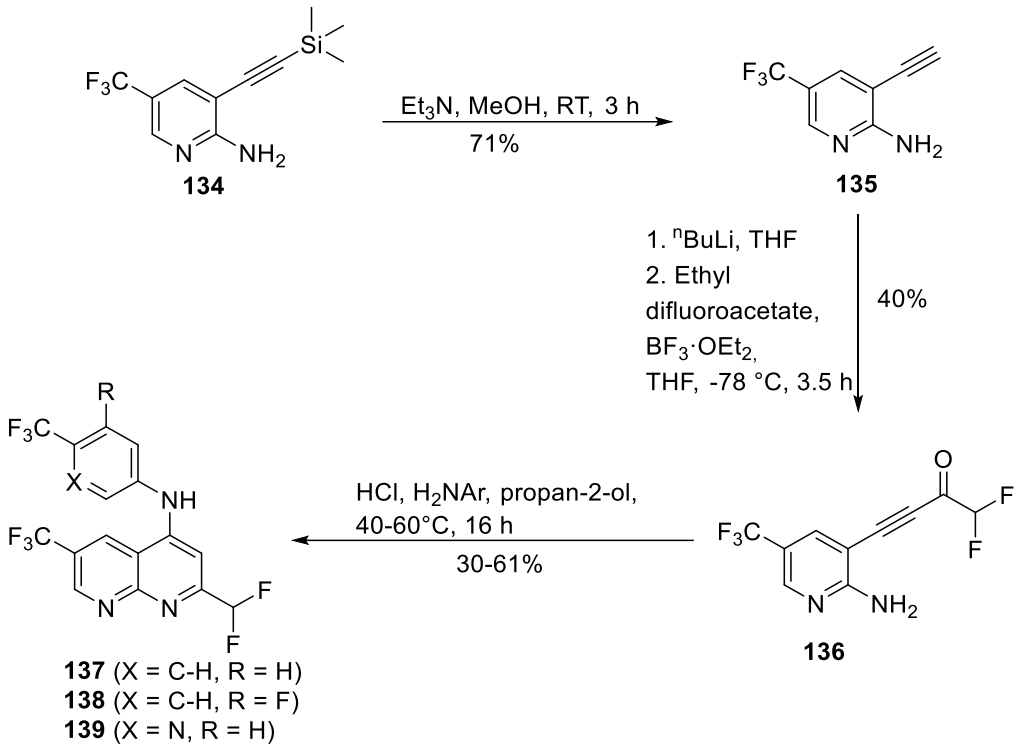
The 6-fluoro- compounds could be made by adapting the synthetic route developed previously to make the 6-chloro- analogues, starting from the commercially available 5-fluoropyridin-2-amine (**126**). This synthesis proceeded in similar yields to those obtained for the 6-chloro- analogues, with the final nucleophilic addition-cyclisation step once again resulting in varied yields depending on how nucleophilic the aniline is, with less reactive anilines and aminopyridines also requiring higher temperatures (Scheme 5.1).



Scheme 5.1 Synthesis of 6-fluoro-1,8-naphthyridines

6-Trifluoromethyl- derivatives were synthesised using an analogous method (Scheme 5.2), starting from **134,** which was provided by Professor Philip Kocienski.

In total, a small library of 6 compounds was synthesised in this way.



Scheme 5.2 Synthesis of 6-(trifluoromethyl)-1,8-naphthyridines

5.1.2 Biological evaluation

These compounds were sent to collaborators at UT Southwestern for testing against DHODH enzyme, both from parasites (*P. falciparum* and *P. vivax*) and human, and to collaborators at Dundee for testing against *P. falciparum* (isolate 3D7) cells (Table 5.1). All of these molecules were potent inhibitors of both *P. falciparum* and *P. vivax* enzyme, but only **132** was as good as the best 6-chloro- analogues, with IC_{50} values of 17 nM (*P. falciparum*) and 13 nM (*P. vivax*). As with most other 1,8-naphthyridines, all these compounds showed excellent selectivity for parasite enzyme with no inhibition of human DHODH observed even at the highest concentration tested (100 µM). There was more variation between the 6 compounds in the EC_{50} values obtained in cells than in the enzyme assay. In this assay, both compounds containing the 3-fluoro-4-(trifluoromethyl)aniline showed excellent potency, comparable to **55** (8 nM). **137** was also very potent (35 nM) but both aminopyridine derivatives were less potent in this assay than against the DHODH enzyme, as was **131**.

Solubility was measured in PBS (pH 7.4), FaSSIF and FaSSGF (Table 5.2).

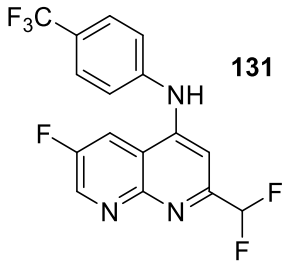
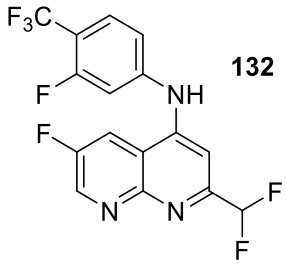
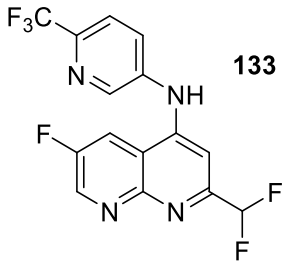
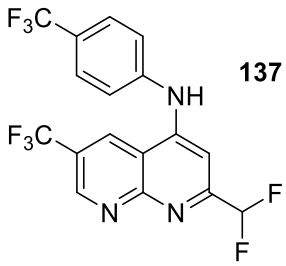
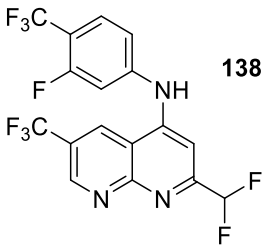
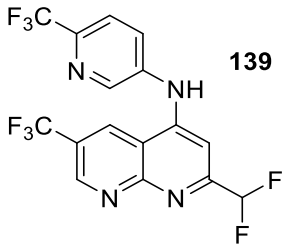
Compound structure	PfDHODH IC_{50}/nM	PvDHODH IC_{50}/nM	*P. falciparum* cell EC_{50}/nM
 **131**	47 ± 8	74 ± 21	103 ± 12
 **132**	17 ± 2	13 ± 2	13 ± 5
 **133**	47 ± 4	46 ± 3	76 ± 28
 **137**	48 ± 4	63 ± 6	35 ± 8
 **138**	46 ± 7	40 ± 5	12 ± 1
 **139**	68 ± 8	69 ± 9	111 ± 21

Table 5.1 Inhibition values for compounds with either 6-fluoro- or 6-(trifluoromethyl)-substituents against DHODH enzyme *Plasmodium falciparum* cells

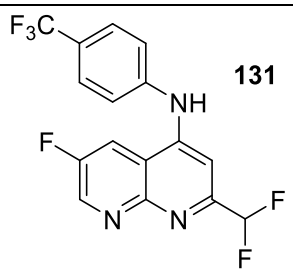
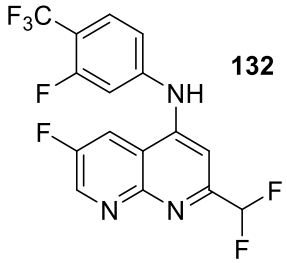
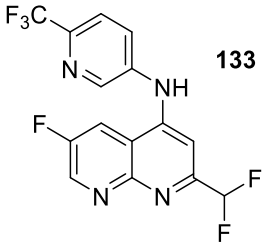
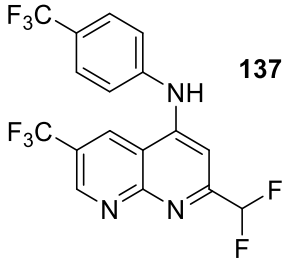
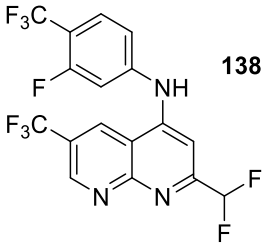
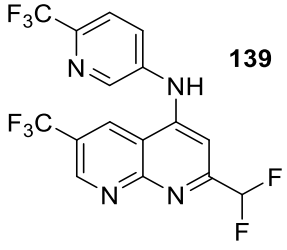
Compound structure	PBS (pH 7.4)/μM	FaSSIF (pH 6.5)/μM	FaSSGF (pH 1.6)/μM
 **131**	12	50	1193
 **132**	20	79	651
 **133**	132	177	466
 **137**	4.1	17	240
 **138**	58	40	56
 **139**	1.8	2.1	43

Table 5.2 Solubility of compounds with different 6-substituents in three media.

As with most 1,8-naphthyridines synthesised so far, solubility in PBS is low for all of these except **133** and **138**. As expected, these hydrophobic molecules are more soluble in FaSSIF solution, with 6-fluoro- analogues showing moderate solubility, as good or better than the 6-chloro- analogues. The result for aminopyridyl variant **133** (177 μM) was especially good. However, the 6-(trifluoromethyl)- derivatives were less soluble, showing low to moderate solubility in FaSSIF. All of these six basic molecules were much more soluble in the acidic FaSSGF solution, with the 6-fluoro compounds remaining more soluble than the 6-(trifluoromethyl) analogues.

Overall, these compounds appeared promising, with **132** in particular showing a good balance between high potency against both the parasite enzyme and cells, excellent selectivity and an improvement in solubility over **55**.

Metabolic stability of four promising compounds was assessed by collaborators at Monash University. The compounds were incubated in rat liver microsomes and half-life, CL_{int} and predicted *in vivo* CL_{int}, CL_{blood} and E_H values were calculated using equations 2.1–2.5 (Table 5.3).

Compound	**132**	**137**	**138**	**139**
Degradation half-life/min	>255	150	>255	>255
In vitro CL_{int}/ μg min^{-1} mg $protein^{-1}$	<7	12	<7	<7
Predicted CL_{int}/mL min^{-1} kg^{-1}	<12	20	<12	<12
Predicted CL_{blood}/mL min^{-1} kg^{-1}	<10	15	<10	<10
Predicted E_H	<0.15	0.23	<0.15	<0.15

Table 5.3 Metabolic stability parameters for frontrunner 6-F and 6-CF_3 1,8-naphthyridines in rat liver microsomes

These compounds were also incubated in human liver microsomes and the same values were calculated (Table 5.4).

Compound	**132**	**137**	**138**	**139**
Degradation half-life/min	>255	>255	>255	>255
In vitro CL_{int}/ µg min^{-1} mg $protein^{-1}$	<7	<7	<7	<7
Predicted CL_{int}/mL min^{-1} kg^{-1}	<6	<6	<6	<6
Predicted CL_{blood}/mL min^{-1} kg^{-1}	<5	<5	<5	<5
Predicted E_H	<0.22	<0.22	<0.22	<0.22

Table 5.4 Metabolic stability parameters for frontrunner 6-F and 6-CF_3 1,8-naphthyridines in human liver microsomes

All of these compounds showed very good metabolic stability in microsomes with all having a half-life too long to be measured in this study and hence very low clearance in human microsomes. In rat microsomes, **137** had a long half-life (150 minutes) with low clearance and predicted E_H (0.23) and the other three compounds had a half-life too long to be determined under the time-frame of this experiment and therefore very low clearance and hepatic extraction ratio. This means that none of these compounds is expected to be metabolised quickly by cytochrome P450 *in vivo*.

The molecules were also incubated in human liver S9 fraction to test for metabolism by aldehyde oxidase (Table 5.5). All of the 6-(trifluoromethyl)-analogues had a very long half-life and hence very low clearance and E_H values under all conditions, although a small amount of oxidised metabolite was detected in each case. This shows that these three compounds are not expected to be significantly metabolised by either cytochrome P450 or aldehyde oxidase. **132**, however, had a measurable but still quite long half-life of 98 minutes with NADPH present and 151 minutes without, and corresponding reasonably low clearance and hepatic extraction ratio values. The similarity in the values obtained both with and without NADPH, and the large decrease in clearance when the aldehyde oxidase inhibitor raloxifene is added, indicates that this enzyme is responsible for the majority of metabolism

for this compound. This is further supported by the mono-oxygenated metabolite which was detected. Although **132** is still subject to metabolism by aldehyde oxidase, this is significantly reduced compared to **55**.

Compound	Condition	$T_{1/2}$/ min	*In vitro* CL_{int}/ µg min^{-1} mg $protein^{-1}$	Predicted CL_{int}/mL min^{-1} kg^{-1}	Predicted CL_{blood}/ mL min^{-1} kg^{-1}	Predicted E_H
132	+ NADPH	98	3	9	6	0.30
	− NADPH	151	2	6	4	0.22
	− NADPH + raloxifene	>510	<1	<2	<2	<0.08
137	+ NADPH	>510	<1	<2	<2	<0.08
	− NADPH	>510	<1	<2	<2	<0.08
	− NADPH + raloxifene	>510	<1	<2	<2	<0.08
138	+ NADPH	>510	<1	<2	<2	<0.08
	− NADPH	>510	<1	<2	<2	<0.08
	− NADPH + raloxifene	>510	<1	<2	<2	<0.08
139	+ NADPH	>510	<1	<2	<2	<0.08
	− NADPH	>510	<1	<2	<2	<0.08
	− NADPH + raloxifene	>510	<1	<2	<2	<0.08

Table 5.5 Metabolic stability parameters for frontrunner 6-F and 6-CF_3 1,8-naphthyridines in human liver S9 fraction

In conclusion, the 6-trifluoromethyl- analogues were very stable to metabolism by both cytochrome P450 and aldehyde oxidase but were less potent and less soluble than the 6-chloro-compounds. 6-Fluoro- analogues had moderate solubility and retained more potency, especially **132**, but this compound was still liable to some oxidation by aldehyde oxidase, albeit at lower levels than

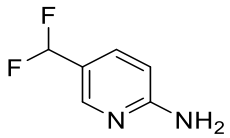
55. This lower level of metabolism by AO would be acceptable so **132** is a potential lead compound. However, an ideal lead compound would have AO metabolism that was reduced even more and would also have a further increase in solubility while retaining the excellent potency and selectivity that **132** possesses.

5.2 6-(Difluoromethyl)-1,8-naphthyridines

Changing the substituent at the 6-position from a trifluoromethyl to a smaller, more polar and less electron-withdrawing difluoromethyl group was proposed. This was intended to result in compounds with similar metabolic stability to the 6-trifluoromethyl-1,8-naphthyridines but with increased potency and solubility.

5.2.1 Synthesis of 6-(difluoromethyl)-1,8-naphthyridines

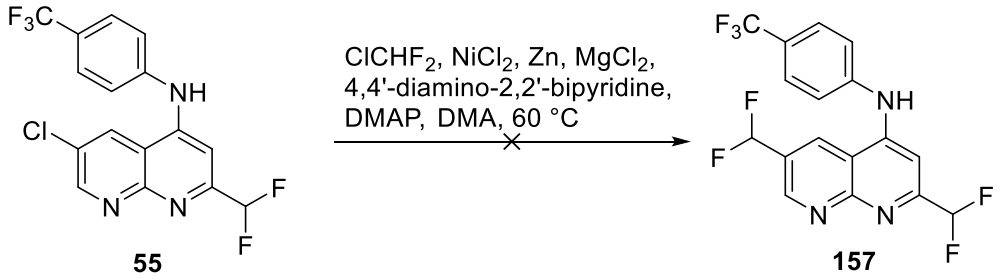
One way to make a small library of 6-(difluoromethyl)-1,8-naphthyridines would be to use a similar synthetic route to that used for the 1,8-naphthyridines with other substituents at the 6-position. That would require starting from 5-(difluoromethyl)pyridin-2-amine (**140**), which unfortunately is not commercially available so would have to be synthesised.



140

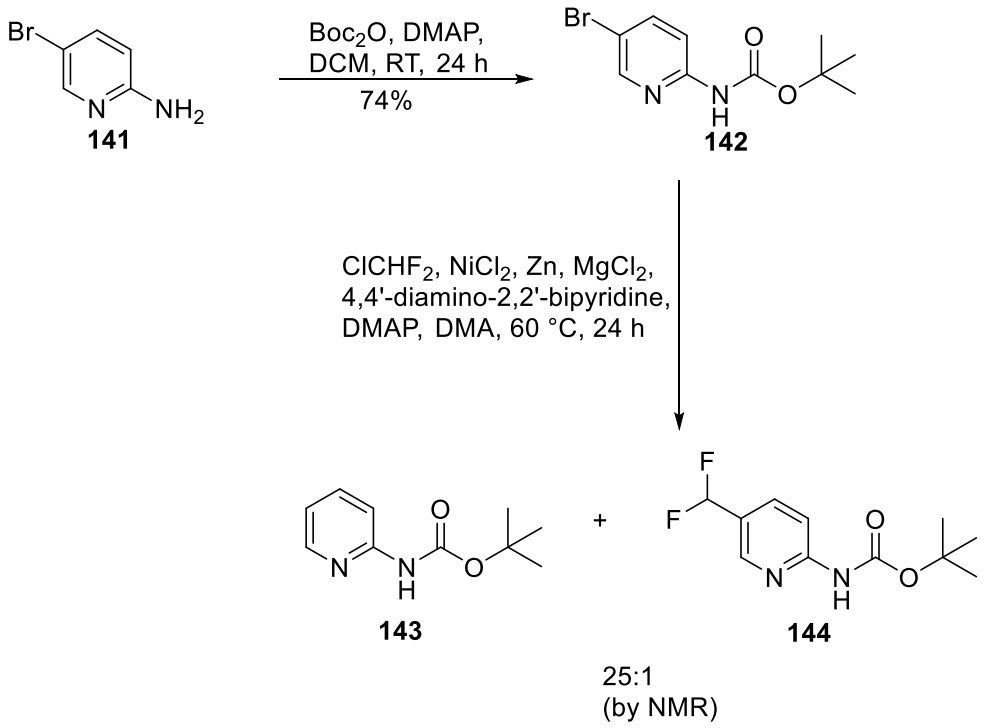
Another option might be to introduce the difluoromethyl group at a later stage, from either one of the existing final products or an advanced intermediate. This was the more attractive option because, with a lot of these intermediates and final compounds already in hand, it would allow the desired 6-(difluoromethyl)-1,8-naphthyridine final compounds to be synthesised very quickly in only a couple of steps.

One method reported to convert either a chloro- or bromo- group to difluoromethyl is a nickel-catalysed reaction with chlorodifluoromethane.[128] This was attempted on **55** (Scheme 5.3) but only unreacted starting material was obtained.



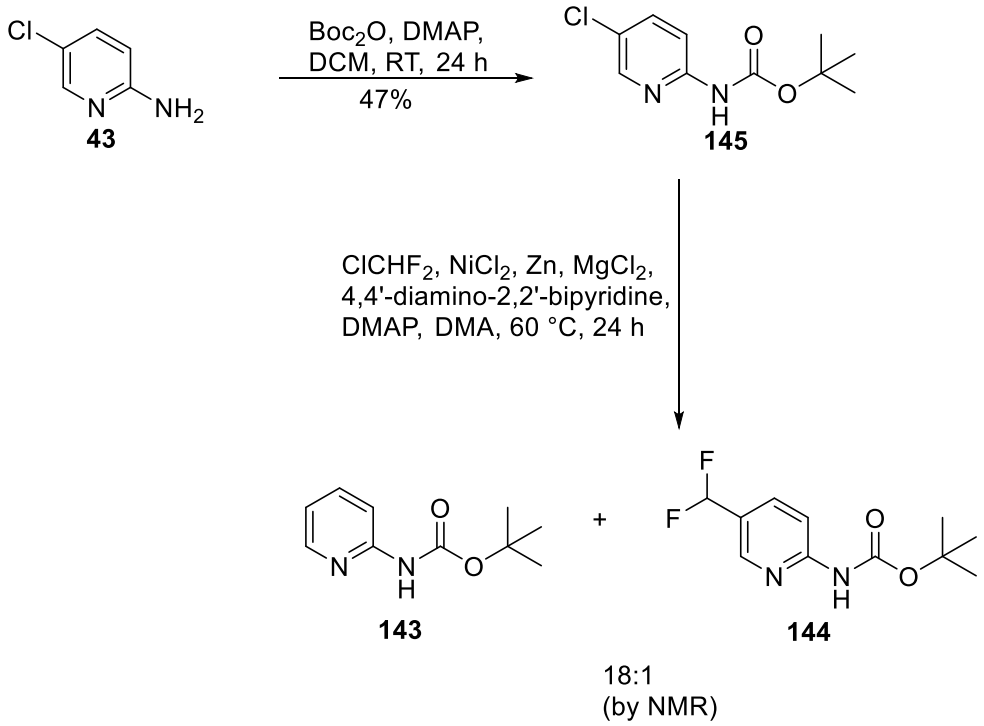
Scheme 5.3 Attempted synthesis of 6-(difluoromethyl)-1,8-naphthyridines from **55** using a nickel-catalysed reaction with chlorodifluoromethane

This reaction was also tried on 5-bromo-pyridin-2-amine but again no product was obtained and the same outcome was observed with 5-bromo-2-nitropyridine. Repeating the reaction with the BOC-protected amine resulted in the observation of a trace amount of the desired product **144** by NMR but the major product was the dehalogenated compound **143** (Scheme 5.4).



Scheme 5.4 BOC protection of **141** followed by attempted difluoromethylation using a nickel-catalysed reaction with chlorodifluoromethane

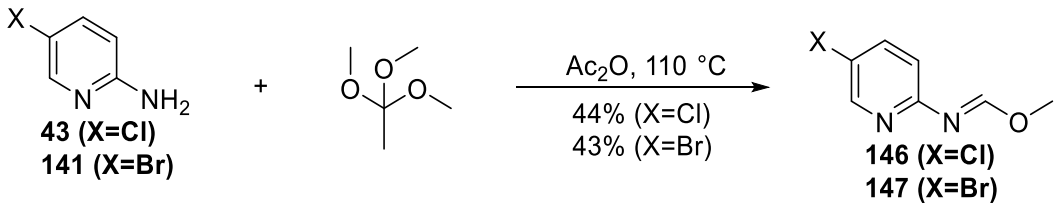
Changing the halogen from a bromine to a less reactive chlorine could reduce the rate of dehalogenation observed in this reaction. Therefore, the same route was attempted with this change to the starting molecule (Scheme 5.5).



Scheme 5.5 BOC protection of **43** followed by attempted difluoromethylation using a nickel-catalysed reaction with chlorodifluoromethane

Although this reduced the amount of dehalogenated product, there was also less of the desired product because some starting material remained unreacted. This means that the product ratio did not improve significantly.

The relatively acidic proton present in **142** and **145** could be accelerating the dehalogenation reaction. Therefore, a different protecting group with no such protons was used and both the chloro- and bromo- variants were synthesised (Scheme 5.6). Initially this was attempted by simply refluxing in trimethyl orthoacetate but this was unsuccessful so acetic anhydride was added[129] which gave a moderate yield of the desired products **146** and **147**.



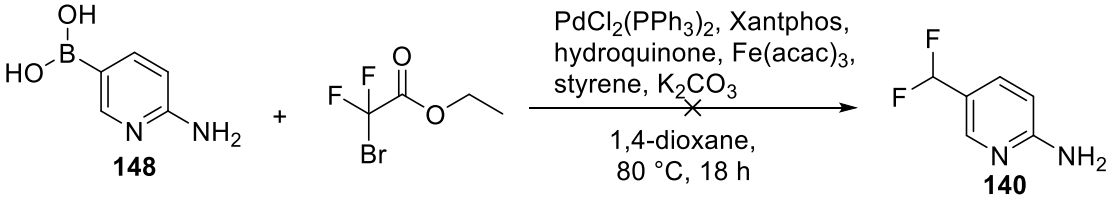
Scheme 5.6 Protection of **43** and **141** as an imidate

However, these compounds did not undergo the nickel-catalysed difluorination reaction, with most of the imidate recovered unchanged.

Another method to introduce a difluoromethyl group uses a difluoromethylated silver heterocyclic carbene complex, (SIPr)Ag(CF_2H), in a palladium-catalysed reaction.[130] However, this was unsuccessful when

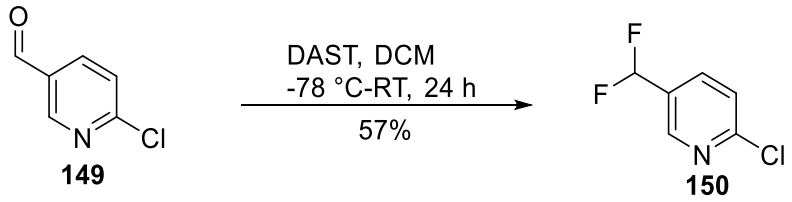
attempted on a 1,8-naphthyridine, with only unreacted starting material obtained. It would not be feasible to use this reaction in an early step of the synthetic route due to the high cost of the silver reagent which is needed in stoichiometric quantities.

A palladium-catalysed difluoromethylation reaction starting from a boronic acid and ethyl bromodifluoroacetate has also been reported.[131] This was attempted using 6-aminopyridine-3-boronic acid (Scheme 5.7) but no reaction was observed.



Scheme 5.7 Attempted synthesis of **140** from **148** using a palladium-catalysed difluoromethylation reaction

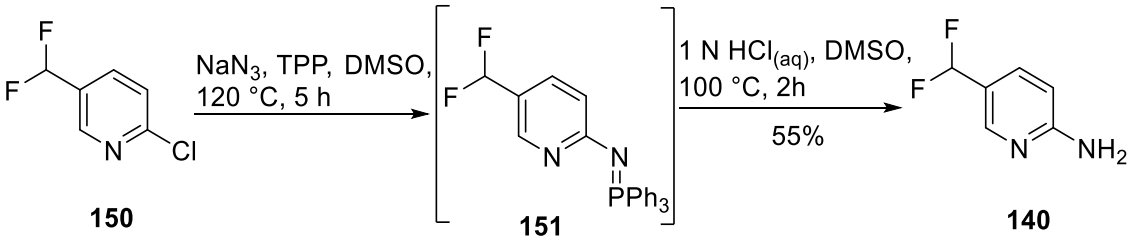
Finally, a difluoromethylation of aldehyde **149** using diethylaminosulphur trifluoride (DAST)[132] was performed which proceeded to give **150** in moderate yield (Scheme 5.8) and was scalable. The reaction was also attempted with the similar reagents Deoxofluor[133] and XtalFluor-E[134] but no product was obtained with these.



Scheme 5.8 Difluoromethylation of **149** using DAST

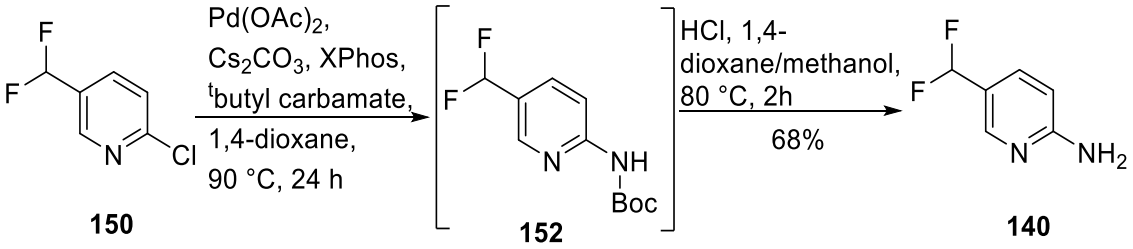
Next, it was necessary to convert the 2-chloropyridine **150** to the 2-aminopyridine **140**. At first, this was attempted using a simple S_NAr reaction, heating with microwave radiation at 120 °C in aqueous ammonia. However, this was unsuccessful, with the starting material remaining unchanged. Repeating this reaction with copper(I) chloride added to the reaction mixture gave the same result.

Therefore a one-pot Staudinger reaction[135] was used (Scheme 5.9). Initially yields were poor due to the hydrolysis of the difluoromethyl group back to the aldehyde but, by decreasing the temperature of the acid hydrolysis step from 120 °C to 100 °C, production of this undesired product was reduced and the yield of **140** increased to 55%.



Scheme 5.9 Amination of **150** in a one-pot Staudinger reaction

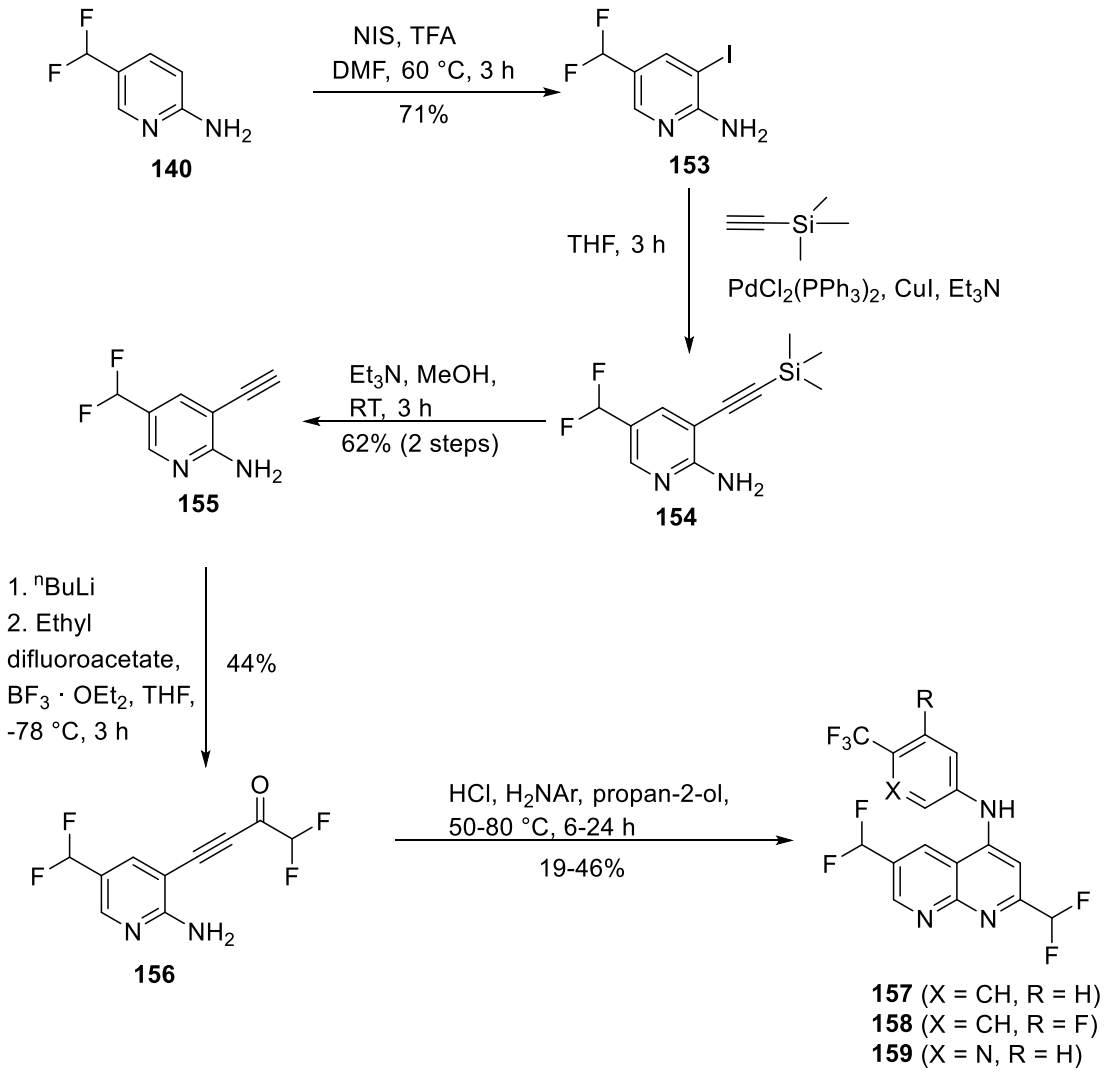
Another way to achieve this transformation would be to use a Buchwald-Hartwig amination with ^{t}butyl carbamate[136] followed by removal of the BOC group with acid (Scheme 5.10).



Scheme 5.10 Buchwald-Hartwig amination followed by removal of the BOC group to give **140**

This reaction proceeded in higher overall yield than the one-pot Staudinger reaction (Scheme 5.9) and uses safer conditions, so was chosen to be used for scale-up and good yields were obtained on multi-gram scale.

With the required 2-aminopyridine **140** in hand, synthesis of the desired 1,8-naphthyridines could be performed using an analogous route to the one developed for other 6-substituents. The steps proceeded in moderate to good yield and cyclisation with three different anilines or aminopyridines gave the 1,8-naphthyridines **157**, **158** and **159** (Scheme 5.11).



Scheme 5.11 Synthesis of 6-(difluoromethyl)-1,8-naphthyridines from **140**

5.1.2 Biological evaluation

These compounds were sent to collaborators at UT Southwestern for testing against DHODH enzyme, both from parasites (*P. falciparum* and *P. vivax*) and human, and to collaborators at Dundee for testing against *P. falciparum* (isolate 3D7) cells (Table 5.6).

All three compounds showed quite high levels of inhibition of DHODH enzyme from both *P. falciparum* and *P. vivax*, with slightly higher potency than the corresponding 6-(trifluoromethyl)-1,8-naphthyridines (Table 5.1). Two of these compounds, **157** and **158**, showed excellent potency against the *P. falciparum* cells, with EC_{50} values of 12 nM and 7 nM respectively. The more polar aminopyridine-containing compound **159** was not quite as potent with an EC_{50} of 30 nM. Excellent selectivity for the parasite enzyme over human was also

observed, with none of these molecules showing any inhibition of human DHODH at concentrations of up to 100 μM.

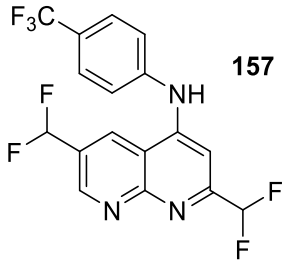
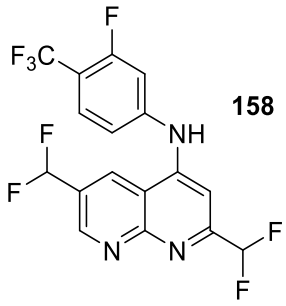
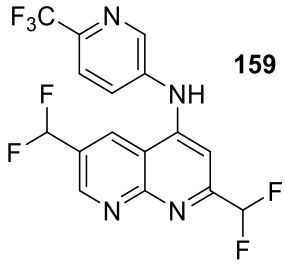
Compound structure	PfDHODH IC_{50}/nM	PvDHODH IC_{50}/nM	*P. falciparum* cell EC_{50}/nM
 **157**	31 ± 5	43 ± 4	12 ± 4
 **158**	42 ± 3	36 ± 3	7 ± 3
 **159**	47 ± 5	35 ± 2	30 ± 16

Table 5.6 Inhibition values for 6-(difluoromethyl)-1,8-naphthyridines against DHODH enzyme from *P. falciparum* and *P. vivax*, and *P. falciparum* 3D7 cells

Solubility was measured in PBS, FaSSIF and FaSSGF (Table 5.7). In all three media, **157** was the least soluble, with the addition of a fluorine at the 3-position of the aniline (**158**) causing a significant increase in solubility and the aminopyridine derivative **159** in between. Solubility in PBS was generally quite low, as with the other 1,8-naphthyridines. In FaSSIF, moderate solubility was observed, with the best compound **158** being soluble at concentrations of up to 50 μM. Solubility in the acidic FaSSGF was quite good, with values of over 100 μM obtained for **158** and **159**. Compared to the 6-(trifluoromethyl)-1,8-naphthyridine series (Table 5.2), changing to a difluoromethyl group generally resulted in a small increase in solubility in all conditions tested.

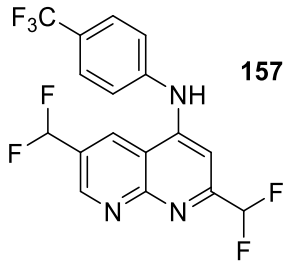
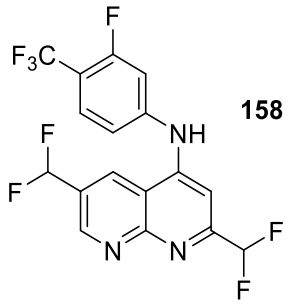
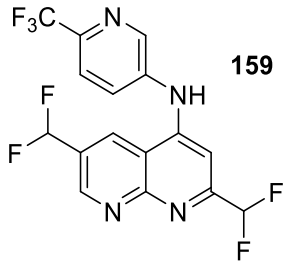
Compound structure	PBS (pH 7.4)/µM	FaSSIF (pH 6.5)/µM	FaSSGF (pH 1.6)/µM
 **157**	7.6	14	69
 **158**	29	50	195
 **159**	13	22	114

Table 5.7 Solubility of 6-(difluoromethyl)-1,8-naphthyridines in 3 media.

Metabolic stability of **157** and **158** was assessed by TCG Lifesciences. The compounds were incubated for 1 h in human liver microsomes at a concentration of 1 µM, and half-life and CL_{int} values were determined (Table 5.8). **157** had a very long half-life and low clearance while **158** was even more stable, remaining essentially unchanged during the incubation period. This demonstrates that these molecules are not metabolised to a significant extent by cytochrome P450.

The two compounds were also incubated in human liver cytosol for 2 h at a concentration of 1 µM, and half-life and CL_{int} values were determined (Table 5.9). Disappointingly, a relatively short half-life was observed in this assay, with moderately high clearance of these molecules. This suggests that the 6-difluoromethyl-1,8-naphthyridines are susceptible to metabolism by aldehyde oxidase.

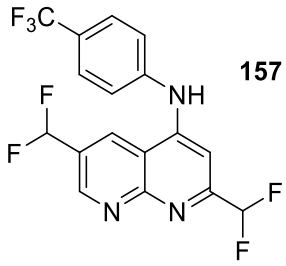
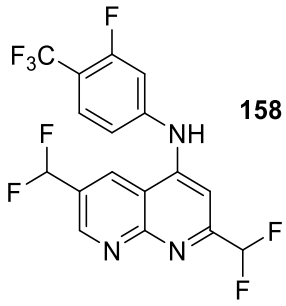
Compound structure	Degradation half-life/min	$CL_{int,\ app}$/µL min^{-1} mg^{-1}
 **157**	2259	0.6
 **158**	64043	0.0

Table 5.8 Metabolic stability data for 6-(difluoromethyl)-1,8-naphthyridines in human liver microsomes.

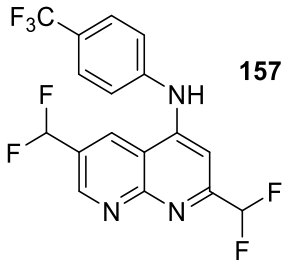
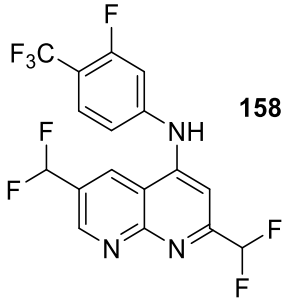
Compound structure	Degradation half-life/min	$CL_{int,\ app}$/µL min^{-1} mg protein^{-1}
 **157**	44.0	15.8
 **158**	36.8	18.8

Table 5.9 Metabolic stability data for 6-(difluoromethyl)-1,8-naphthyridines in human liver cytosol.

Overall, the 6-(difluoromethyl)-1,8-naphthyridines showed very good potency and selectivity, and moderate solubility, clearly better than the 6-(trifluoromethyl) derivatives in these areas. The most potent compounds, **157** and **158**, show cellular inhibition values that are among the very best

observed for any 1,8-naphthyridine. These molecules were not significantly metabolised by cytochrome P450 but, unlike the 6-(trifluoromethyl) series, were metabolised to a moderate extent by aldehyde oxidase. This means that, considering all of the desired properties for a lead compound, none of the 6-(difluoromethyl)-1,8-naphthyridines look as promising as the 6-fluoro analogue **132**.

Nevertheless, the 6-(difluoromethyl)- compounds **157** and **158,** as well as **132**, are currently undergoing further studies to investigate their suitability as drug candidates for the treatment of malaria.

Chapter 6 1,8-Naphthyridines as Inhibitors of *Toxoplasma gondii* DHODH

Toxoplasma gondii, the causative agent of toxoplasmosis, is another parasite which is similar to *Plasmodium*. Therefore it may be possible to use what has been learned from targeting *Plasmodium* DHODH to create inhibitors for *T. gondii* DHODH and ultimately new treatments for toxoplasmosis.

6.1 Investigating whether anti-malarial 1,8-naphthyridines also have efficacy against *T. gondii*

Three frontrunner compounds (**40**, **55** and **132**) were sent to collaborators at the University of Chicago to be tested against *T. gondii* cells in a Yellow Fluorescent Protein (YFP) assay.[137] The necessary control experiments were performed to ensure that the change in fluorescence observed was due to the compounds and the compounds were found to be active at concentrations greater than 1 μM (Fig. 6.1).

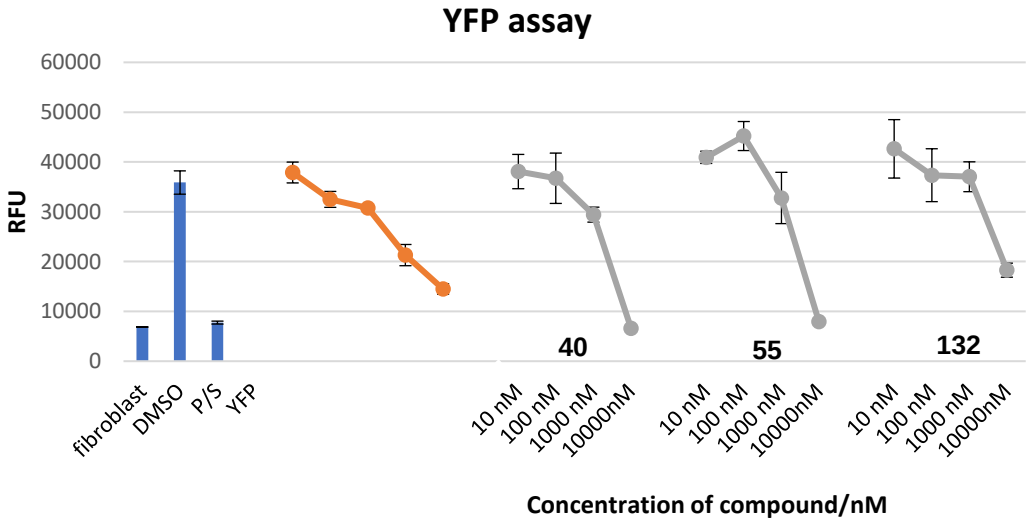


Figure 6.1 Results of YFP assay against *T. gondii* showing relative fluorescence, and hence cell survival, at different concentrations of three compounds: **40**, **55** and **132**.

The compounds are not cytotoxic (Fig. 6.2) so the cell death is likely to be caused by inhibition of DHODH rather than off-target effects.

Docking studies were performed to help understand why these compounds are less potent against *T. gondii* than *P. falciparum*. First, a homology model of the *T. gondii* enzyme was created, because there is no X-ray crystal structure available of DHODH from this species. This was done by using

Swiss-model[138-140] to create a predicted protein structure for *T. gondii* based on that of the *P. falciparum* enzyme.

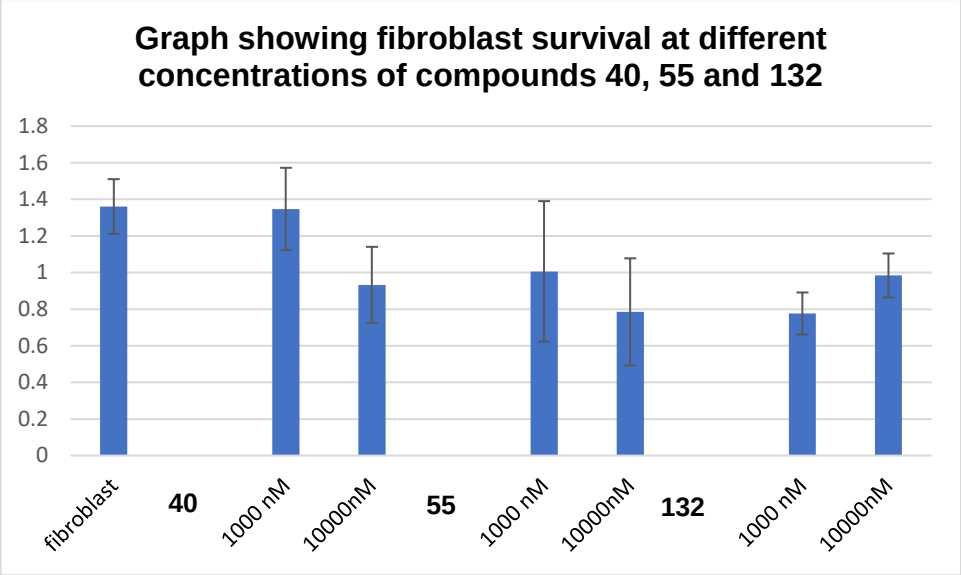


Figure 6.2 Fibroblast survival at different concentrations **40**, **55** and **132** to show that these compounds are not cytotoxic

Molecules were docked into their known binding site in the *P. falciparum* enzyme using Glide without constraints and the predicted poses consistently replicated the binding observed in the crystal structure of a 1,8-naphthyridine bound to DHODH. The frontrunner compounds also all showed very good docking scores: **132** for example had a score of −11.488. However, when this was repeated in the *T. gondii* homology model, the docking score was much worse (−7.755), reflecting the results of the cellular assays.

Comparing the predicted binding pose of **132** between the species (Fig. 6.3), a marked difference can be observed. Although the key arginine and histidine residues that the 1,8-naphthyridines form hydrogen bonds to are conserved between the species, the Phe-188 in *P. falciparum* is mutated to Thr-211 in *T. gondii*. This is predicted to clash with where the aniline group of **132** sits in *P. falciparum*, forcing the molecule to move away in the *T. gondii* enzyme and hence resulting in the loss of a hydrogen bond to arginine. This suggests that the 1,8-naphthyridine core could still bind strongly in *T. gondii*, with the hydrogen bonds to the arginine and histidine residues maintained, if the aniline was replaced with a different group that did not clash with Thr-211.

To test this hypothesis, the 1,8-naphthyridine with a simple amino group at the 4-position (**160**) was docked into the *T. gondii* homology model. This had a docking score of −6.947, similar to that of **132** despite being a much smaller molecule and hence demonstrating that the aniline group does not aid binding

to the *T. gondii* enzyme. It reverted to binding in a similar way to that in *P. falciparum*, with strong hydrogen bonds to the arginine and histidine residues (Fig. 6.4).

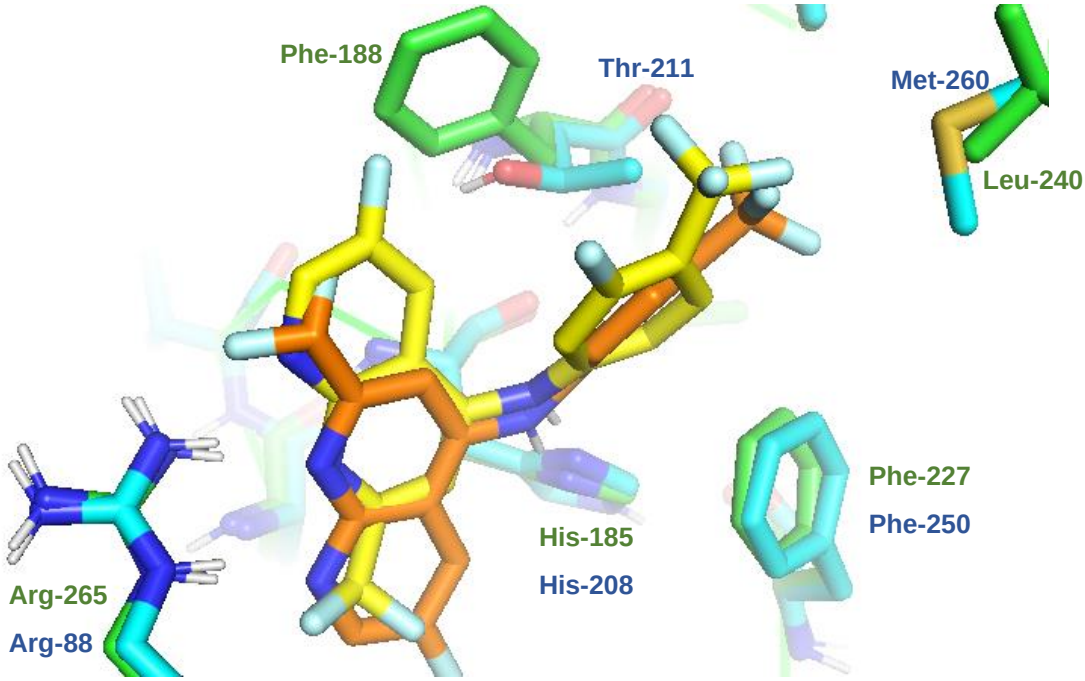


Figure 6.3 Predicted binding of **132** into DHODH using Glide. The *P. falciparum* enzyme is shown in green and overlaid with the *T. gondii* enzyme in blue. The predicted binding pose of **132** in *P. falciparum* is shown in orange, and in *T. gondii* in yellow.

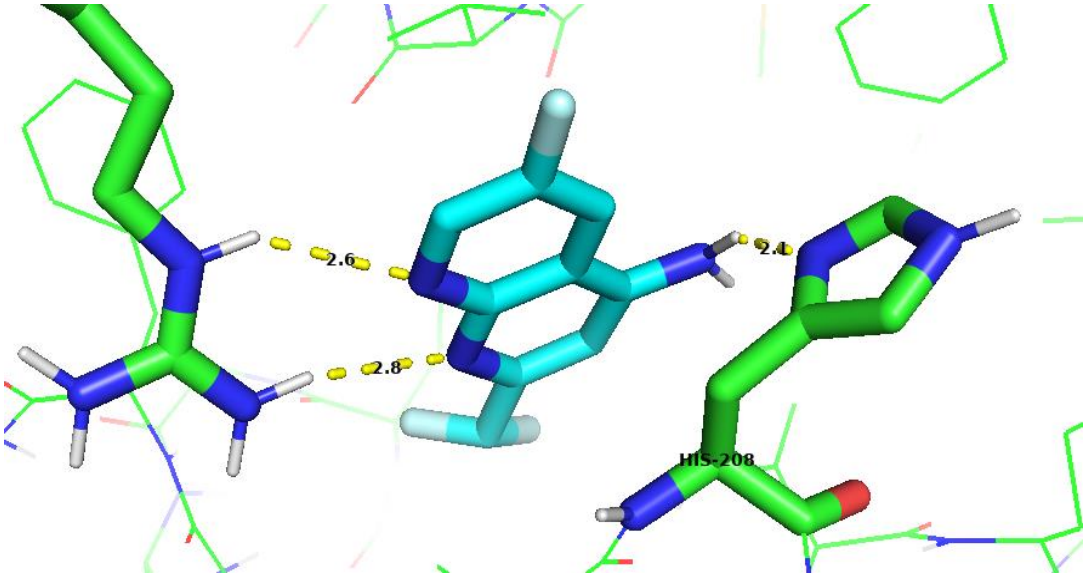


Figure 6.4 Predicted binding pose of a 1,8-naphthyridine with an amino group at the 4-position (**160**) in *T. gondii* DHODH generated by Glide. Hydrogen bonds to Arg-288 and His-208 are shown by yellow dashes along with distances (Å).

6.2 *In silico* design of 1,8-naphthyridines with improved binding to *T. gondii* DHODH

A library of 1,8-naphthyridines with different amines at the 4-position was docked into the *T. gondii* binding site to investigate which groups could be tolerated in this species (Table 6.1). Increasing the size of the amine from methyl up to cyclobutyl increased the predicted binding as these were still able to bind with strong hydrogen bonds to Arg-288 and His-208 (Fig. 6.5) and increasingly filled the hydrophobic cavity but larger groups such as cyclohexyl were pushed further away from Arg-288 with worse scores.

Introducing an ethylene or amide linker between the amine and phenyl group appears to restore some potency by moving the aromatic group away from the clashing threonine residue.

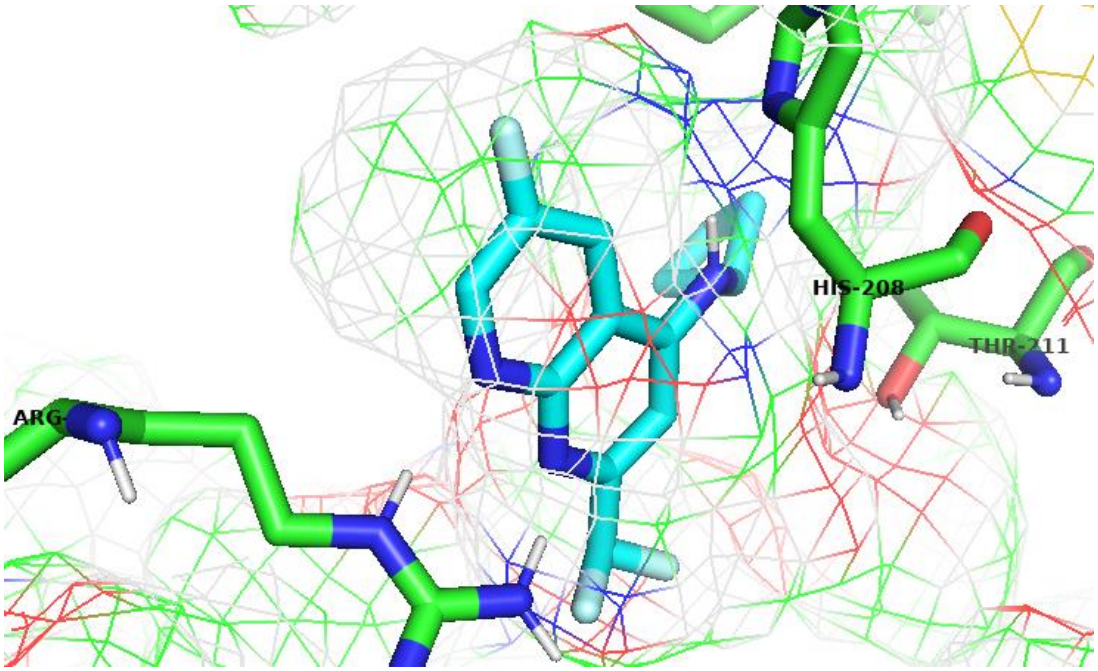


Figure 6.5 Predicted binding pose of **164** (blue) in *T. gondii* DHODH (green) generated by Glide. The binding pocket is shown as a mesh.

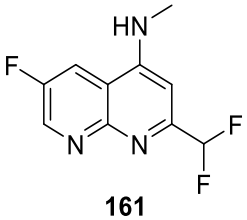
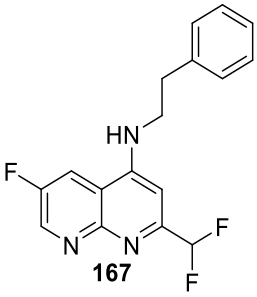
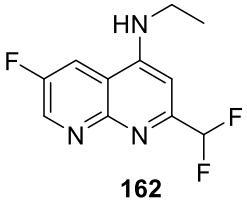
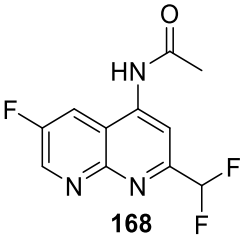
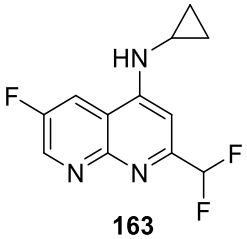
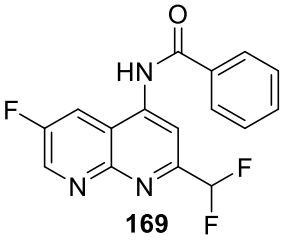
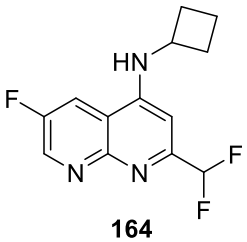
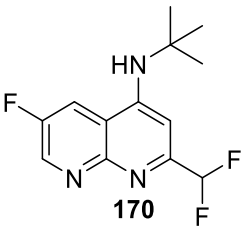
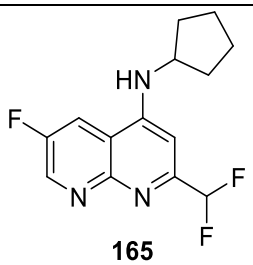
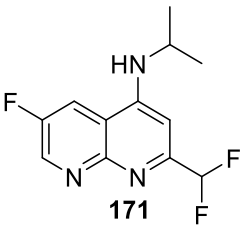
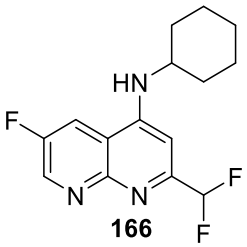
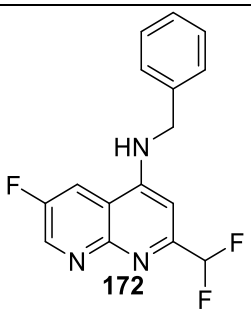
Structure	Docking score	Structure	Docking score
 **161**	−6.941	 **167**	−8.301
 **162**	−7.236	 **168**	−7.399
 **163**	−7.420	 **169**	−8.292
 **164**	−7.524	 **170**	−6.694
 **165**	−7.060	 **171**	−7.429
 **166**	−6.463	 **172**	−7.515

Table 6.1 Docking scores (generated using Glide) of a library of 1,8-naphthyridines with different amines at the 4-position into a *T. gondii* DHODH homology model.

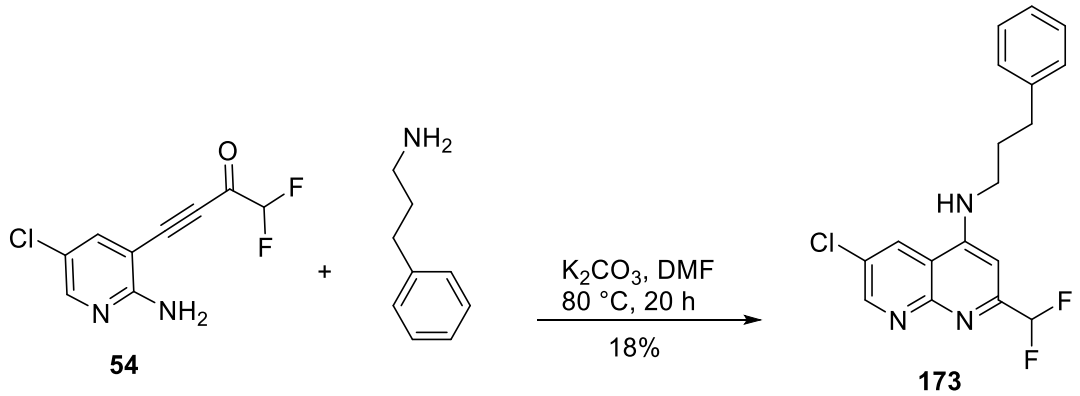
A *de novo* molecular design program, SPROUT[141], was used to aid with the design of inhibitors of *T. gondii* DHODH. However, it was unable to grow the 1,8-naphthyridine core into any putative inhibitors of this enzyme, possibly due to the very tight confines of this binding pocket.

Therefore, it was decided to make a small library of compounds with different length linkers in order to test the hypothesis that increasing the linker length between the phenyl group and the 1,8-naphthyridine core would restore potency.

6.3 Synthesis of putative inhibitors of *T. gondii* DHODH

Due to the high availability of precursors to the 6-chloro-1,8-naphthyridines, it was decided to use this scaffold to investigate the inhibition of *T. gondii* DHODH. Since the aim was to investigate inhibition only, it did not matter if these inhibitors had any metabolic liability; more stable variants, such as the 6-fluoro- analogues of any very potent compounds, could later be synthesised if necessary.

Compounds were synthesised from the acetylenic ketone intermediate **54** (Scheme 6.1). Unlike with the 4-anilino-1,8-naphthyridines made previously, base was used to catalyse this reaction rather than acid so that the primary amine would not get protonated. Yields were poor but sufficient material was obtained for testing against the parasite.



Scheme 6.1 Synthesis of **173** from **54** by an addition-cyclisation reaction

Other compounds such as **174** were made in an analogous way, as were **175**, **176**, **177**, **178** and **179** (Fig. 6.6) which were synthesised by MChem student Omar Allam.

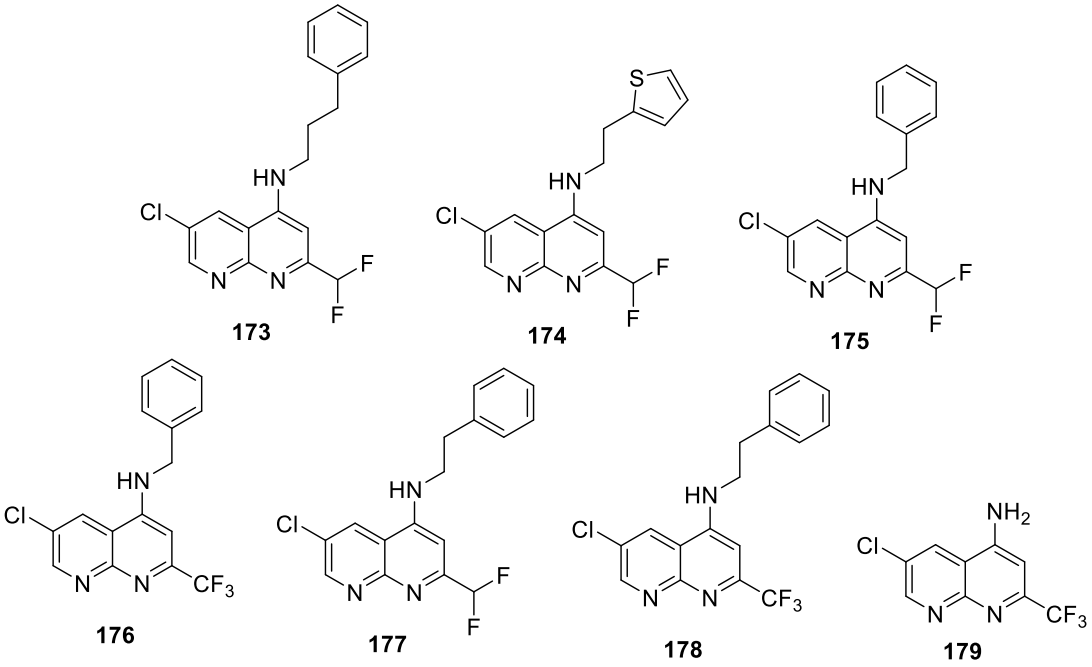


Figure 6.6 Chemical structures of newly synthesised *T. gondii* DHODH inhibitors

6.4 Assessment of potency against *T. gondii*

These newly synthesised compounds were sent to collaborators at the University of Chicago to be tested against *T. gondii* cells in the YFP assay[137] (Fig. 6.7–6.9).

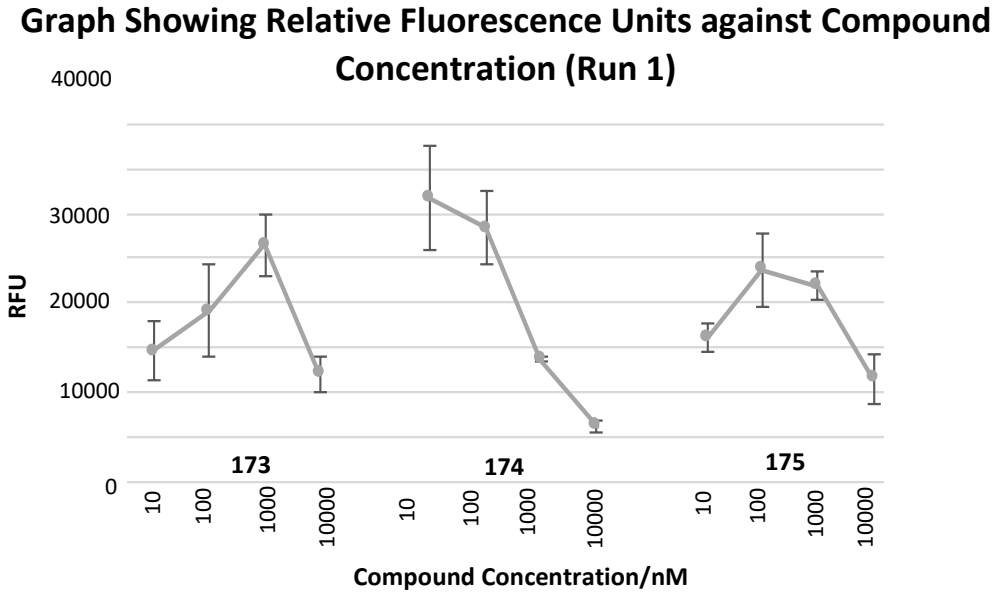


Figure 6.7 Results of cellular assay (run 1) against *T. gondii* showing relative fluorescence, and hence cell survival, at different concentrations of three compounds, **173**, **174** and **175**.

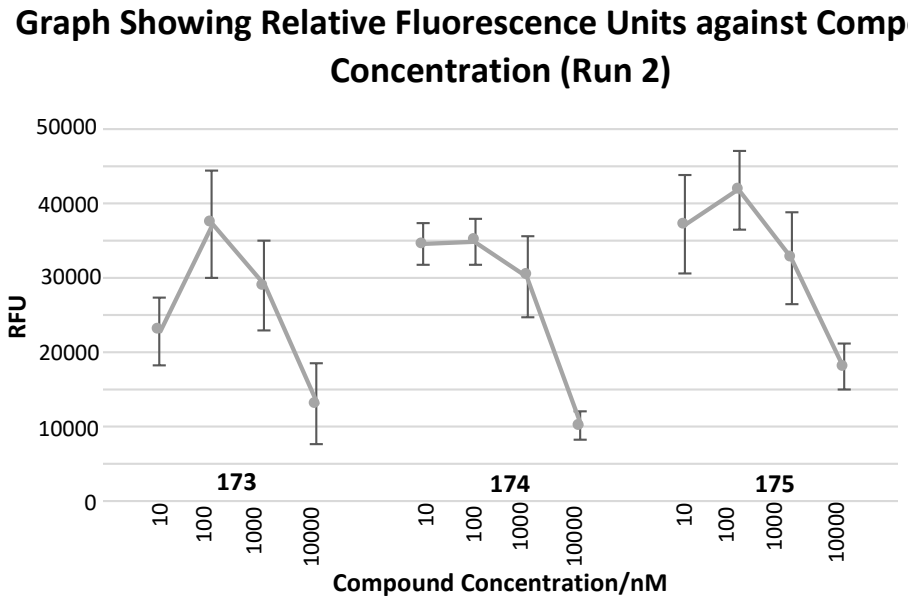


Figure 6.8 Results of cellular assay (run 2) against *T. gondii* showing relative fluorescence, and hence cell survival, at different concentrations of three compounds, **173**, **174** and **175**.

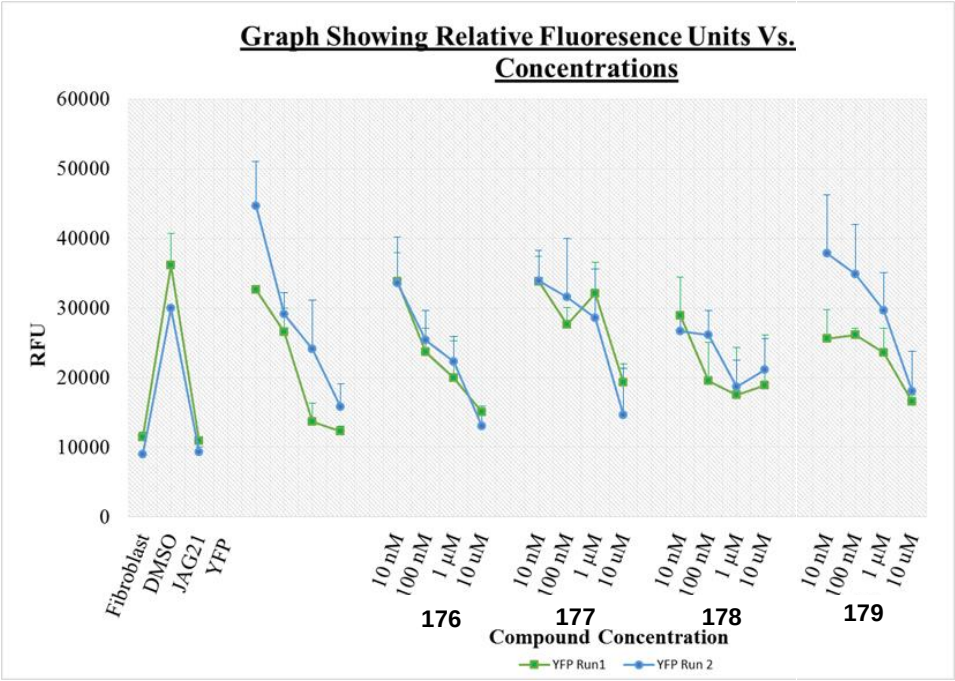


Figure 6.9 Results of cellular assay against *T. gondii* showing relative fluorescence, and hence cell survival, at different concentrations of four compounds, **176**–**179**.

All these compounds were also tested for cytotoxicity and did not show any siginificant effects on cell viability, indicating that the decrease in fluorescence, and hence death of the *T. gondii* cells, is likely to be caused by inhibition of DHODH rather than any off-target effects.

From the graphs of relative fluorescence against compound concentration, an estimate of the EC_{50} values for these compounds against *T. gondii* can be

obtained (Figure 6.10). The most potent compound, **178**, has an EC_{50} value of around 0.5 μM.

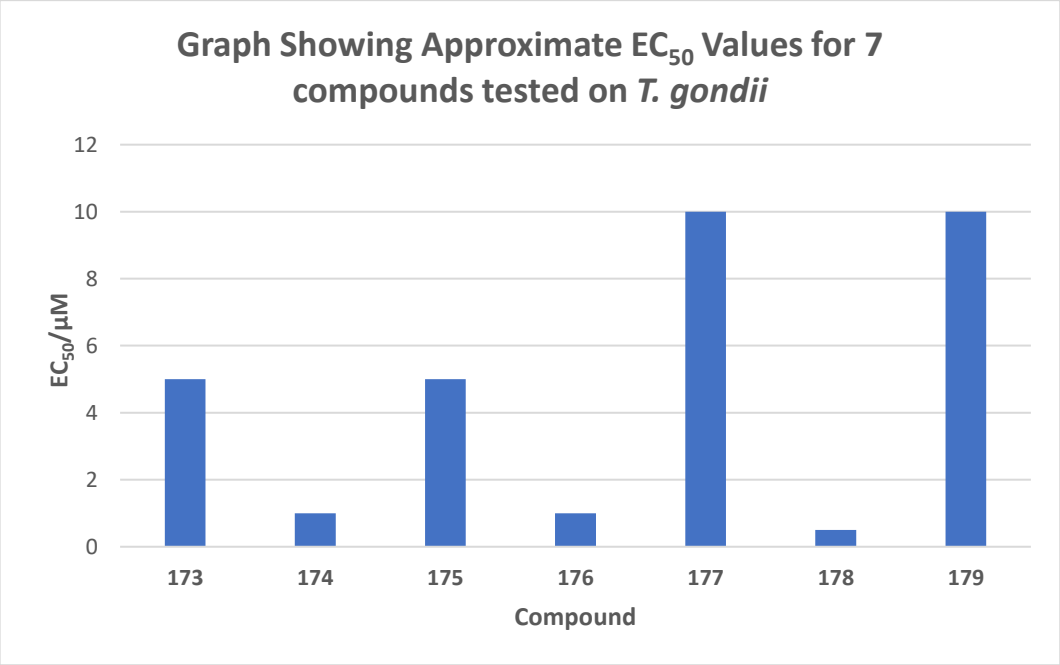


Figure 6.10 Approximate EC_{50} values for the seven newly synthesised compounds tested against *T. gondii*

Comparing the matched pairs **175** and **176**, and **177** and **178**, the 2-trifluoromethyl analogues have slightly higher potency than the 2-difluoromethyl analogues, opposite to the trend observed for the 1,8-naphthyridines in the *Plasmodium* assays.

Varying the linker length between the core and the phenyl group from one carbon to three seems to make little difference to the potency of the compounds. However, it is not possible to draw firm conclusions on this due to the odd shape of the graphs (Fig. 6.7 and Fig. 6.8) obtained with **173**. The relative fluorescence would be expected to decrease as compound concentration increases because fewer cells survive but, with this compound in particular, the fluorescence increases initially before decreasing at the highest concentrations. This may be due to the yellow colour of this molecule interfering with the assay readout.

Switching the phenyl group in **177** for a thiophene (**174**) resulted in a small improvement in docking score and around a tenfold increase in potency on *T.gondii*. It may be that this slightly smaller group can fit better into the tight binding pocket.

One compound (**179**) lacking an aromatic group was tested and this was less potent than the other 2-(trifluoromethyl)-1,8-naphthyridines (**176** and **178**), showing that this aromatic group does contribute to the potency of the molecule.

6.5 1,8-Naphthyridines as inhibitors of *T. gondii* DHODH – conclusions

The anti-malarial 1,8-naphthyridines were also active against *T. gondii* but to a much lesser degree. Computational modelling showed this to be due to a mutation of a phenylalanine residue to a threonine which clashes with the aniline group. Therefore, molecules were synthesised with alkyl linkers moving the phenyl group away from this residue and some improvement in potency was achieved, up to around 500 nM on *T. gondii* cells.

In the future, other linkers such as amide or urea, could be tried to investigate whether further improvement in potency is possible.

Overall, it has been challenging to adapt the 1,8-naphthyridine series into potent compounds against *T. gondii*, with the changes in the binding site in this species causing clashes with these compounds that cannot be easily overcome without disrupting the important hydrogen bonds made by the 1,8-naphthyridine core.

Chapter 7 Conclusion

7.1 1,8-Naphthyridines as DHODH inhibitors for the treatment of malaria

A library of 1,8-naphthyridines has been synthesised using the two synthetic routes which were developed to allow access to 1,8-naphthyridines with various different substituents.

Initial efforts were focussed on 6-chloro-1,8,naphthyridines with the first frontrunner compound **55** showing excellent potency (IC_{50} 14 nM against PfDHODH, EC_{50} 7 nM against *P. falciparum* cells) and selectivity over the human enzyme. Oral bioavailability in rats was also good, as was the stability of the compound to metabolism by cytochrome P450. However, metabolism by aldehyde oxidase was observed and the solubility in FaSSIF (31 μM) was also not as high as desired. Attempts to increase solubility with more polar groups at the 4-position were unsuccessful because this caused the potency of these compounds to decrease too much. Overall, this first series was useful for showing that a difluoromethyl group at the 2-position gave greater potency and higher solubility than a trifluoromethyl group but improvements in solubility and metabolic stability were needed.

Adding a substituent at the 7-position, the proposed site of oxidation by AO, was the first modification tried to avoid this metabolic liability. Unfortunately, an amino, methylamino or dimethylamino group here resulted in a significant decrease in potency. A smaller loss in potency was observed with a difluoromethyl group but this analogue showed some inhibition of human DHODH and was also very difficult to synthesise. Therefore, none of the 7-substituted 1,8-naphthyridines had a good enough combination of properties to be considered as lead compounds.

Next, compounds were investigated which contained different substituents at the 6-position, because experiments using the Baran ‘litmus test’ had indicated that changing the group at this position could affect susceptibility to AO metabolism. A small library of nine molecules with either a fluoro, trifluoromethyl or difluoromethyl group at this position was synthesised.

The 6-trifluoromethyl- analogues were very stable to metabolism by both cytochrome P450 and aldehyde oxidase but were less potent and less soluble than the 6-chloro-1,8-naphthyridines. 6-Fluoro- analogues had moderate solubility and retained more potency, especially **132**, but this compound was still liable to some oxidation by aldehyde oxidase, albeit at lower levels than

55. Compared to the competitor compound **26** which is currently in clinical trials, **132** shows similar potency against *P. falciparum* enzyme and cells, and greater inhibition of PvDHODH (Table 7.1). Importantly, it is also much more soluble which could remove the need for difficult and expensive formulations which are a major problem with **26**.

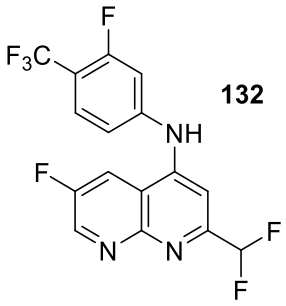
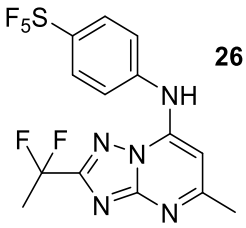
Assay	 **132**	 **26**
PfDHODH IC_{50}/nM	17	13
PvDHODH IC_{50}/nM	13	26
P. falciparum 3D7 cells EC_{50}/nM	13	11
FaSSIF solubility/µM	79	10

Table 7.1 A comparison of potency and solubility between **132** and the competitor compound **26**

The lower level of metabolism by AO on **132** would be acceptable so this is a potential lead compound. However, an ideal lead compound would have AO metabolism that was reduced even more while retaining the excellent potency and selectivity that **132** possesses.

The 6-difluoromethyl compounds also looked promising, with very good potency and selectivity and moderate solubility. They are clearly superior to the 6-(trifluoromethyl)-1,8-naphthyridines in both potency and solubility and the most potent compounds, **157** and **158**, had excellent cellular inhibition EC_{50} values of around 10 nM. However, although resistant to metabolism by cytochrome P450, these molecules are metabolised moderately quickly by aldehyde oxidase.

Therefore, **132** remains the lead compound and, in the future, could be progressed further and subjected to further assays to test for efficacy, pharmacokinetics and toxicity with the goal of entering clinical trials as a potential new drug for the treatment or prevention of malaria.

7.2 1,8-Naphthyridines as DHODH inhibitors for the treatment of toxoplasmosis

The anti-malarial 1,8-naphthyridines were also active against *T. gondii* but to a much lesser degree, thought to be due to a mutation of a phenylalanine residue to a threonine which clashes with the aniline group. This clash cannot be easily overcome without disrupting the hydrogen bonds made by the 1,8-naphthyridine core to histidine and arginine residues so it has been challenging to adapt the 1,8-naphthyridine series into potent compounds against *T. gondii.* A library of molecules was synthesised with different alkyl linkers moving the phenyl group away from the Thr-211 residue and some improvement in potency was achieved, up to around 500 nM on *T. gondii* cells for the best compound **178**, but it was not possible to achieve potency at similar levels to that observed against *P. falciparum*.

In the future, other linkers could be investigated and it would also be desirable to test these compounds in a TgDHODH enzyme assay to check how strongly they inhibit the enzyme and how this correlates to potency against *T. gondii* cells. However, with such a tight binding site in *T. gondii* DHODH, it may not be possible to make 1,8-naphthyridines which strongly inhibit this enzyme.

Chapter 8 Experimental

8.1 Biological assays

8.1.1 DHODH enzyme assays

DHODH enzyme assays were performed by collaborators from the Phillips group at UT Southwestern.

8.1.1.1 Protein expression

PfDHODH

PCR was used to amplify a truncated segment of the pyrD gene from *P. falciparum* strain C2B genomic DNA encoding the DHODH enzyme. Primers 1 (CCTGAATTTTTTTCCATGGATATATTTTTAAAATTC) and 2 (CACTTATGTGTCGACCGTGTTTAATTAACTTTTGC), which introduce NcoI and SalI restriction sites respectively, were used to create a 1244 bp DNA fragment. This was ligated into a pProEX HTa prokaryotic expression vector (Invitrogen) which produces protein fused to an N-terminus His_6 sequence. The cloned gene was sequenced to check that no unintentional mutations had been introduced.

The 42 amino acid insert unique to *P. falciparum* was verified by PCR amplification of a pyrD gene fragment encoding this region using the cDNA library from strain Dd2 (MR4/ATCC, Manassas). Primers 3 (GGAAGATACGCTGATTATATAGC) and 2 were used to amplify a fragment of ~750 bp that was ligated into ZeroBlunt TOPO vector (Invitrogen). The cloned pyrD fragment was sequenced with the M13/reverse primer following amplification and purification of plasmid DNA.[142]

Chemically competent *E. coli* DH5α cells were cotransformed with the His_6-DHODH expression vector and the RIG plasmid which encodes the tRNAs for Arg, Ile, and Gly residues.[143] The overexpressed enzyme of ~45 kDa molecular mass lacks 168 amino acids from the N-terminus, removing the hydrophobic membrane-associated domain. Cells were grown in LB medium to an $A_{600\,nm}$ of 0.5, supplemented with 0.1 mM FMN and induced with 0.6 mM isopropyl β-d-thiogalactopyranoside (Fisher). They were harvested using centrifugation 3 h after induction, then frozen in liquid nitrogen. Typically, 5 g of cell paste was obtained per litre of culture.

The frozen cell paste was resuspended in lysis buffer (50 mM Tris, pH 8.5, 5 mM 2-mercaptoethanol, 2% Triton X-100, 0.5 mM FMN, and 10% glycerol) at

5 mL per gram of cells. A protease inhibitor mixture of phenylmethylsulfonyl fluoride (200 µM), leupeptin (1 µg/mL), antipain (2 µg/mL), benzamidine (10 µg/mL), pepstatin (1 µg/mL), and chymostatin (1 µg/mL) was added. Lysozyme was added to a final concentration of 0.1% (w/v) and the solution was incubated for 2 h on ice. The mixture was frozen in liquid nitrogen, thawed, then sonicated for 2 minutes. The lysate was centrifuged for 30 minutes at 85000 g.

The supernatant was loaded onto nickel agarose resin (Qiagen, 10 mL) equilibrated with buffer A (50 mM Tris, pH 8.5, 300 mM NaCl, 5 mM 2-mercaptoethanol, 20 mM imidazole, 0.1 mM FMN, and 10% glycerol). The column was washed with ten volumes of buffer A, followed by elution of bound protein with buffer B (50 mM Tris, pH 8.5, 300 mM NaCl, 5 mM 2-mercaptoethanol, 300 mM imidazole, 0.1 mM FMN, and 10% glycerol). The eluted material was concentrated to ~1 mL using a Centri-Prep 10 ultrafiltration device (Amicon Millipore Corp.) and applied to a Hi-Load 16/60 gel filtration column packed with Superdex 200 resin (Amersham Biosciences), equilibrated with 100 mM HEPES, pH 8.0, 150 mM NaCl and 10% glycerol. The flow rate was maintained at 0.5 mL/min and 3 mL fractions were collected.[142]

Fractions were analysed for purity using SDS-PAGE,[144] and the appropriate fractions were combined and concentrated. Protein concentration was determined using the Bradford assay with bovine serum albumin as a standard.[145] The yield of purified protein was generally 1.5–2 mg per gram of cell paste for the 45 kDa DHODH. All measurements were performed on enzyme preparations containing the His_6 tag, except where noted. In such cases, the tagged enzyme was incubated overnight at 4 °C with TEV-protease immobilised on glutathione-agarose.[146] DHODH was eluted from the reduced glutathione beads, and the enzyme with the His_6 tag remaining was removed using chromatography on Ni^{2+}-agarose resin.[142]

PvDHODH

A 1266 bp DNA fragment of PvDHODH was amplified by PCR from genomic *P. vivax* strain Belem DNA provided by John Barnwell (CDC) using primers 5′-GTAGTAGCTCTATACATGTATTTCGAGTCCTACGACCCCG-3′ and 5′-CTCGAGGGCGGCCCGCCGGTGGGCCCGCCCGACGGCGT-3′ and ligated into Zero Blunt TOPO vector (Invitrogen). QuickChange site-directed mutagenesis kit (Stratagene) was used to remove an internal NdeI site from PvDHODH using primers 5′-TGTCTACTCACATGATTTCTCAAATG-3′ and 5′-CATTTGAGAAATCATGTGAGTAGACA-3′. The resultant clone was used

to amplify the PvDHODH region by PCR, introducing XhoI and NdeI restriction sites, with primers 5′-TGGAATTCGCCCTCGAGGGCGGCCCGCCGG-3′ and 5′-GTAGTAGCTCTACATATGTATTTCGAGTCCTACGAC-3′ respectively. This PCR product was ligated into Zero Blunt TOPO vector (Invitrogen) and digested with XhoI and NdeI to excise the DHODH coding sequence, which was then ligated to pET22b expression vector (Novagen) to create the C-terminal His_6-tagged truncated PvDHODH sequence used for recombinant protein expression. The protein was expressed as a soluble truncated protein without the N-terminal domain which includes the membrane-spanning region of the protein.[95] The enzyme was purified using Ni^{2+}-agarose chromatography and gel filtration as above.

Human DHODH

The gene for an N-terminal truncated human DHODH (Met-30—Arg-396) was amplified from a cDNA library derived from human pituitary gland (Clontech) using PCR. Primers hD1 (GCCTCCTACCATATGGCCACGGGAG) and hD2 (ACGCTGGAATTCCTCCGATGATCTGCTCC) were used, which introduce NdeI and EcoRI restriction sites respectively. The 1125 bp PCR product was ligated into pCR-Blunt II-TOPO vector (Invitrogen). Recombinant plasmids were digested with NdeI and EcoRI restriction enzymes and separated using electrophoresis. The PCR product was extracted using gel purification (Qiagen) and ligated into a similarly restricted and purified pET22b (Novagen) protein overexpression vector to generate a protein with a His_6 C-terminal tag. Transformed BL21(DE3) cells were grown at 37 °C in rich LB medium [tryptone (35 g/L), yeast extract (20 g/L), NaCl (5 g/L), and glycerol (10%)] to an $A_{600\,nm}$ of 0.6. FMN (100 µM) and 2-isopropyl-1-thio-β-d-galactopyranoside (200 µM) were added and the cells were incubated for 4 h at 30 °C then harvested. The rest of the purification was identical to the procedure for isolation of PfDHODH. Approximately 2 mg of purified HsDHODH was obtained per litre of medium.[142]

8.1.1.2 Enzymatic assay

Steady-state kinetic analysis was performed using a dye-based spectrophotometric method in assay buffer (100 mM HEPES, pH 8.0, 150 mM NaCl, 10% glycerol, 0.05% Triton X-100). All buffers were degassed prior to use. Enzyme and substrate concentrations were: DHODH – 5 nM, L-dihydroorotate – 0.2 mM, decylubiquinone – 0.02 mM, DCIP – 0.12 mM. Inhibitor stock solutions (100 mM) were made in DMSO and protected from light in dark amber vials. Serial dilutions were performed to generate a threefold dilution series of 100× stocks in DMSO which were used in the final

assay (final inhibitor concentration range 0.01−100 μM). Enzyme stocks were diluted into assay buffer containing 0.1 mM BSA to make a 100× working stock solution, which was kept on ice. Assays were initiated by adding 5 μL of this stock solution to 500 μL of assay buffer containing substrates and inhibitors. The temperature was maintained at 25 °C using a circulating water bath and the reduction of DCIP was followed at 600 nm (ε = 18.8 mM^{-1} cm^{-1}).[147] Data were collected in triplicate for each concentration. Data were fitted to the log[I] against response (three parameters) equation to determine the concentration which resulted in 50% enzyme inhibition (IC_{50}) except for compounds where the IC_{50} > 10μM, which were instead fitted to the standard IC_{50} equation $Y = \frac{1}{1+\frac{X}{IC_{50}}}$ (8.1) in Graphpad Prism.[95]

8.1.2 *P. falciparum* asexual blood stage assay

Assay on *P. falciparum* 3D7 asexual blood stage cells was performed by collaborators at the University of Dundee.

P. falciparum cells were cultured according to the following protocol:

Preparation of red blood cells

1. 1 unit of A-positive blood was obtained from Ninewells Hospital.

2. 1 litre of incomplete media was made up – RPMI 1640 (25 mM HEPES, L-glutamine) [Gibco] (15.9 g), $NaHCO_3$ [Sigma] (1 g), glucose [Sigma] (2 g), gentamicin (50 mg/mL) [Gibco] (400 μL), hypoxanthine solution (13.6 g/L in 0.1 M NaOH, pH to 7.3 using NaOH) [Sigma] (2 mL).

3. Blood (35 mL per tube) was dispensed into 50 mL falcon tubes then stored in the fridge.

4. Blood was washed with incomplete media just before assay set up.

5. Volume was made up to 50 mL with incomplete media then centrifuged at 1800 g for 5 minutes, and the supernatant removed.

6. Cells were washed 4 times with incomplete media.

7. Cells were resuspended in complete media (incomplete media with additional Albumax II [Gibco, 5 g]), then sedimented by centrifuging at 1800 g for 3 minutes. Sedimented cells were treated as 100% haematocrit.

Resuscitation of parasites from stabilate

1. Stabilate was removed from liquid nitrogen and defrosted at 37 °C.

2. Stabilate was transferred to a 10 mL tube and the volume noted.

3. Thawing solution 1 (12% NaCl [VWR], 0.2 × volume) was added slowly to the parasites, with gentle mixing.

4. The solution was left to stand at RT for 2 minutes.

5. Thawing solution 2 (1.6% NaCl, 5 × volume) was added slowly to the parasites, with gentle mixing.

6. The solution was centrifuged at 1500 g for 5 minutes and the supernatant removed.

7. Thawing solution 3 (0.9% NaCl [VWR], 0.2% glucose [Sigma], 10 × volume) was added slowly to the parasites, with gentle mixing.

8. The solution was centrifuged at 1500 g for 5 minutes and the supernatant removed.

9. The pellet was resuspended in 3 mL of complete media and transferred to a T25 vented flask containing compete media (5 mL) with 5% haematocrit. The flask was flushed with 1% O_2, 3% CO_2, balance N_2, and incubated at 37 °C.

10. Media were changed daily, smearing on day 2, and, if the parasitaemia was >2.5%, flask volume was increased to 10 mL with the addition of 2 mL of 5% haematocrit blood/complete media mixture.

11. Culture was maintained at 2–6% parasitaemia.

Preparation of stabilate

1. Blood cells were sedimented by centrifuging at 1800 g for 5 minutes and the supernatant removed.

2. Cells were resuspended in 1 × volume of freezing solution – (28% v/v glycerol [Sigma], 3% w/v sorbitol [Sigma], 0.65% w/v NaCl [VWR]) then aliquoted into 2 mL cryotubes (500 μL per tube).

3. The solution was immediately placed into liquid nitrogen.

Assay

Day 1: 250 nL of 10 mM mefloquine was added to columns 12 and 24 of sterile 384-well black, clear bottom, cell culture assay plates. Compound curves were added to assay plates using Echo, programmed to stamp 250 nL (for 50 μM top concentration). *P. falciparum* 3D7 culture was counted and 20 mL of culture was prepared for each plate at 5% haematocrit, 0.3% parasitaemia. Culture (50 μL) was added to all wells of all plates using WellMate with small

bore tubing on full (S-1) speed. Plates were incubated for 72 h at 37 °C under an atmosphere of special gas mix (1% O_2, 3% CO_2, balance N_2).

Day 4: SybrGreen aliquots (10000× in DMSO, Invitrogen) were diluted to 3× with lysis buffer – Tris HCl (Sigma, 20 mM, 3.15 g/L), EDTA (BDH, 5 mM, 1.86 g/L), saponin (Fluka, 0.16% w/v, 1.6 g/L), TX100 (Fisher, 1.6% v/v, 16 mL/L), pH to 7.9. SybrGreen/lysis buffer (10 μL) was added to each well of each assay plate. Plates were incubated in the dark at room temperature overnight then read on a Victor plate reader using '384sybrgreen' protocol (excitation 485 nm, emission 528 nm). Inhibition for each test compound was calculated using equation 8.2:

$$Inhibition\ (\%) = \frac{100 \times (Test\ compound - blank)}{No\ inhibtion - blank} \quad (8.2)$$

EC_{50} values were calculated using non-linear regression curve-fitting within ActivityBase data. The assay was run in duplicate for each compound.

8.1.3 *T. gondii* assays

Assays to assess the potency of compounds against *T. gondii* were performed by collaborators in the MacLeod group at the University of Chicago.[137]

Compounds were initially prepared as 10 mM stock solutions in DMSO [Sigma-Aldrich], and working dilutions were made with IMDM-C (1×, glutamine, 25 mM HEPES, 10% FBS) [Gibco]).

8.1.3.1 Tachyzoite assays

Human foreskin fibroblasts (HFF) were cultured on a flat, clear-bottomed, black 96-well plate to 90–100% confluence. IMDM (1×, glutamine, 25 mM HEPES, Phenol red, 10% FBS [Gibco]) was removed from each well and replaced with IMDM-C (1×, glutamine, 25 mM HEPES, 10% FBS) [Gibco]). Type I RH parasites expressing Yellow Fluorescent Protein (RH-YFP) were lysed from host cells by double passage through a 27-gauge needle then counted and diluted to 32000/mL in IMDM-C. Fibroblast cultures were infected with 3200 tachyzoites of the Type I RH-YFP strain and returned to the incubator at 37 °C for 1–2 h to allow for infection. Diluted solutions of the compounds were made using IMDM-C, and 20 μL was added to each designated well. Controls included: pyrimethamine/sulfadiazine, DMSO only, fibroblast only and an untreated YFP gradient with twofold dilutions of the parasite. Cells were incubated at 37 °C for 72 h. The plates were read using a fluorometer (Synergy H4 Hybrid Reader, BioTek) to determine the amount of yellow fluorescent protein, in relative fluorescence units (RFU), as a measure of parasite burden after treatment. Data were collected using Gen5

software and analysed with Excel. The assay was run in duplicate for each compound tested.

8.1.3.2 Cytotoxicity assay

Lack of toxicity for mammalian host cells was demonstrated first by visual inspection of monolayers following giemsa staining, in separate methods by incorporation of WST, a mitochondrial cell death reagent. Toxicity assays were conducted using WST-1 cell proliferation reagent (Roche). HFF were grown on a flat, clear-bottomed, black 96-well plate. Confluent HFF were treated with inhibitory compounds at concentrations equal to those being tested in tachyzoite assays. Compounds diluted in IMDM-C (20 µL) were added to each designated well, in triplicate for each condition. A gradient of twofold decreasing concentrations of DMSO in IMDM-C was used as a control. The plate was incubated for 72 h at 37 °C. WST-1 reagent (10 µL) (Roche) was added to each well and the cells were incubated for 30–60 minutes. Absorbance was read using a fluorometer at 420 nm. A higher degree of colour change (and absorbance) indicated mitochondrial activity and hence cell viability.

8.1.4 Metabolic stability

Assays were performed by the Charman group at Monash University.

Microsome

Compound (0.5 µM) was incubated with human, rat, mouse and dog liver microsomes (Xenotech) at 37 °C and 0.4 mg/mL protein concentration. The reaction was initiated by the addition of an NADPH-regenerating system and quenched at various time points over a 60 minute incubation period by the addition of acetonitrile containing diazepam. Control samples (containing no NADPH) were included and quenched at 2, 30 and 60 minutes to check for potential degradation in the absence of cofactor.

Liver S9 fraction

Each test compound (1 µM) was incubated with human liver S9 fraction (Xenotech) at 37 °C and 2.5 mg/mL protein concentration. The reaction was initiated by the addition of compound to matrix either with NADPH, without NADPH, or without NADPH but with raloxifene (10 µM) which had been pre-incubated for 30 minutes. Reactions were quenched at various time points over a 120 minute incubation period by the addition of acetonitrile containing diazepam. Following protein precipitation with acetonitrile, samples were

centrifuged for 4 minutes at 4,500 rpm. The supernatant was removed and analysed using LCMS (see below).

Carbazeran, O^6-benzylguanine and zoniporide were used as positive control compounds for aldehyde oxidase activity and raloxifene inhibition, and the corresponding mono-oxygenated metabolites were monitored and detected based on accurate mass. Dextromethorphan, diclofenac, omeprazole, phenacetin and verapamil were all included as positive control compounds for cytochrome P450 mediated metabolism. The results for the compounds included in the stability assay for CYP450-mediated degradation were consistent with historical data.

Analytical Conditions

Instrument – Waters Xevo G2 QTOF coupled to a Waters Acquity UPLC

Detection – Positive electrospray ionisation multiple-reaction monitoring mode

Cone Voltage – 30 V

Column – Ascentis Express C8 column (50 x 2.1 mm, 2.7 □m)

LC conditions – Gradient cycle time: 4 minutes; Injection volume: 5 µL; Flow rate: 0.4 mL/min

Mobile phase – Acetonitrile-water gradient with 0.05% formic acid in each solvent

8.1.5 Cytochrome P450 inhibition

Performed by the Charman group at Monash University.

Multiple concentrations of each test compound (0.25–20 µM) and positive control inhibitors were incubated at 37 °C, along with each substrate, in human liver microsomes (XenoTech LLC., USA) with total organic solvent concentration of 0.47% (v/v). The reactions were initiated by the addition of an NADPH-regenerating system and the samples were quenched by ice-cold acetonitrile containing diazepam (0.15 µg/mL) as an analytical internal standard. The concentration of metabolites in quenched samples was determined using UPLC-MS (Waters/Micromass Xevo TQD triple-quadrupole) relative to calibration standards prepared in quenched microsomal matrix. Control samples confirmed that the UPLC-MS assay of the specific metabolites was not affected by the presence of each test compound. The inhibitory effect of each test compound and positive control inhibitor was based on the reduction in the formation of the metabolite relative to metabolite formation in the absence of inhibitor. When the inhibition of metabolite

formation exceeded 50%, the inhibitor concentration resulting in 50% inhibition (IC_{50}) was obtained by non-linear curve fit of log(inhibitor concentration) against percentage inhibition using a 4-parameter sigmoidal function with minimum and maximum inhibition values constrained to 0% and 100%, respectively. Where less than 50% inhibition was observed at the highest concentration tested (20 μM), the IC_{50} value was reported as >20 μM.

8.1.6 Permeability

Performed by the Charman group at Monash University.

Caco-2 cells (passage 37) were seeded onto 0.3 cm^2 polycarbonate filter transwells at a density of 60000 cells per well. The transport experiment was conducted using cell monolayers on day 24 post-seeding. Permeability experiments were performed using transport buffer (pH 7.4 Hanks balanced salt solution containing 20 mM HEPES) in both the apical and basolateral chambers. Donor solutions were prepared by adding an aqueous (for propranolol and Lucifer Yellow) or a DMSO (for test compounds and rhodamine 123) stock solution into transport buffer at 20 μM (for test compounds), 50 μM (for propranolol) or 100 μM (for Lucifer Yellow and rhodamine 123). The final DMSO concentration in the donor solution was 0.1% v/v for all compounds and all donor solutions were equilibrated at 37 °C for 4 h before centrifuging at 4000 rpm for 5 minutes to remove any compound which had precipitated. The supernatant buffer solution was used in the permeability experiment and compound concentration in aliquots of this bulk solution was measured. Compound flux was assessed over 120 minutes, with samples taken from the acceptor chamber at multiple time points (for test compounds, propranolol and rhodamine 123) or at the end (for Lucifer Yellow) of the transport experiment. At each sample time, the volume of acceptor solution removed was replaced with blank transport buffer and acceptor concentrations were corrected for the dilution that occurred with buffer replacement in the data analysis. Samples from the donor chamber were taken at the start (approximately 2 minutes) and end of the experiment. Donor and acceptor samples for all test compounds and propranolol were stored frozen at −80 °C until analysis by LCMS (Waters Micromass Quattro Premier coupled to a Waters Acquity UPLC, detection by positive electrospray ionisation multiple-reaction monitoring mode, Supelco Ascentis Express RP Amide column (50×2.1 mm, 2.7 μm), gradient cycle time 4 minutes, injection volume 3 μL, flow rate 0.4 mL/min, mobile phase acetonitrile-water gradient with 0.05% formic acid). Donor and acceptor samples for Lucifer Yellow and

rhodamine 123 were analysed using a fluorescence assay (FLUOstar OPTIMA plate reader; BMG Lab Technologies) with excitation/emission wavelengths set to 430/535 nm for Lucifer Yellow and 500/525 nm for rhodamine 123.

8.1.7 *In vivo* pharmacokinetics

All animal studies were performed by collaborators at Monash University using established procedures in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the study protocols were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee.

8.1.7.1 Mouse

Systemic exposure was studied in non-fasted male Swiss outbred mice weighing 19–30 g. Mice had access to food and water throughout. The compound was formulated by dispersing in a CMC-SV vehicle. The sample was vortexed and sonicated, producing a uniform fine yellow suspension and mixed by inverting the tubes prior to each dosing. All animals were dosed within 1.6 h of formulation preparation.

Compounds were dosed to mice by gavage (10 mL/kg) for oral administration. After oral administration, blood samples were collected up to 24 h (n = 3 mice per time point) with a maximum of two samples from each mouse. Samples were collected either via submandibular bleed (120 µL of conscious sampling) or terminal cardiac puncture (0.4 mL under anaesthesia using inhaled isoflurane). Blood was collected into Eppendorf tubes containing heparin as an anticoagulant and stabilisation cocktail (containing Complete®, a protease inhibitor cocktail, and potassium fluoride) to minimise *ex vivo* compound degradation in blood/plasma samples. Blood samples were centrifuged immediately, supernatant plasma was removed, and the sample was stored at −80°C until analysis by LCMS:

Instrument – Waters Micromass Quattro Premier coupled to a Waters Acquity UPLC

Detection – Positive electrospray ionisation multiple-reaction monitoring mode

Column – Supelco Ascentis Express RP Amide column (50 × 2.1 mm, 2.7 µm)

LC conditions – Gradient cycle time: 4 min; Injection vol: 3 µL; Flow rate: 0.4 mL/min.

Mobile phase – Acetonitrile-water gradient with 0.05% formic acid

Extraction (plasma) – Protein precipitation for plasma using acetonitrile (2-fold volume ratio).

8.1.7.2 Rat

Pharmacokinetics were studied in overnight-fasted male Sprague-Dawley rats weighing 250–285 g. Rats had access to water throughout, and access to food from 4 h post-dose. The compound was administered intravenously, formulation by dissolving in DMSO (5% v/v) with 5% Solutol HS-15 in saline, at a 10 minute constant rate infusion via an indwelling jugular vein cannula (1 mL per rat, n = 3 rats) and orally, formulation by wet-milling with HPMC-SV, by gavage (3 mL/kg per rat, n = 3 rats). Samples of arterial blood and total urine were collected until 24 h post-dose. Arterial blood was collected directly into vials at 4 °C containing heparin, Complete® (a protease inhibitor cocktail) and potassium fluoride to minimise *ex vivo* degradation in blood/plasma samples. Blood samples were centrifuged, supernatant plasma was removed and samples were stored frozen (−20 °C) until analysis by LCMS:

Instrument – Waters Micromass Quattro Premier coupled to a Waters Acquity UPLC

Detection – Positive electrospray ionisation multiple-reaction monitoring mode

Column – Supelco Ascentis Express RP Amide column (50 × 2.1 mm, 2.7 µm)

LC conditions – Gradient cycle time: 4 min; Injection vol: 3 µL; Flow rate: 0.4 mL/min.

Mobile phase – Acetonitrile-water gradient with 0.05% formic acid

Extraction (plasma/blood) – Protein precipitation using acetonitrile (twofold volume ratio)

Extraction (urine) – Urine treated with acetonitrile and further diluted with 50% acetonitrile-water (total dilution 20 & 200-fold).

Determination of Whole Blood-to-Plasma Ratio

Fresh heparinised whole blood was collected from male Sprague-Dawley rats. After 30 minutes, the test compound (1000 ng/mL) was added. Aliquots were transferred into microcentrifuge tubes and kept at 37 °C under an atmosphere of 5% CO_2 to maintain the pH at 7.4. At 240 minutes, 4 aliquots of blood were collected. The remaining blood was centrifuged (Eppendorf, Mini Spin plus; 9500 g) for 2 minutes and 4 aliquots of the plasma fraction were collected for assessment of the whole blood-to-plasma partitioning ratio. The whole blood

and plasma fraction samples were matrix matched by addition of an equal volume of blank blood or plasma to result in 1:1 blood:plasma then snap frozen in dry ice. All samples were stored frozen at −80 °C until analysis by LCMS. Whole blood-to-plasma partitioning ratios (B/P) were obtained by dividing the measured concentration in the blood control sample by the concentration measured in plasma following centrifugation of whole blood.

8.2 Solubility assay

FaSSIF and FaSSIF-v2 powder were bought from biorelevant.com. Fresh FaSSIF or FaSSGF buffer was made for each experiment as follows: FaSSIF – sodium hydroxide (0.1392 g, 3.48 mmol), maleic acid (0.222 g, 1.91 mmol) and sodium chloride (0.401 g, 6.86 mmol) were dissolved in water (90 mL). The pH was adjusted to 6.5 with 2 M sodium hydroxide$_{(aq)}$ or 2 M hydrochloric acid$_{(aq)}$ then water was added until the volume reached 100 mL. To this buffer solution was added FaSSIF-v2 powder (0.179 g) and the mixture stirred until all the solid had dissolved, then allowed to stand for 1 h.

FaSSGF – Sodium chloride (0.1999 g, 3.42 mmol) was dissolved in water (90 mL). The pH was adjusted to 1.6 using 2 M hydrochloric acid$_{(aq)}$ then water was added until the volume reached 100 mL. To this buffer solution was added FaSSIF powder (6 mg) and the mixture stirred until all the solid had dissolved.

Calibration

A dilution series of each compound in 50% aq. MeOH was prepared for HPLC calibration (0.05–20 µM) and peak area against concentration was plotted. A straight line of best fit was added and the gradient determined.

Measurement procedure:

A 1 mg/mL suspension of each test compound was prepared in each buffer: PBS (pH 7.4), FaSSIF (pH 6.5) and FASSGF (pH 1.6). The suspension was mixed for 20 h at room temperature with magnetic stirring (PBS) or at 37 °C in an incubator (FaSSIF, FaSSGF). Samples were centrifuged (10000 rpm, 3 minutes) then 300 µL of the supernatant was transferred to a fresh tube and centrifuged again (10000 rpm, 3 minutes). 100 µL of the supernatant was diluted tenfold with 50% aq. MeOH to make a sample which was analysed by HPLC.Two HPLC samples were measured for each compound in each buffer and compared with the calibration plot to determine the concentration.

8.3 Computational

8.3.1 Generation of TgDHODH homology model

A homology model for TgDHODH was created using Swiss-model,[138-140] using as a template the structure of *P. falciparum* DHODH (sequence identity 41%) and as the target the primary amino acid sequence for *T. gondii* DHODH:
MAALTVHFQGRFALLRLPISSGKPLCREARVRRSGTRPVSADNLSHARCV
LPKCHSFCPAGGMQESPEARVTLSRGTSRNFGTFLTALGNDVHWKSAFP
GALLRTQIRKLSVSLHPRPGSAESSGPSAGLPPKDVDPEEIERIVRERTTRE
RKANRRLVFLVLLLGTGVYCYSALQDVSSMIYSFYEPVTSVLFRYFSSGPL
DPETAHGYTMELAKRGWLPVDYDREESALNVDINGLKFLSPIGLAAGFDKH
AEAPAALLRMGFSFLEVGSITPKPQPGNPKPRLFRLYEDRSVINRFGFNSN
GADYAQTQLEAFSEARLRDPFTAQGVLGVSLGKNKTSEDAVADLREGVKK
LGRFADFLVVNLSSPNTPGLRSLQSASHLAAIIDGVQEELDALDRQAQAAS
QKQRNERRRHGGNPEETKAFYANQTGRRPLFFVKIAPDLSMEEKESIAKV
ALEKNLDGFVVSNTTIQRPETLKSPAKSETGGLSGRALKHLSTACVSDMYK
LTQGKLAIIATGGVESGRDALDKIEAGASLVELYSSMVYIGPQVARRVKNEL
YQALNEKGYKDVAAAVGRKHKHVPEKKLQAPKFD

Model Building

Models were built using ProMod3. Coordinates which are conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodelled using a fragment library. Side chains were then rebuilt. Finally, the geometry of the resulting model was regularised by using a force field. If loop modelling with ProMod3 failed, an alternative model was built with PROMOD-II[148].

Model Quality Estimation

The global and per-residue model quality was assessed using the QMEAN scoring function[139].

Ligand Modelling

Ligands present in the template structure (orotate and FMN) were not transferred to the homology model because the binding site was not conserved.

Oligomeric State Conservation

Homo-oligomeric structure of the target protein was predicted based on the analysis of pairwise interfaces of the identified template structures. For each relevant interface between polypeptide chains (interfaces with more than 10

residue-residue interactions), the $Qscore_{Oligomer}$[149] was predicted from features such as similarity to target and frequency of observing this interface in the identified templates. The prediction was performed with a random forest regressor using these features as input parameters to predict the probability of conservation for each interface. The $Qscore_{Oligomer}$ of the whole complex was then calculated as the weight-averaged $Qscore_{Oligomer}$ of the interfaces. The oligomeric state of the target is predicted to be the same as in the template when $Qscore_{Oligomer}$ is predicted to be higher or equal to 0.5.

8.3.2 Glide docking

Docking using Glide required preparation of the receptor using the Maestro protein preparation tool.[150] This process involves assigning protonation states at pH 7.4 then minimising the energy of the protein using the OPLS3 forcefield.[151] A docking grid was generated by alignment of the homology model with the previously obtained co-crystal structure of a 1,8-naphthyridine bound to PfDHODH (Fig. 1.12). Docking was generally performed using standard precision (SP) settings without constraints.

8.4 General information

Dry solvents were dried and purified using a Pure Solv MD solvent Purification System (Innovative Technology Inc.) or obtained from commercial suppliers. All other solvents used were HPLC or analytical grade. Solvents and reagents were obtained from commercial suppliers and used without further purification.

Thin layer chromatography was performed using aluminium backed silica gel 60 plates from Merck with visualisation using an ultraviolet lamp. Flash chromatography was performed using silica gel 60 (40–63 μm particles).

Proton, carbon and fluorine NMR data were collected on Bruker Avance 300, AV3 400, AV 500 or Jeol 600. All shifts were referenced against the solvent peak. Solvents ($CDCl_3$, DMSO-d_6, D_2O and methanol-d_4) used for NMR were obtained from Sigma-Aldrich. NMR data were reported in the following format: ppm (splitting pattern, coupling constant (Hz), number of protons, proton ID). Signal assignments were made by the aid of COSY, DEPT 135 and HMQC.

LCMS data were recorded on a Donex Ultimate 3000 LC system with an acetonitrile/water + 0.1% formic acid gradient.

High resolution mass spectra (HRMS) were recorded using a Bruker MaXis Impact spectrometer using electron spray (ES) ionisation.

Infrared spectra were recorded on a Perkin-Elmer one FTIR spectrometer.

HPLC was performed on an Agilent 1290 Infinity series equipped with a UV detector and a Hyperprep C_{18} reverse-phase column.

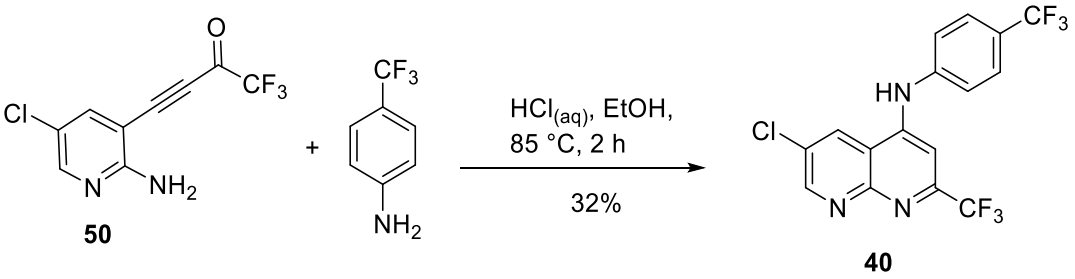
Optical rotation was performed using a Schmidt + Haensch Polartronic H532. All measurements were taken at 20 °C with a wavelength of 589 nm and a path length of 0.5 dm.

8.5 Synthetic procedures

General procedure for 'litmus test'[124]

Test compound (0.031 mmol), bis (((difluoromethyl)sulfinyl)oxy)zinc (12 mg, 0.041 mmol), trifluoroacetic acid (2 µL, 0.026 mmol) and ^{t}butyl hydroperoxide (70% aq. solution, 10 µL, 0.072 mmol) were dissolved in DMSO (150 µL). The mixture was stirred at room temperature for 2 h then diluted with methanol (1 mL) and analysed by LCMS.

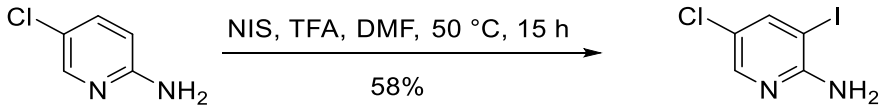
6-Chloro-2-(trifluoromethyl)-N-(4-(trifluoromethyl)phenyl)-1,8-naphthyridin-4-amine (**40**)



To a solution of **50** (76.8 mg, 0.309 mmol) in ethanol (3 mL) was added 4-(trifluoromethyl)aniline (32 µL, 0.406 mmol) followed by 2 M hydrochloric acid (0.20 mL, 0.40 mmol). The reaction was stirred at 85 °C under reflux for 2 h then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave an orange/brown solid which was partitioned between ethyl acetate (20 mL) and saturated aqueous sodium hydrogen carbonate (20 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:4). The solvent was removed *in vacuo* to leave the title compound as yellow needles (38.5 mg, 0.0983 mmol, 32%); m. p. 200–220 °C (from DMSO); Rf 0.17 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.99 (s, 1H, N-H), 9.15 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H), 9.12 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.83 (d, *J* = 8.5 Hz, 2H, aniline 3-H), 7.63 (d, *J* = 8.5 Hz, 2H, aniline 2-H), 7.39 (s, 1H, naphthyridine 3-H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.0 (sp^2 C), 153.7 (naphthyridine 7-CH), 150.3 (sp^2 C), 149.9

(q, *J* = 33.5 Hz, naphthyridine 2-C), 143.0 (sp^2 C), 131.1 (naphthyridine 5-CH), 128.3 (sp^2 C), 126.9 (q, *J* = 3.5 Hz, aniline 3-CH), 124.7 (q, *J* = 32.4 Hz, aniline 4-C), 124.2 (q, *J* = 272 Hz, CF_3), 122.1 (aniline 2-CH), 121.3 (q, *J* = 276 Hz, CF_3).115.6 (sp^2 C), 98.6 (sp^2 C); ^{19}F NMR (282 MHz, DMSO-d_6) δ –60.6 (s, CF_3), –67.2 (s, CF_3); ν_{max}/cm^{-1} (solid); 3224, 3187, 3063, 2927, 2252, 2127, 1591, 1563, 1534, 1518, 1447, 1417, 1386, 1360, 1322, 1284, 1253 and 1231; m/z (ES) found MH^+ 392.030317, $C_{16}H_8{}^{35}ClF_6N_3$ requires MH^+ 392.038369; HPLC 3.58 min., 70%, 3.61 min., 30%.

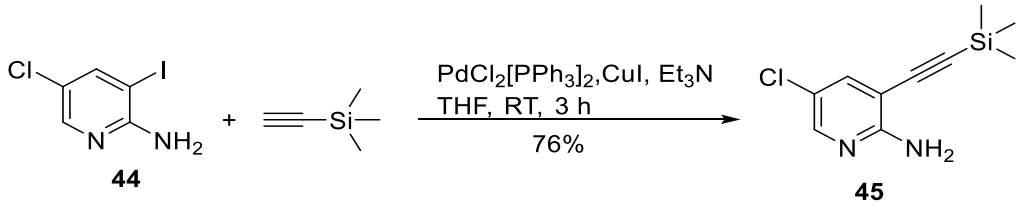
5-Chloro-3-iodopyridin-2-amine (**44**)



43 **44**

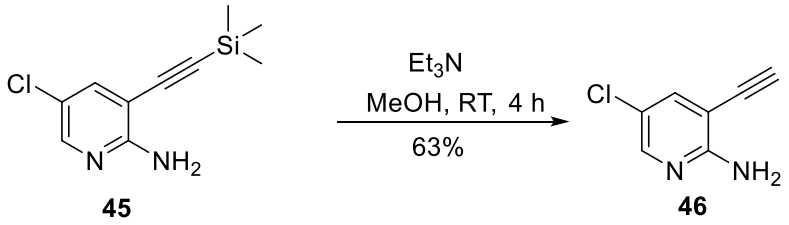
N-Iodosuccinimide (24.805 g, 110.26 mmol) was added to DMF (100 mL) portionwise at room temperature with stirring. To the resultant solution was added 5-chloro-pyridin-2-amine (12.796 g, 99.535 mmol) and the mixture stirred under nitrogen for 30 minutes. To the resultant suspension was added trifluoroacetic acid (9.2 mL, 120 mmol). After 10 minutes at room temperature the mixture was stirred at 50 °C under nitrogen for 15 h. The reaction mixture was poured into water (350 mL) and diethyl ether (250 mL) was added with rapid stirring. Excess *N*-iodosuccinimide was destroyed by adding solid sodium bisulphite (10 g) followed by solid sodium bicarbonate until effervescence ceased. The aqueous layer was extracted with diethyl ether (2 × 100 mL) and the combined organic extracts were washed successively with water (100 mL) and brine (50 mL) then dried over magnesium sulphate and evaporated *in vacuo*. The residue was triturated with pet. ether 40–60 to give an orange solid which was purified using column chromatography. Elution with diethyl ether-pet. ether 40–60 (1:4) gave a yellow solid which was recrystallised from MTBE-pet. ether 40–60 (1:1) to give the title compound (14.801 g, 58.166 mmol, 58%) as pale yellow needles, m. p. 97–99 °C (from MTBE-pet. ether 40–60) (lit. 93–97 °C[152]); Rf 0.40 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.98 (d, *J* = 1.9 Hz, 1H, 6-H), 7.84 (d, *J* = 1.9 Hz, 1H, 4-H), 4.94 (br s, 2H, NH_2) (consistent with lit.[97]); ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.1 (pyridine C), 146.2 (6-CH), 145.9 (4-CH), 120.3 (pyridine C), 77.2 (pyridine C); ν_{max}/cm^{-1} (solid); 3445, 3289, 3130 (br), 1632, 1569, 1454, 1384 and 1236; m/z (ES) found MH^+ 254.917701, $C_5H_4{}^{35}ClIN_2$ requires MH^+ 254.918050; HPLC 1.64 min., 100%.

5-Chloro-3-((trimethylsilyl)ethynyl)pyridin-2-amine (**45**)



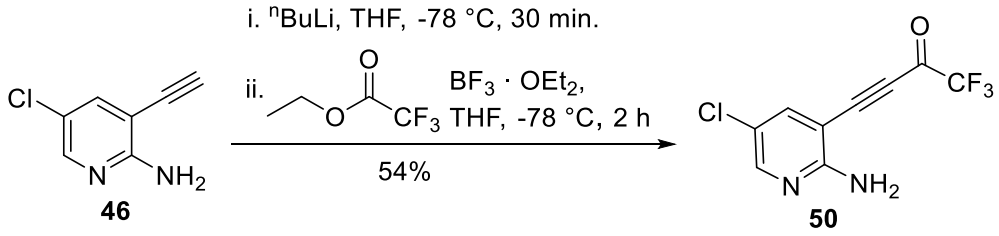
44 (12.757 g, 50.134 mmol), copper(I) iodide (0.201 g, 1.057 mmol**)** and bis(triphenylphosphine)palladium(II) dichloride (0.675 g, 0.962 mmol) were dissolved in THF (110 mL) at room temperature and flushed with nitrogen for 30 minutes. To the resultant grey solution was added with stirring triethylamine (34 mL, 242 mmol) followed immediately by ethynyltrimethylsilane (22 mL, 159 mmol). An orange solution formed which gradually turned yellow upon stirring in a water bath at room temperature. After stirring for 3 h, the black suspension was partitioned between pet. ether 40–60 (200 mL) and water (100 mL). The layers were separated and the organic layer washed with water (2 × 100 ml) and brine (50 mL). The organic layer was dried over magnesium sulphate and evaporated to give a dark brown solid. Pet. ether 40–60 (50 mL) was added and the suspension stirred at 0 °C for 30 minutes. The solid was collected using vacuum filtration and washed with cold pet. ether 40–60 to give the product **45** (8.514 g, 37.880 mmol, 76%) as a grey solid. A sample of product (0.136 g, 0.603 mmol) was dissolved in pet. ether 40–60 (10 mL). APDTC (12.4 mg, 0.075 mmol) was added followed by water (2 mL). The mixture was stirred at room temperature for 2 h then filtered to remove the solid. The layers were then separated and the organic layer dried over magnesium sulphate. The solvent was removed *in vacuo* to leave the pure product as colourless needles (0.102 g, 0.453 mmol, 75%), m. p. 109–110 °C (from pet. ether 40–60) (lit. 108 °C[153]); Rf 0.76 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.96 (d, *J* = 2.5 Hz, 1H, 6-H), 7.50 (d, *J* = 2.5 Hz, 1H, 4-H), 5.00 (s, 2H, NH_2), 0.26 (s, 9H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.6 (pyridine C), 146.7 (6-CH), 139.4 (4-CH), 120.0 (pyridine C), 104.2 (pyridine C), 102.7 (C≡C), 99.0 (C≡C), 0.0 (CH_3) (consistent with lit.[97]); ν_{max}/cm^{-1} (solid); 3456, 3288, 3160, 2959, 2923, 2149, 1622, 1554, 1456, 1405, 1288 and 1249; m/z (ES) found MH^+ 225.054746, $C_{10}H_{13}{}^{35}ClN_2Si$ requires MH^+ 225.060931. HPLC 1.25 min., 86%.

5-Chloro-3-ethynylpyridin-2-amine (**46**)



To a solution of **45** (4.197 g, 18.673 mmol) in methanol (60 mL) was added triethylamine (8.0 mL, 57 mmol). The solution was stirred at room temperature for 4 h then the solvent was removed *in vacuo*. The solid residue was dissolved in diethyl ether and filtered through a plug of silica gel to remove polar black impurities. The resultant yellow solution was concentrated *in vacuo* and the solid residue dissolved in acetone (15 mL). Pet. ether 40–60 (30 mL) was added and the solution was left at −20 °C for 16 h. The yellow solid was collected by suction filtration and washed with cold pet. ether 40–60 to give the title compound as yellow needles (1.801 g, 11.806 mmol, 63%), m. p. 101–106 °C decomposed (from acetone-pet. ether 40–60); Rf 0.24 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00 (d, *J* = 2.5 Hz, 1H, 6-H), 7.53 (d, *J* = 2.5 Hz, 1H, 4-H), 5.06 (s, 2H, NH_2), 3.45 (s, 1H, C≡C-H) (consistent with lit.[154]); ^{13}C NMR (126 MHz, $CDCl_3$) δ 157.8 (pyridine C), 147.1 (pyridine 6-CH), 139.8 (pyridine 4-CH), 119.9 (pyridine C), 102.8 (pyridine C), 84.5 (C≡C-H), 78.1 (C≡C); ν_{max}/cm^{-1} (solid); 3474, 3289, 3136, 2918, 2105, 1818, 1633, 1552, 1474, 1402, and 1246; m/z (ES) found MH^+ 153.021074, $C_7H_5{}^{35}ClN_2$ requires MH^+ 153.021402. HPLC 1.24 min., 100%.

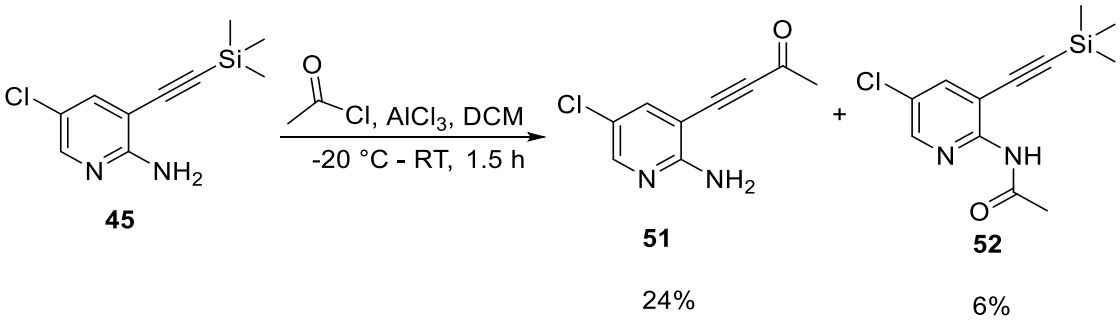
4-(2-Amino-5-chloropyridin-3-yl)-1,1,1-trifluorobut-3-yn-2-one (**50**)



One crystal of triphenylmethane and **46** (0.148 g, 0.970 mmol) were dissolved in dry THF (10 mL) under an atmosphere of nitrogen. A 1.6 M solution of ^{n}butyllithium in hexanes (1.5 ml, 2.4 mmol) was added and a dark red colour formed. The solution was stirred at −78 °C for 30 minutes then a solution of ethyl trifluoroacetate (0.13 mL, 1.1 mmol) in dry THF (10 mL) was added followed by boron trifluoride diethyl etherate (0.15 mL, 1.2 mmol). The mixture was stirred at −78 °C for 2 h then quenched with saturated aqueous ammonium chloride solution (2 mL), causing the red colour to disappear. The

slurry was allowed to warm to room temperature then the THF was removed *in vacuo*. The residue was diluted with diethyl ether (40 mL), washed with brine (2 × 10 mL) and dried over magnesium sulphate. The solvent was removed *in vacuo* to leave a black residue which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:1). The solvent was removed *in vacuo* to leave the title compound as a brown solid (0.129 g, 0.519 mmol, 54%), m. p. 240 °C decomposed (from ethyl acetate-pet. ether 40–60); Rf 0.19 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (s, 1H, pyridine 6-H), 7.70 (s, 1H, pyridine 4-H), 5.32 (s, 2H, NH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.2 (C=O), 159.0 (pyridine C), 152.0 (pyridine 6-CH), 141.7 (pyridine 4-CH), 132.6 (CF_3), 120.5 (pyridine C), 112.3 (pyridine C), 97.5 (C≡C), 90.1 (C≡C); ^{19}F NMR (282 MHz, $CDCl_3$) δ −77.4 (s, CF_3); ν_{max}/cm^{-1} (solid); 3437, 3301, 3152, 2925, 2182, 1694, 1638, 1545, 1470, 1413, 1288 and 1219; m/z (ES) found MH^+ 249.003323, $C_9H_4{}^{35}ClF_3N_2O$ requires MH^+ 249.003702; HPLC 1.82 min., 100%.

4-(2-Amino-5-chloropyridin-3-yl)but-3-yn-2-one (**51**)

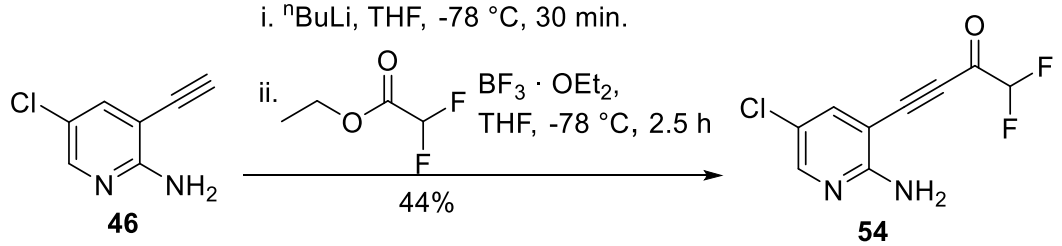


45 (0.502 g, 2.233 mmol) was dissolved in dry DCM (2 mL) at −20 °C under an atmosphere of nitrogen. Acetyl chloride (0.19 mL, 2.67 mmol) was added to give a brown solution then aluminium chloride (0.992 g, 7.437 mmol) was added with vigorous stirring. After stirring for 30 minutes at −20 °C, the reaction mixture was allowed to warm to room temperature and stirred for a further 1 h. The reaction mixture was diluted with DCM (30 mL) and washed sequentially with 1 M aqueous sodium hydroxide (20 mL), water (20 mL) and brine (20 mL). The organic layer was separated, dried over magnesium sulphate and the solvent removed *in vacuo*. The black solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (3:1). Fractions containing product were combined and the solvent was removed *in vacuo* to give the title compound **51** as yellow needles (0.102 g, 0.524 mmol, 24%), m. p. 140–143 °C decomposed (from DCM); Rf 0.17 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, *J* = 2.5 Hz, 1H, 6-H), 7.61 (d, *J* = 2.5 Hz, 1H, 4-H), 5.20 (s, 2H, NH_2), 2.46 (s, 3H, CH_3); ^{13}C NMR

(101 MHz, $CDCl_3$) δ 183.5 (C=O), 158.3 (pyridine C), 149.7 (pyridine 6-CH), 140.9 (pyridine 4-CH), 120.2 (pyridine C), 99.9 (pyridine C), 94.9 (C≡C), 84.1 (C≡C) 32.6 (CH_3); ν_{max}/cm^{-1} (solid); 3413, 3300, 3162, 2922, 2853, 2175, 1662, 1632, 1578, 1548, 1472, 1407, 1356, 1335, 1283 and 1228; m/z (ES) found MH^+ 195.031027, $C_9H_7{}^{35}ClN_2O$ requires MH^+ 195.031967; HPLC 1.91 min., 100%.

Also isolated as an orange solid was a minor N-substituted product, *N*-(5-chloro-3-((trimethylsilyl)ethynyl)pyridin-2-yl)acetamide **52** (37.5 mg, 0.141 mmol, 6%), m. p. 51–54 °C decomposed (from DCM); Rf 0.37 (1:3 ethyl acetate-pet. ether 40–60); ^1H NMR (300 MHz, $CDCl_3$) δ 8.25 (d, *J* = 2.6 Hz, 1H, 6-H), 8.06 (s, 1H, N-H), 7.69 (d, *J* = 2.6 Hz, 1H, 4-H), 2.46 (s, 3H, amide CH_3), 0.29 (s, 9H, Si-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 150.6 (pyridine C), 146.6 (pyridine 6-CH), 140.2 (pyridine 4-CH), 126.0 (pyridine C), 109.6 (pyridine C), 106.3 (C≡C), 97.4 (C≡C), 25.3 (CH_3), 0.0 (Si-CH_3); ν_{max}/cm^{-1} (solid); 3176, 2958, 2163, 1671, 1563, 1511, 1426, 1392, 1371, 1290 and 1250; m/z (ES) found MNa^+ 289.051712, $C_{12}H_{15}{}^{35}ClN_2OSi$ requires MNa^+ 289.053440; HPLC 3.07 min., 93%.

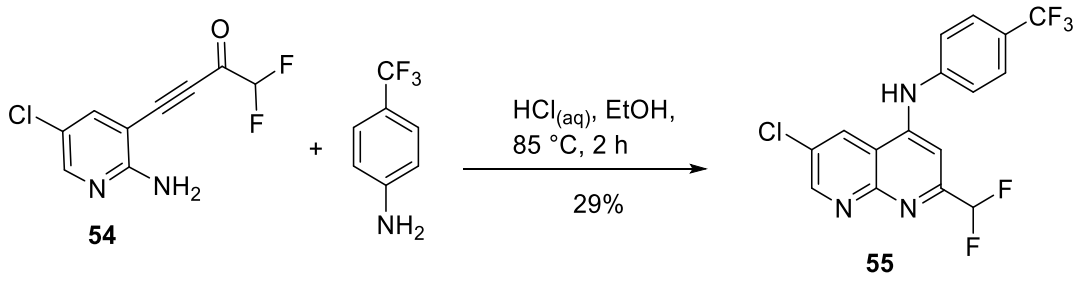
4-(2-Amino-5-chloropyridin-3-yl)-1,1-difluorobut-3-yn-2-one (**54**)



One crystal of triphenylmethane and **46** (0.338 g, 2.22 mmol) were dissolved in dry THF (20 mL) under an atmosphere of nitrogen. A 1.6 M solution of ^{n}butyllithium in hexanes (3.3 mL, 5.3 mmol) was added and a dark red colour formed. The solution was stirred at −78 °C for 30 minutes then a solution of ethyl difluoroacetate (0.26 mL, 2.47 mmol) and boron trifluoride diethyl etherate (0.33 mL, 2.67 mmol) in dry THF (20 mL) was added. The mixture was stirred at −78 °C for 2.5 h then quenched with saturated aqueous ammonium chloride solution (4 mL), causing the red colour to disappear. The slurry was allowed to warm to room temperature then the THF was removed *in vacuo*. The residue was diluted with diethyl ether (30 mL), washed with brine (2 × 30 mL) and dried over magnesium sulphate. The solvent was removed *in vacuo* to leave a brown residue which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3). The solvent was removed *in vacuo* to leave the title compound as an orange solid

(0.226 g, 0.979 mmol, 44%), m. p. 130–140 °C decomposed (from ethyl acetate-pet. ether 40–60); Rf 0.38 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.19 (d, *J* = 2.6 Hz, 1H, 6-H), 7.93 (d, *J* = 2.6 Hz, 1H, 4-H), 6.99 (s, 2H, NH_2), 6.57 (t, *J* = 53.5 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.9 (C=O), 160.1 (pyridine C), 151.5 (pyridine 6-CH), 141.8 (pyridine 4-CH), 117.7 (pyridine C), 109.4 (t, *J* = 249 Hz, CHF_2), 102.8 (pyridine C), 97.2 (C≡C), 91.2 (C≡C); ^{19}F NMR (376 MHz, DMSO-d_6) δ −127.0 (d, *J* = 52.6 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3437, 3301, 3152, 2925, 2182, 1694, 1638, 1545, 1470, 1413, 1288 and 1219; m/z (ES) found MH^+ 231.012707, $C_9H_5{}^{35}ClF_2N_2O$ requires MH^+ 231.013123; HPLC 2.01 min., 96%.

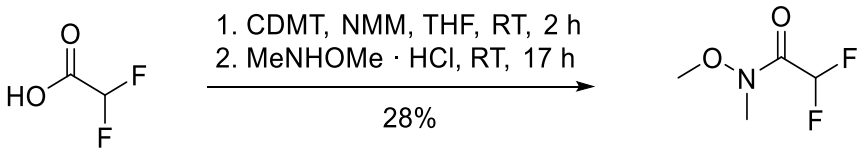
6-Chloro-2-(difluoromethyl)-N-(4-(trifluoromethyl)phenyl)-1,8-naphthyridin-4-amine (**55**)



To a brown solution of **54** (80.4 mg, 0.349 mmol) in ethanol (3 mL) was added 4-(trifluoromethyl)aniline (32 µL, 0.41 mmol) followed by 2 M hydrochloric acid (0.20 mL, 0.40 mmol). The reaction was heated to reflux at 85 °C for 2 h then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave a brown solid which was partitioned between ethyl acetate (20 mL) and saturated aqueous sodium hydrogen carbonate (20 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:6–1:2). The solvent was removed *in vacuo* to leave the title compound as an orange solid (37.9 mg, 0.101 mmol, 29%), m. p. > 270 °C (from ethyl acetate-pet. ether 40–60); Rf 0.08 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.98 (d, *J* = 2.3 Hz, 1H, naphthyridine 7-H), 8.56 (d, *J* = 2.3 Hz, 1H, naphthyridine 5-H), 7.71 (d, *J* = 8.4 Hz, 2H, aniline 3-H), 7.61 (s, 1H, N-H), 7.45 (s, 1H, naphthyridine 3-H), 7.43 (d, *J* = 8.4 Hz, 2H, aniline 2-H), 6.62 (t, *J* = 55.2 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.7 (t, *J* = 26.5 Hz, naphthyridine 2-C), 154.4 (sp^2 C), 153.5 (naphthyridine 7-CH), 149.1 (sp^2 C), 142.2 (sp^2 C), 129.4 (sp^2 C), 129.2 (naphthyridine 5-CH), 127.3 (q, *J* = 3.6 Hz, aniline 3-CH), 127.2 (q, *J* = 33.1 Hz, aniline 4-C), 123.9 (q, *J* = 272 Hz, CF_3), 121.8 (aniline 2-CH), 115.6

(sp^2 C), 114.1 (t, *J* = 242 Hz, CHF_2), 100.1 (naphthyridine 3-CH); ^{19}F NMR (376 MHz, $CDCl_3$) δ –62.3 (s, CF_3), –115.4 (d, *J* = 56.5 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3258, 1591, 1557, 1519,1411, 1384, 1361 and 1321; m/z (ES) found MH^+ 374.047912, $C_{16}H_9{}^{35}ClF_5N_3$ requires MH^+ 374.047793; HPLC 1.41 min., 100%.

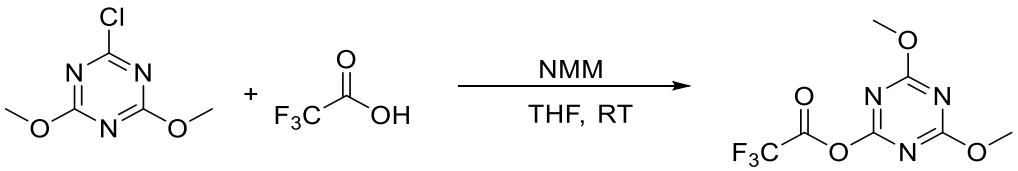
2,2-Difluoro-N-methoxy-N-methylacetamide (**60**)



60

To a solution of difluoroacetic acid (0.230 mL, 3.65 mmol) in THF (11 mL) were added 2-chloro-4,6-dimethoxy-1,3,5-triazine (0.755 g, 4.303 mmol) and *N*-methylmorpholine (1.2 mL, 11 mmol). The mixture was stirred at room temperature for 2 h then *N*,*O*-dimethylhydroxylamine hydrochloride (0.362 g, 3.712 mmol) was added. The mixture was stirred for a further 17 h then quenched with water (15 mL) and extracted with diethyl ether (2 × 7 mL). The combined organic extracts were washed with saturated aqueous sodium carbonate solution (15 mL), 1 M aqueous HCl (15 mL) and brine (15 mL). The organic layer was dried over sodium sulphate and the solvent evaporated to give the product as a colourless oil (0.141 g, 1.016 mmol, 28%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.29 (t, *J* = 53.7 Hz, 1H, CHF_2), 3.78 (s, 3H, O-CH_3), 3.27 (s, 3H, N-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.3 (t, *J* = 27.1 Hz, C=O), 106.3 (t, *J* = 245 Hz, CHF_2), 62.2 (O-CH_3), 55.3 (N-CH_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ –126.9 (d, J = 53.7, CHF_2) (consistent with lit.[155]); ν_{max}/cm^{-1} (liquid); 2949, 1695, 1563, 1467, 1398, 1362 and 1200.

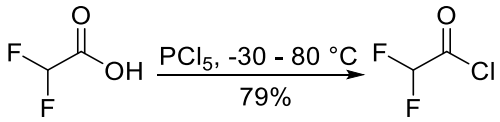
4,6-Dimethoxy-1,3,5-triazin-2-yl 2,2,2-trifluoroacetate (**61**)



61

To a THF (5 mL) solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (0.912 g, 5.19 mmol) and *N*-methylmorpholine (0.532 g, 5.26 mmol) was added dropwise trifluoroacetic acid (0.40 mL, 5.2 mmol) in THF (5 mL). The reaction mixture was stirred at room temperature for 18 h. No product was obtained.

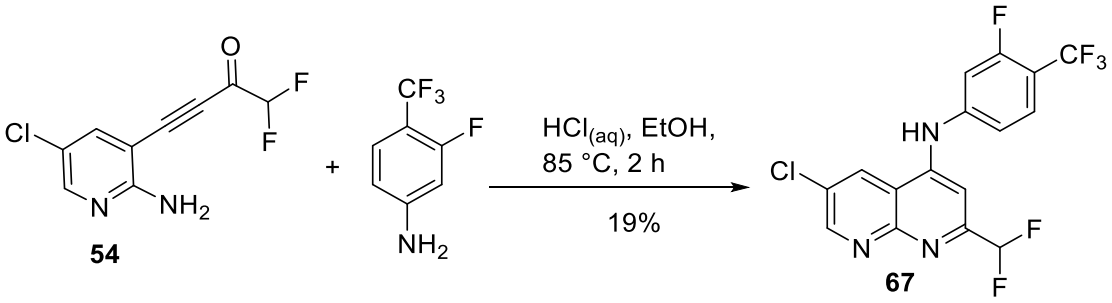
2,2-Difluoroacetyl chloride (**65**)



65

To difluoroacetic acid (0.40 mL, 6.36 mmol) was added phosphorous pentachloride (1.504 g, 7.222 mmol) in several portions at −30 °C. The temperature was raised to 80 °C and the product distilled off and collected at −78 °C as a colourless liquid (0.5737 g, 5.011 mmol, 79%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.97 (t, *J* = 53.5 Hz, 1H) (consistent with lit.[156]); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.6 (t, *J* = 36.2 Hz, C=O), 107.4 (t, *J* = 257 Hz, CHF_2); ^{19}F NMR (282 MHz, $CDCl_3$) δ −122.3 (d, *J* = 53.5 Hz, CHF_2) (consistent with lit.[157]); ν_{max}/cm^{-1} ($CDCl_3$); 1815, 1791 and 1296.

6-Chloro-2-(difluoromethyl)-N-(3-fluoro-4-(trifluoromethyl)phenyl)-1,8-naphthyridin-4-amine (**67**)

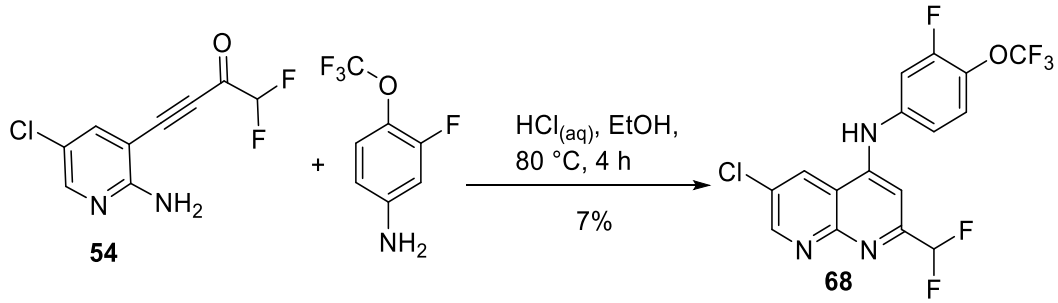


54 **67**

To a brown solution of **54** (0.165 g, 0.714 mmol) in ethanol (10 mL) was added 4-amino-2-trifluorobenzotrifluoride (0.140 g, 0.781 mmol) followed by 2 M hydrochloric acid (0.39 mL, 0.78 mmol). The reaction was heated to reflux at 85 °C for 2 h then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave a brown solid which was partitioned between ethyl acetate (20 mL) and saturated aqueous sodium hydrogen carbonate (20 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:6–1:2). The solvent was removed *in vacuo* to leave the title compound as an orange solid (52.0 mg, 0.133 mmol, 19%), m. p. 75–78 °C (from DCM); Rf 0.24 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.92 (s, 1H, naphthyridine 7-H), 8.64 (s, 1H, naphthyridine 5-H), 8.20 (s, 1H, NH), 7.64 (t, *J* = 8.0 Hz, 1H, aniline 5-H), 7.52 (s, 1H, naphthyridine 3-H), 7.17 (m, 2H, aniline 2-H, 6-H), 6.61 (t, *J* = 55.1 Hz, 1H, CHF_2); ^{13}C NMR (75 MHz, $CDCl_3$)

δ 158.9 (d, *J* = 258 Hz, C-F), 154.2 (sp^2 C), 153.5 (naphthyridine 7-CH), 148.7 (sp^2 C), 144.8 (sp^2 C), 135.6 (sp^2 C), 129.9 (naphthyridine 5-CH), 129.6 (sp^2 C), 128.7 (d, *J* = 31.2 Hz, aniline 2-CH), 123.0 (q, *J* = 251 Hz, CF_3), 116.1 (sp^2 C), 115.9 (d, *J* = 3.4 Hz, aniline 6-CH), 114.1 (sp^2 C), 113.8 (t, *J* = 242 Hz, CHF_2), 109.2 (d, *J* = 23.7 Hz, aniline 5-CH), 101.3 (naphthyridine 3-CH); ^{19}F NMR (376 MHz, $CDCl_3$) δ –60.8 (d, *J* = 12.3 Hz, CF_3), –110.8 (q, *J* = 12.3 Hz, C-F), –115.1 (d, *J* = 55.1 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3198, 2962, 2928, 1629, 1592, 1561, 1451, 1414, 1363, 1318 and 1234; m/z (ES) found MH^+ 392.038891, $C_{16}H_8{}^{35}ClF_6N_3$ requires MH^+, 392.038371; HPLC 3.47 min., 100%.

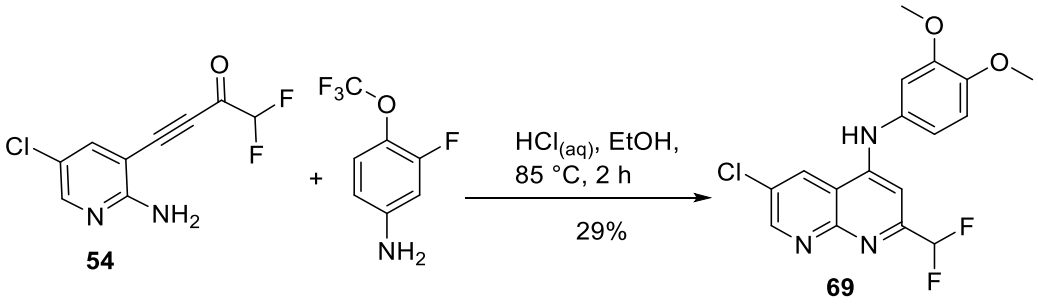
6-Chloro-2-(difluoromethyl)-N-(3-fluoro-4-(trifluoromethoxy)phenyl)-1,8-naphthyridin-4-amine (**68**)



To a brown solution of **54** (61.2 mg, 0.265 mmol) in ethanol (10 mL) was added 3-fluoro-4(trifluoromethoxy)aniline (74.5 mg, 0.382 mmol) followed by 2 M hydrochloric acid (0.16 mL, 0.32 mmol). The reaction was stirred at 80 °C under reflux for 4 h then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave a brown liquid which was partitioned between ethyl acetate (30 mL) and saturated aqueous sodium hydrogen carbonate (30 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with DCM-methanol (50:1). The solvent was removed *in vacuo* to leave the title compound as an orange solid (7.8 mg, 0.019 mmol, 7%), m. p. 134–137 °C (from DCM); Rf 0.42 (30:1 DCM-methanol); ^1H NMR (400 MHz, $CDCl_3$) δ 8.99 (s, 1H, naphthyridine 7-H), 8.52 (d, *J* = 1.6 Hz, 1H, naphthyridine 5-H), 7.50 (s, 1H, N-H), 7.40 (d, *J* = 8.5 Hz, 1H, aniline 6-H), 7.37 (s, 1H, naphthyridine 3-H), 7.20 (dd, *J* = 10.6, 2.4 Hz, 1H, aniline 2-H), 7.15 (d, *J* = 8.5 Hz, 1H, aniline 5-H), 6.63 (t, *J* = 55.1 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.7 (t, *J* = 4.5 Hz, naphthyridine 2-C), 155.2 (d, *J* = 255 Hz, aniline C-F), 154.3 (naphthyridine C), 153.5 (naphthyridine 7-CH), 149.2 (naphthyridine C), 138.8 (d, *J* = 8.6 Hz, aniline C), 133.7 (d, *J* = 12.6 Hz, aniline C), 129.4 (naphthyridine C), 129.0 (naphthyridine 5-CH), 125.2 (aniline 6-CH), 120.5 (q, *J* = 262 Hz, CF_3), 118.4

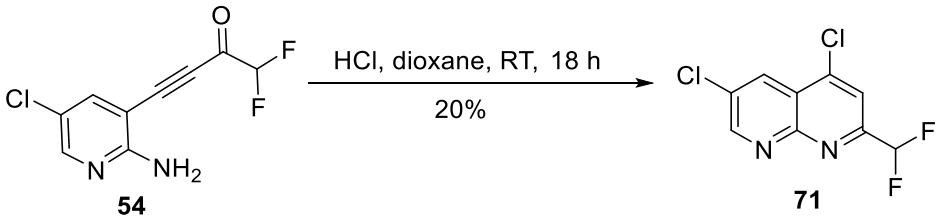
(d, *J* = 3.6 Hz, aniline 5-CH), 115.3 (naphthyridine C), 114.0 (t, *J* = 243 Hz, CHF_2), 111.6 (d, *J* = 21.2 Hz, aniline 2-CH), 99.8 (naphthyridine C); ^{19}F NMR (376 MHz, $CDCl_3$) δ –58.9 (d, *J* = 4.6 Hz, CF_3), –115.3 (d, *J* = 55.2 Hz, CHF_2), –124.6 (s, C-F) ; ν_{max}/cm^{-1} (solid); 3256, 2916, 1590, 1560, 1509, 1387 and 1250; m/z (ES) found MH+ 408.032911, $C_{16}H_8{}^{35}ClF_6N_3O$ requires MH^+ 408.033284; HPLC 3.34 min., 95%.

6-Chloro-2-(difluoromethyl)-N-(3,4-dimethoxyphenyl)-1,8-naphthyridin-4-amine (**69**)



To a brown solution of **54** (44.0 mg, 0.191 mmol) in ethanol (10 mL) was added 3,4-dimethoxyaniline (70.7 mg, 0.462 mmol) followed by 2 M hydrochloric acid (0.25 mL, 0.50 mmol). The reaction was stirred at 85 °C under reflux for 2 h then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave a brown solid which was partitioned between ethyl acetate (30 mL) and saturated aqueous sodium hydrogen carbonate (30 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:1). The solvent was removed *in vacuo* to leave the title compound as an orange solid (25.7 mg, 0.0703 mmol, 29%), m. p. 139–141 °C (from DCM); Rf 0.11 (1:2 ethyl acetate-pet. ether 40–60); ^1H NMR (300 MHz, $CDCl_3$) δ 8.95 (d, *J* = 2.4 Hz, 1H, naphthyridine 7-H), 8.56 (d, *J* = 2.4 Hz, 1H, naphthyridine 5-H), 7.54 (br s, 1H, N-H), 7.09 (s, 1H, naphthyridine 3-H), 6.92 (s, 1H, aniline 2-H), 6.90 (d, *J* = 2.1 Hz, 1H, aniline 6-H), 6.84 (d, *J* = 2.1 Hz, 1H, aniline 5-H), 6.57 (t, *J* = 55.3 Hz, 1H), 3.92 (s, 3H, methoxy), 3.84 (s, 3H, methoxy); ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.5 (t, *J* = 26.2 Hz, naphthyridine 2-C), 154.3 (sp^2 C), 153.0 (naphthyridine 7-CH), 151.4 (sp^2 C), 150.0 (sp^2 C), 147.8 (sp^2 C), 131.1 (sp^2 C), 129.0 (naphthyridine 5-CH), 128.5 (sp^2 C), 117.0 (sp^2 CH), 114.5 (sp^2 C), 114.3 (t, *J* = 241 Hz, CHF_2), 112.0 (sp^2 CH), 108.7 (sp^2 CH), 98.0 (sp^2 CH), 56.1 (CH_3), 56.0 (CH_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ –115.4 (d, *J* = 55.3 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 1751, 1685, 1604, 1457,1419, 1365, 1304 and 1218; m/z (ES) found MH^+ 366.074896, $C_{17}H_{14}{}^{35}ClF_2N_3O_2$ requires MH^+ 366.081537; HPLC 2.02 min., 100%.

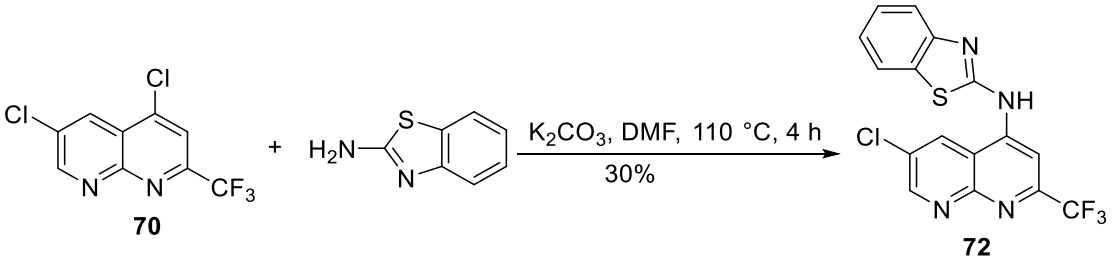
4,6-Dichloro-2-(difluoromethyl)-1,8-naphthyridine (**71**)



54 **71**

To a brown solution of **54** (0.241 g, 0.978 mmol) in 1,4-dioxane (10 mL) was added 4 M HCl in 1,4-dioxane (0.60 mL, 2.4 mmol). The solution was stirred under an atmosphere of nitrogen at room temperature for 18 h. The solvent was removed *in vacuo* to leave a brown solid which was neutralised with saturated aqueous sodium hydrogen carbonate solution (20 mL) and extracted with ethyl acetate (20 mL). The organic layer was dried over magnesium sulphate and the solvent removed in vacuo to leave a dark brown solid which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:5–1:2). The title compound was obtained as yellow needles (48.0 mg, 0.193 mmol, 20%), m. p. 86–90 °C (from chloroform); Rf 0.78 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.14 (d, *J* = 2.5 Hz, 1H, 7-H), 8.63 (d, *J* = 2.6 Hz, 1H, 5-H), 7.95 (s, 1H, 3-H), 6.81 (t, *J* = 54.8 Hz, 1H, CHF_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.8 (t, *J* = 28.0 Hz, naphthyridine 2-C), 154.8 (naphthyridine 7-CH), 153.5 (naphthyridine C), 144.1 (naphthyridine C), 132.1 (naphthyridine C), 131.8 (naphthyridine 5-CH), 122.7 (naphthyridine C), 119.1 (naphthyridine 3-CH), 113.6 (t, *J* = 242 Hz, CHF_2); ^{19}F NMR (282 MHz, $CDCl_3$) δ –114.8 (d, *J* = 54.8 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3074, 2925, 2851, 1599, 1544, 1453, 1368, 1297 and 1250; m/z (ES) found MH^+ 248.978425, $C_9H_4{}^{35}Cl_2F_2N_2$ requires MH^+ 248.979236; HPLC 3.01 min., 100%.

N-(6-Chloro-2-(trifluoromethyl)-1,8-naphthyridin-4-yl)benzo[d]thiazol-2-amine (**72**)

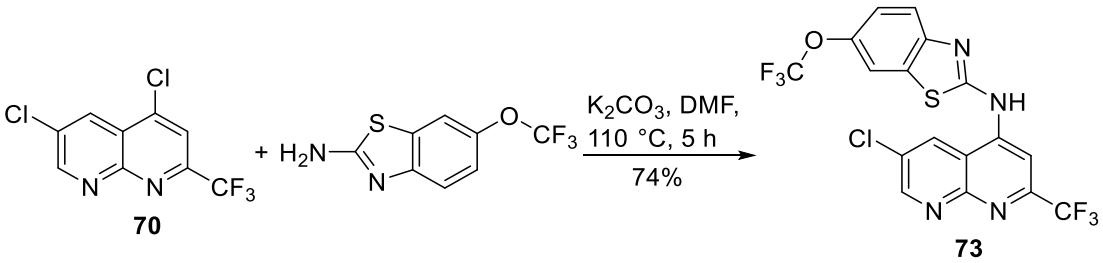


70 **72**

70 (102.9 mg, 0.385 mmol), 2-amino-benzothiazole (56.1 mg, 0.374 mmol) and potassium carbonate (0.195 g, 1.412 mmol) were dissolved in anhydrous DMF (3 mL) under an atmosphere of nitrogen. The reaction mixture was

heated at 110 °C for 4 h then allowed to cool to room temperature. Water (20 mL) was added and an orange precipitate formed which was isolated using vacuum filtration. The precipitate was purified using column chromatography; elution with DCM-methanol (50:1–1:1). The solvent was removed *in vacuo* to leave the title compound as an orange solid (42.3 mg, 0.111 mmol, 30%), m. p. > 270 °C (from DCM-methanol); Rf 0.55 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.43 (br s, 1H, N-H), 9.32 (br s, 2H, naphthyridine 3-H, 5-H,), 9.20 (s, 1H, naphthyridine 7-H), 7.99 (d, *J* = 7.8 Hz, 1H, benzothiazole 4-H), 7.81 (d, *J* = 7.8 Hz, 1H, benzothiazole 7-H), 7.46 (t, *J* = 7.6 Hz, 1H, benzothiazole 5-H), 7.33 (t, *J* = 7.6 Hz, 1H, benzothiazole 6-H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 153.9 (naphthyridine 7-CH), 131.4 (naphthyridine 5-CH), 126.8 (benzothiazole 5-CH), 124.2 (benzothiazole 6-CH), 122.0 (benzothiazole 4-CH), 120.8 (benzothiazole 7-CH), 104.2 (naphthyridine 3-CH), other peaks not resolved; ^{19}F NMR (376 MHz, DMSO-d_6) δ –67.0 (s, CF_3); ν_{max}/cm^{-1} (solid); 2967, 2924, 1681, 1573, 1519, 1488, 1444, 1412, 1383, 1344 and 1263; m/z (ES) found MNa^+ 402.999918, $C_{16}H_8{}^{35}ClF_3N_4S$ requires MNa^+ 403.000250; HPLC 2.44 min., 100%.

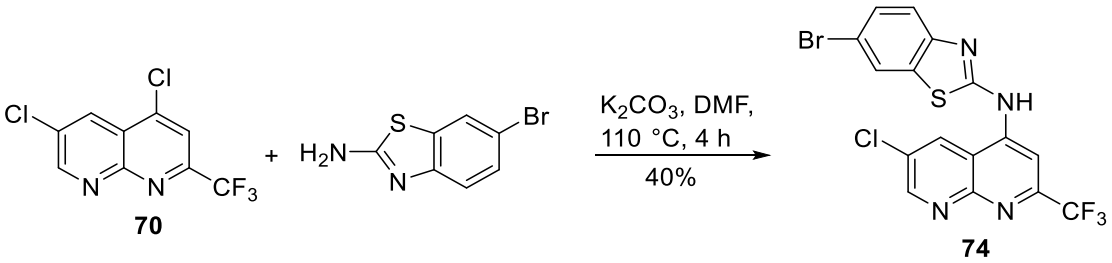
N-(6-Chloro-2-(trifluoromethyl)-1,8-naphthyridin-4-yl)-6-(trifluoromethoxy)benzo[d]thiazol-2-amine (**73**)



70 (0.132 g, 0.493 mmol), 2-amino-6-trifluoromethoxy-benzothiazole (0.112 g, 0.477 mmol) and potassium carbonate (0.200 g, 1.451 mmol) were dissolved in anhydrous DMF (3 mL) under an atmosphere of nitrogen. The reaction mixture was heated at 110 °C for 5 h then allowed to cool to room temperature. Water (10 mL) was added and an orange precipitate formed which was isolated using vacuum filtration. The precipitate was recrystallised from methanol and the filtrate purified using column chromatography; elution with DCM-methanol (50:1–1:1). The solvent was removed *in vacuo* to leave the title compound as an orange solid (0.164 g, 0.352 mmol, 74%), m. p. > 270 °C (from methanol); Rf 0.33 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4–DCM) δ 9.19 (s, 1H, benzothiazole 7-H), 9.02 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 8.98 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H),

7.72 (m, 2H, benzothiazole 4-H, naphthyridine 3-H), 7.25 (dd, *J* = 8.8, 1.6 Hz, 1H, benzothiazole 5-H), 4.47 (s, 1H, N-H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 162.8 (sp^2 C), 154.3 (sp^2 C), 153.6 (naphthyridine 7-CH), 150.9 (sp^2 C), 150.6 (sp^2 C), 150.3 (sp^2 C), 144.3 (sp^2 C), 132.6 (q, *J* = 1.5 Hz, sp^2 C), 131.6 (naphthyridine 5-CH), 122.1 (d, *J* = 278 Hz, CF_3), 121.7 (sp^2 C), 120.1 (benzothiazole 5-CH), 119.7 (sp^2 C), 117.7 (q, *J* = 279 Hz, CF_3), 117.7 (sp^2 C), 115.1 (benzothiazole 7-CH), 104.3 (naphthyridine 3-CH); ^{19}F NMR (376 MHz, methanol-d_4) δ −59.8 (s, CF_3), −69.7 (s, CF_3); ν_{max}/cm^{-1} (solid); 1660, 1575, 1528, 1436, 1251 and 1216; m/z (ES) found MH$^+$ 465.000255, $C_{17}H_7{}^{35}ClF_6N4OS$ requires MH$^+$ 465.000605; HPLC 2.41 min., 95%.

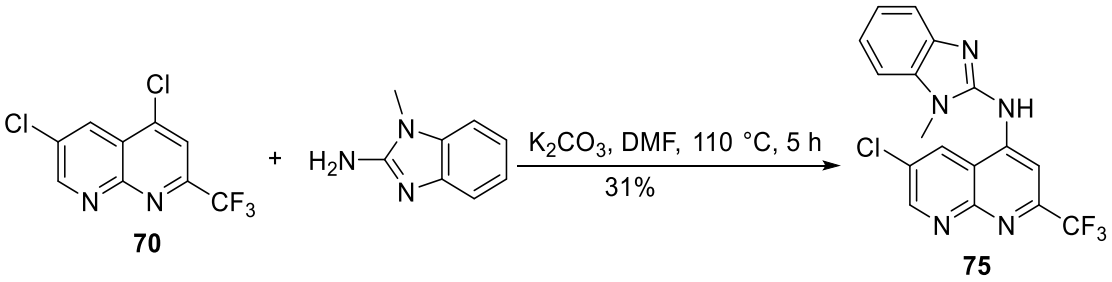
6-Bromo-N-(6-chloro-2-(trifluoromethyl)-1,8-naphthyridin-4-yl)benzo[d]thiazol-2-amine (**74**)



70 (0.138 g, 0.515 mmol), 2-amino-6-bromobenzothiazole (0.117 g, 0.509 mmol) and potassium carbonate (0.154 g, 1.112 mmol) were dissolved in anhydrous DMF (3 mL) under an atmosphere of nitrogen. The reaction mixture was stirred at 110 °C for 4 h then allowed to cool to room temperature. Water (10 mL) was added and an orange precipitate formed which was isolated using vacuum filtration. The precipitate was purified using column chromatography; elution with DCM-methanol (50:1–1:1). The solvent was removed *in vacuo* to leave the title compound as a yellow solid (93.0 mg, 0.202 mmol, 40%). An analytical sample was recrystallised from methanol with 59% recovery, m. p. > 270 °C (from methanol); Rf 0.64 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.47 (br s, 1H, N-H), 9.30 (br s, 2H, naphthyridine 5-H, 7-H), 9.21 (d, *J* = 2.0 Hz, 1H, benzothiazole 7-H), 8.25 (s, 1H, naphthyridine 3-H), 7.75 (d, *J* = 8.3 Hz, 1H, benzothiazole 4-H), 7.59 (dd, *J* = 8.3, 2.0 Hz, 1H, benzothiazole 5-H); ^{13}C NMR (101 MHz, $CDCl_3$/TFA) δ 167.8 (sp^2 C), 152.6 (sp^2 C), 152.2 (naphthyridine 7-CH), 150.8 (q, *J* = 38.8 Hz), 146.1 (sp^2 C), 139.7 (naphthyridine 5-CH), 135.8 (sp^2 C), 133.4 (sp^2 C), 133.3 (benzothiazole 4-CH), 125.2 (benzothiazole 7-CH), 125.2 (sp^2 C), 120.9 (sp^2 C), 120.6 (sp^2 C), 116.8 (benzothiazole 5-CH), 114.7 (q, *J* = 284 Hz, CF_3), 107.8 (q, *J* = 2.1 Hz, naphthyridine 3-CH); ^{19}F NMR (282 MHz, DMSO-d_6) δ −67.0 (s, CF_3); ν_{max}/cm^{-1} (solid); 1592, 1568, 1555,

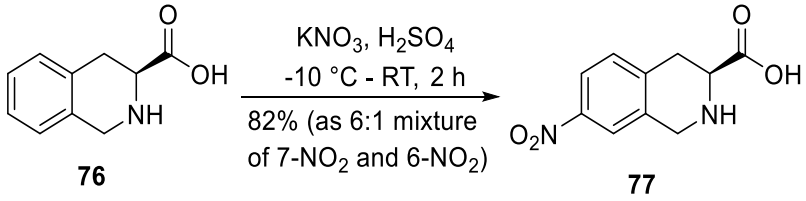
1519, 1446, 1410, 1366, 1268, 1250 and 1232; m/z (ES) found MH^+ 458.927843, $C_{16}H_7{}^{79}Br^{35}ClF_3N_4S$ requires MH^+ 458.928818; HPLC 2.37 min., 100%.

6-Chloro-N-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-(trifluoromethyl)-1,8-naphthyridin-4-amine (**75**)



70 (0.176 g, 0.659 mmol), 2-amino-1-methylbenzimidazole (94.9 mg, 0.645 mmol) and potassium carbonate (0.243 g, 1.762 mmol) were dissolved in anhydrous DMF (3 mL) under an atmosphere of nitrogen. The reaction mixture was stirred at 110 °C for 5 h then allowed to cool to room temperature. Water (10 mL) was added and an orange precipitate formed which was isolated by vacuum filtration. The precipitate was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:1). The solvent was removed *in vacuo* to leave the title compound as a yellow solid (75.9 mg, 0.201 mmol, 31%). An analytical sample was recrystallised from ethyl acetate-pet. ether 40–60 with 33% recovery, m. p. > 270 °C (from methanol); Rf 0.48 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.14 (br s, 1H, N-H), 9.06 (s, 1H, naphthyridine 7-H), 8.94 (s, 1H, naphthyridine 5-H), 7.53 (s, 1H, naphthyridine 3-H), 7.43 (d, *J* = 6.7 Hz, 1H, benzimidazole 4-H), 7.29 – 7.15 (m, 3H, benzimidazole 5-H, 6-H, 7-H), 3.69 (s, 3H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.5 (sp^2 C), 155.1 (sp^2 C), 153.5 (naphthyridine 7-CH), 150.7 (sp^2 C), 150.5 (sp^2 C), 150.2 (sp^2 C), 132.6 (naphthyridine 5-CH), 129.9 (sp^2 C), 128.2 (sp^2 C), 122.8 (benzimidazole CH), 122.7 (benzimidazole CH), 122.3 (q, *J* = 276 Hz, CF_3), 121.5 (sp^2 C), 110.9 (benzimidazole CH), 109.4 (benzimidazole 4-CH), 104.3 (naphthyridine 3-CH), 29.0 (CH_3); ^{19}F NMR (376 MHz, DMSO-d_6) δ –66.7 (s, CF_3); ν_{max}/cm^{-1} (solid); 2920, 2851, 1628, 1607, 1562, 1530, 1493, 1482, 1448, 1430, 1388, 1356, 1325, 1304, 1279, 1265 and 1222; m/z (ES) found MH^+ 378.072658, $C_{17}H_{11}{}^{35}ClF_3N_5$ requires MH^+ 378.072784; HPLC 2.64 min., 100%.

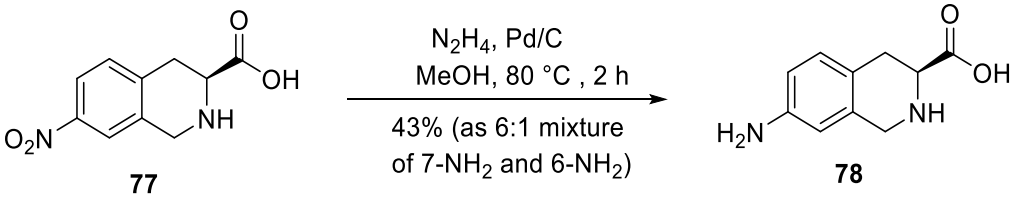
(S)-7-Nitro-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**77**)



76 **77**

(S)-1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (1.252 g, 7.065 mmol) was dissolved in sulfuric acid (98%, 30 mL) at –10 °C. A solution of potassium nitrate (0.754 g, 7.462 mmol) in sulphuric acid (98%, 25 mL) was added dropwise while maintaining the temperature at –10 °C. The mixture was stirred at –10 °C for 10 minutes then allowed to warm to room temperature and stirred for a further 2 h. The mixture was poured onto ice and neutralised with concentrated aqueous ammonia solution, causing a white precipitate to form. The mixture was diluted with water (40 mL) and filtered to obtain the product as a colourless solid (1.290 g, 5.805 mmol, 82% as a mixture of 7-nitro and 6-nitro isomers), m. p. 250–255 °C decomposed (from H_2O) (lit. decomposition from 245 °C[110]) ; ^{1}H NMR (400 MHz, D_2O) δ 8.13 (m, 2H, 6-H, 8-H), 7.49 (m, 1H, 5-H), 4.57 (m, 1H, sp^3 H), 4.52 (m, 1H, sp^3 H), 4.34 – 4.30 (m, 1H, sp^3 H), 3.53 (m, 1H, sp^3 H), 3.29 (m, 1H, sp^3 H) (consistent with lit.[111]); ν_{max}/cm^{-1} (solid); 2600–3000 (br), 1682, 1539, 1490, 1457, 1398, 1352, 1316 and 1276; m/z (ES) found MH^+ 223.070657, $C_{10}H_{10}N_2O_4$ requires MH^+ 223.071333; HPLC 0.92 min., 7%, 0.96 min., 93%.

(S)-7-Amino-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**78**)

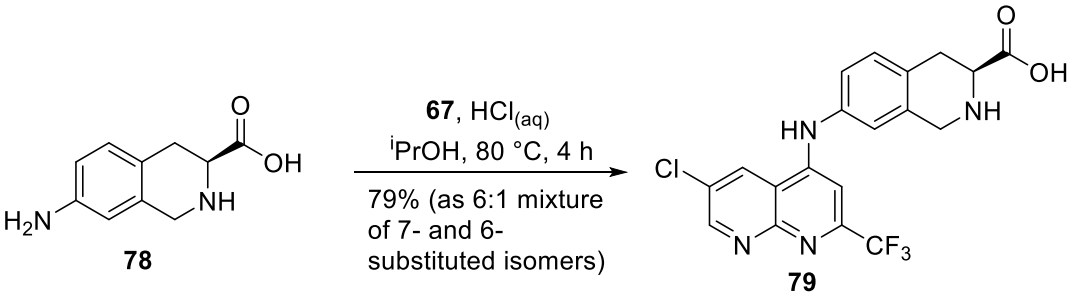


77 **78**

To a solution of **77** (0.234 g, 1.054 mmol) in methanol (30 mL) was added 10% palladium on charcoal (0.112 g) and hydrazine hydrate (0.6 mL). The solution was heated under reflux at 80 °C for 2 h then allowed to cool to room temperature. The mixture was filtered to remove the catalyst then the solvent was removed *in vacuo* to leave the product as an off-white solid (86.7 mg, 0.452 mmol, 43 % as a mixture of 7-amino and 6-amino isomers), m. p. 230–240 °C decomposed (from methanol) (lit. 220–221 °C[111]); ^{1}H NMR (400 MHz, D_2O) δ 7.07 (d, *J* = 8.3 Hz, 1H, 5-H), 6.76 (dd, *J* = 8.3, 2.3 Hz, 1H, 6-H), 6.63 (d, *J* = 2.3 Hz, 1H, 8-H), 4.27 (m, 2H, 1-H), 3.91 (dd, *J* = 11.2, 5.3 Hz, 1H,

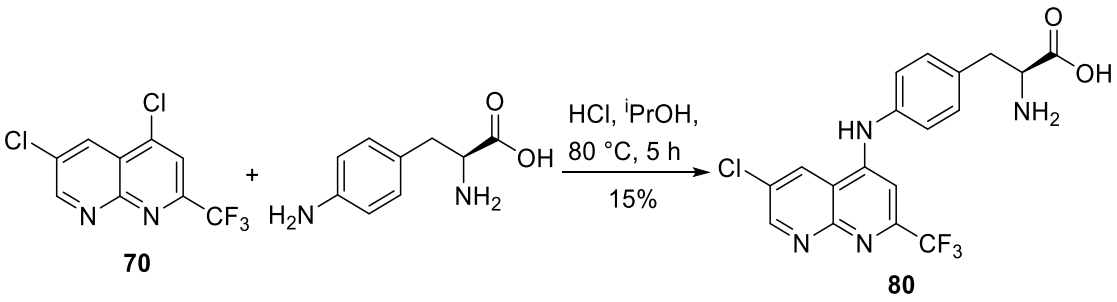
3-H), 3.22 (dd, *J* = 16.9, 5.3 Hz, 1H, 4-Ha), 2.97 (dd, *J* = 16.9, 11.2 Hz, 1H, 4-Hb) (consistent with lit.[110]); ^{13}C NMR (101 MHz, D_2O) δ 174.0 (C=O), 144.5 (aniline C), 129.8 (aniline 5-CH), 128.5 (aniline C), 122.4 (aniline C), 116.8 (aniline 6-CH), 113.7 (aniline 8-CH), 56.5 (3-CH), 44.1 (1-CH_2), 28.2 (4-CH_2); ν_{max}/cm^{-1} (solid); 3316, 3246, 2500–3000 (br), 1628, 1537, 1509, 1453, 1399, 1318, 1299 and 1254; m/z (ES) found MH^+ 193.096876, $C_{10}H_{10}N_2O_4$ requires MH^+ 193.097154; HPLC 1.81 min., 23%, 1.85 min., 70%.

(S)-7-((6-Chloro-2-(trifluoromethyl)-1,8-naphthyridin-4-yl)amino)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**79**)



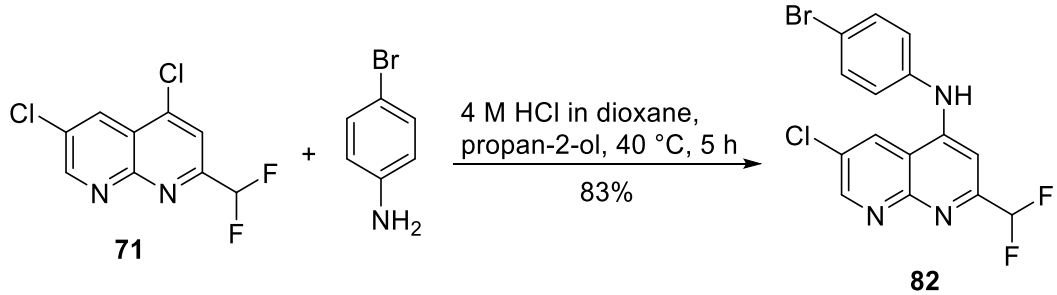
78 (0.178 g, 0.925 mmol) and **71** (0.261 g, 0.977 mmol) were dissolved in propan-2-ol (10 mL). 2 M aqueous HCl (1.0 mL, 2.0 mmol) was added and the mixture stirred under reflux at 80 °C for 4 h. The solvent was removed *in vacuo* to leave an orange solid which was washed with pet. ether 40–60 (50 mL) to remove unreacted **71**, giving the product as an orange/brown solid (0.310 g, 0.733 mmol, 79% as an inseparable 6:1 mixture of 7- and 6- substituted isomers), m. p. 250–255 °C decomposed (from methanol); Rf 0.08 (2:1 DCM-methanol); ^{1}H NMR (300 MHz, methanol-d_4) δ 8.95 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H), 8.86 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.39–7.20 (m, 3H, phenyl H), 7.01 (s, 1H, naphthyridine 3-H), 4.35 (s, 2H, 1-H), 3.87 (dd, *J* = 10.7, 4.6 Hz, 1H, 4-Ha), 3.38 (dd, *J* = 17.3, 4.6 Hz, 1H, 4-Hb), 3.17–3.06 (m, 1H, 3-H); ^{13}C NMR (75 MHz, methanol-d_4) δ 170.6 (C=O), 158.0 (sp^2 C), 156.2 (naphthyridine 7-CH), 149.9 (q, *J* = 34.4 Hz), 148.9 (sp^2 C), 140.6 (sp^2 C), 136.8 (sp^2 C), 135.2 (naphthyridine 5-CH), 132.5 (sp^2 C), 132.2 (phenyl CH), 131.4 (sp^2 C), 125.9 (phenyl CH), 124.5 (phenyl CH), 121.1 (q, *J* = 269 Hz), 115.8 (sp^2 C), 100.2 (naphthyridine 3-CH), 55.1 (3-CH), 45.3 (1-CH_2), 29.3 (4-CH_2), other peaks not resolved; ^{19}F NMR (282 MHz, methanol-d_4) δ −68.8 (s, CF_3); ν_{max}/cm^{-1} (solid); 3378, 2400–3000 (br), 1740, 1649, 1593, 1564, 1506, 1407, 1387, 1272 and 1207; m/z (ES) found MH^+ 423.082791, $C_{19}H_{14}{}^{35}ClF_3N_4O_2$ requires MH^+ 423.083014; HPLC 1.81 min., 13%, 1.84 min., 80%.

(S)-2-Amino-3-(4-((6-chloro-2-(trifluoromethyl)-1,8-naphthyridin-4-yl)amino)phenyl)propanoic acid (**80**)



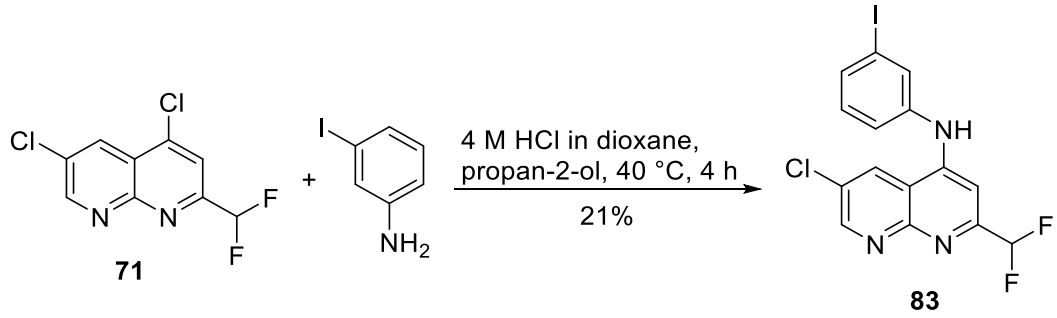
4-Amino-L-phenylalanine (0.102 g, 0.568 mmol) and **70** (0.148 g, 0.552 mmol) were dissolved in propan-2-ol (4 mL). 2 M aqueous HCl (0.60 mL, 1.2 mmol) was added and the mixture stirred under reflux at 80 °C for 5 h. The solvent was removed *in vacuo* to leave an orange solid which was washed with pet. ether 40–60 (50 mL) then DCM (30 mL) and recrystallised from acetonitrile-water to give the product as a yellow solid (34.0 mg, 0.083 mmol, 15%, m. p. 240–250 °C decomposed (from acetonitrile-water); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.94 (d, *J* = 2.6 Hz, 1H, naphthyridine 7-H), 8.87 (d, *J* = 2.6 Hz, 1H, naphthyridine 5-H), 7.36 (d, *J* = 8.2 Hz, 2H, phenyl 2-H), 7.31 (d, *J* = 8.2 Hz, 2H, phenyl 3-H), 7.13 (s, 1H, naphthyridine 3-H), 3.91 (dd, *J* = 7.5, 4.9 Hz, 1H, 2-H), 3.26 (dd, *J* = 14.7, 4.9 Hz, 1H, 3-Ha), 3.05 (dd, *J* = 14.7, 7.5 Hz, 1H, 3-Hb); ^{13}C NMR (101 MHz, methanol-d_4) δ 171.6 (C=O), 154.1 (sp^2 C), 153.3 (naphthyridine 7-CH), 147.8 (sp^2 C), 138.0 (sp^2 C), 132.1 (sp^2 C), 130.7 (phenyl 2-CH), 130.6 (naphthyridine 5-CH), 128.9 (sp^2 C), 124.0 (phenyl 3-CH), 101.1 (sp^2 C), 97.4 (naphthyridine 3-CH), 56.9 (2-CH), 36.0 (3-CH_2), other peaks not resolved; ^{19}F NMR (376 MHz, methanol-d_4) δ −70.0 (s, CF_3); ν_{max}/cm^{-1} (solid); 2700–3300 (br), 1591, 1562, 1512, 1445, 1411, 1385, 1360, 1318, 1284 and 1232; m/z (ES) found MH$^+$ 411.083603, $C_{18}H_{14}{}^{35}ClF_3N_4O_2$ requires MH$^+$ 411.083014; HPLC 1.81 min., 100%.

N-(4-Bromophenyl)-6-chloro-2-(difluoromethyl)-1,8-naphthyridin-4-amine (**82**)



4-Bromoaniline (0.237 g, 1.377 mmol) and **71** (0.359 g, 1.440 mmol) were dissolved in propan-2-ol (5 mL). 4 M HCl in 1,4-dioxane (0.40 mL, 1.6 mmol) was added and the mixture was stirred at 40 °C for 5 h. A yellow precipitate formed which was isolated using vacuum filtration to give the title compound as a yellow solid (0.441 g, 1.148 mmol, 83%), m. p. 180–185 °C decomposed (from propan-2-ol); Rf 0.39 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.25 (br s, 1H, N-H), 9.26 (d, *J* = 2.4 Hz, 1H, naphthyridine 5-H), 9.12 (d, *J* = 2.4 Hz, 1H, naphthyridine 7-H), 7.70 (d, *J* = 8.3 Hz, 2H, aniline 3-H), 7.40 (d, *J* = 8.3 Hz, 2H, aniline 2-H), 7.11 (s, 1H, naphthyridine 3-H), 7.00 (t, *J* = 54.5 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.1 (naphthyridine 7-CH), 153.6 (t, *J* = 24.8 Hz, naphthyridine 2-C), 152.8 (sp^2 C), 152.5 (sp^2 C), 138.2 (sp^2 C), 133.2 (aniline 3-CH), 132.5 (naphthyridine 5-CH), 128.4 (sp^2 C), 126.3 (aniline 2-CH), 118.6 (sp^2 C), 115.1 (sp^2 C), 113.1 (t, *J* = 239 Hz, CHF_2), 98.6 (t, *J* = 4.2 Hz, naphthyridine 3-CH); ^{19}F NMR (282 MHz, DMSO-d_6) δ −116.58 (d, *J* = 54.3 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2572, 1629, 1610, 1578, 1555, 1481, 1449, 1414, 1404, 1352, 1260 and 1225; m/z (ES) found MH^+ 385.967824, $C_{15}H_9{}^{81}Br^{35}ClF_2N_3$ requires MH^+ 385.968873; HPLC 2.88 min., 100%.

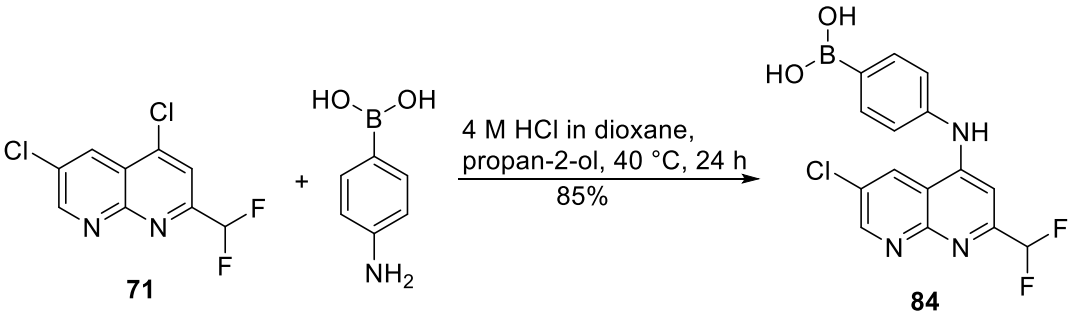
6-Chloro-2-(difluoromethyl)-N-(3-iodophenyl)-1,8-naphthyridin-4-amine (**83**)



3-Iodoaniline (0.10 mL, 0.83 mmol) and **71** (0.198 g, 0.793 mmol) were dissolved in propan-2-ol (5 mL). 4 M HCl in 1,4-dioxane (0.22 mL, 0.88 mmol) was added and the mixture was stirred at 40 °C for 4 h. A yellow precipitate formed which was isolated using vacuum filtration then purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:5–1:1). The title compound was obtained as an orange solid (70.2 mg, 0.163 mmol, 21%), m. p. 207–209 °C (from ethyl acetate-pet. ether 40–60); Rf 0.52 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.96 (s, 1H, naphthyridine 7-H), 8.86 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.76 (t, *J* = 2.0 Hz, 1H, aniline 2-H), 7.62 (d, *J* = 7.7 Hz, 1H, aniline 6-H), 7.43 (ddd, *J* = 8.0, 2.0, 1.0 Hz, 1H, aniline 4-H), 7.24 (t, *J* = 8.0 Hz, 1H, aniline 5-H), 7.18 (s, 1H, naphthyridine 3-H), 6.67 (t, *J* = 55.2 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz,

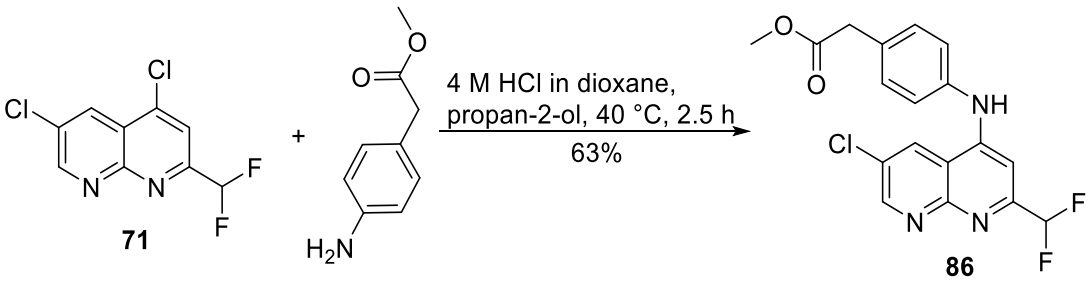
methanol-d_4) δ 156.3 (t, *J* = 25.6 Hz, naphthyridine 2-C), 154.1 (sp^2 C), 152.8 (naphthyridine 7-CH), 151.2 (sp^2 C), 140.5 (sp^2 C), 134.5 (aniline 6-CH), 132.1 (aniline 2-CH), 131.0 (aniline 5-CH), 130.6 (naphthyridine 5-CH), 128.5 (sp^2 C), 122.4 (aniline 4-CH), 115.1 (sp^2 C), 113.7 (t, *J* = 241 Hz, CHF_2), 97.7 (naphthyridine 3-CH), 93.9 (sp^2 C); ^{19}F NMR (376 MHz, methanol-d_4) δ −118.2 (d, *J* = 55.3 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3062, 1599, 1575, 1555, 1472, 1446, 1418, 1388, 1360, 1313, 1242 and 1215; m/z (ES) found MH^+ 431.956507, $C_{15}H_9{}^{35}ClF_2IN_3$ requires MH^+ 431.957056; HPLC 3.06 min., 100%.

(4-((6-Chloro-2-(difluoromethyl)-1,8-naphthyridin-4-yl)amino)phenyl)boronic acid (**84**)



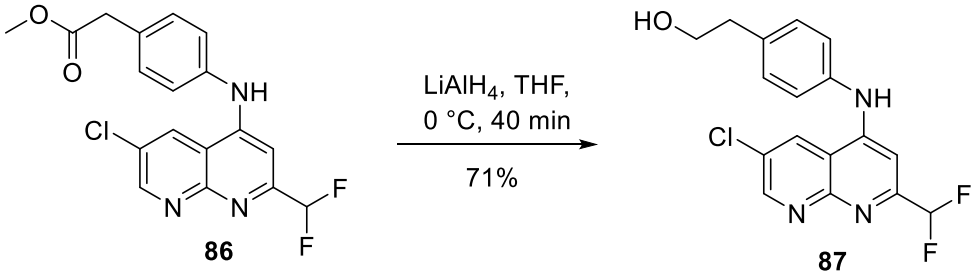
4-Aminobenzene boronic acid hydrochloride (89.4 mg, 0.516 mmol) and **71** (0.132 g, 0.529 mmol) were dissolved in propan-2-ol (3 mL). 4 M HCl in 1,4-dioxane (0.14 mL, 0.56 mmol) was added and the mixture was stirred at 40 °C for 24 h. A yellow precipitate formed which was isolated using vacuum filtration and the title compound was obtained as a yellow solid (0.153 g, 0.437 mmol, 85%), m. p. 180–183 °C decomposed (from propan-2-ol); Rf 0.49 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 9.12 (d, *J* = 2.2 Hz, 1H, naphthyridine 5-H), 9.07 (d, *J* = 2.2 Hz, 1H, naphthyridine 7-H), 7.54 (d, *J* = 7.5 Hz, 2H, aniline 2-H), 7.44 (d, *J* = 7.5 Hz, 2H, aniline 3-H), 7.00 (s, 1H), 6.95 (t, *J* = 53.3 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.6 (naphthyridine 7-CH), 154.3 (sp^2 C), 151.9 (t, *J* = 25.3 Hz, naphthyridine 2-C) 151.1 (sp^2 C), 138.2 (sp^2 C), 133.1 (naphthyridine 5-CH), 130.4 (aniline 2-CH), 128.7 (sp^2 C), 127.3 (sp^2 C), 124.8 (aniline 3-CH), 114.7 (sp^2 C), 112.4 (t, *J* = 241 Hz, CHF_2), 98.3 (t, *J* = 4.0 Hz, naphthyridine 3-CH); ^{19}F NMR (282 MHz, methanol-d_4) δ −121.3 (d, *J* = 53.2 Hz); ν_{max}/cm^{-1} (solid); 3046, 2493 (br), 1609, 1591, 1560, 1489, 1466, 1441, 1406, 1342, 1318, 1256 and 1226; m/z (ES) found MH^+ 350.067524, $C_{15}H_{11}B^{35}ClF_2N_3O_2$ requires MH^+ 350.067368; HPLC 1.27 min., 100%.

Methyl 2-(4-((6-chloro-2-(difluoromethyl)-1,8-naphthyridin-4-yl)amino)phenyl)acetate (**86**)



Methyl-(4-aminophenyl)acetate (0.110 g, 0.666 mmol) and **71** (0.189 g, 0.760 mmol) were dissolved in propan-2-ol (5 mL). 4 M HCl in 1,4-dioxane (0.20 mL, 0.80 mmol) was added and the mixture heated at 40 °C for 2.5 h. A yellow precipitate formed which was isolated using vacuum filtration and the title compound was obtained as a yellow solid (0.158 g, 0.417 mmol, 63%), m. p. 158–160 °C (from propan-2-ol); Rf 0.60 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 9.10 (d, *J* = 2.3 Hz, 1H, naphthyridine 5-H), 9.06 (d, *J* = 2.3 Hz, 1H, naphthyridine 7-H), 7.46 (d, *J* = 8.5 Hz, 2H, aniline 2-H), 7.39 (d, *J* = 8.5 Hz, 2H, aniline 3-H), 7.04 (s, 1H, naphthyridine 3-H), 6.94 (t, *J* = 53.2 Hz, 1H, CHF_2), 3.71 (s, 2H, CH_2), 3.64 (s, 3H, CH_3); ^{13}C NMR (75 MHz, methanol-d_4) δ 173.4 (C=O), 159.9 (sp^2 C), 158.6 (sp^2 C), 157.1 (7-CH), 148.4 (sp^2 C), 136.7 (sp^2 C), 136.3 (sp^2 C), 133.3 (naphthyridine 5-CH), 132.5 (aniline 2-CH), 131.9 (sp^2 C), 126.3 (aniline 3-CH), 114.8 (sp^2 C), 111.5 (t, *J* = 243 Hz, CHF_2), 100.1 (t, *J* = 5.5 Hz, naphthyridine 3-CH), 52.5 (CH_3), 41.0 (CH_2); ^{19}F NMR (282 MHz, methanol-d_4) δ −121.1 (d, *J* = 53.2 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2674, 1752, 1734, 1601, 1587, 1552, 1529, 1510, 1452, 1420, 1400, 1360, 1348, 1324, 1257 and 1222; m/z (ES) found MH^+ 378.081253, $C_{18}H_{14}{}^{35}ClF_2N_3O_2$ requires MH^+ 378.081537; HPLC 2.28 min., 87%.

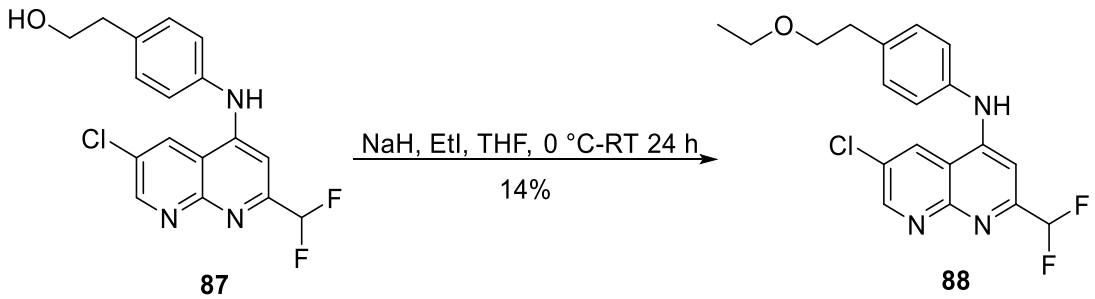
2-(4-{[6-chloro-2-(difluoromethyl)-1,8-naphthyridin-4-yl]amino}phenyl)ethan-1-ol (**87**)



To **86** (0.141 g, 0.373 mmol) in dry THF (2 mL) was added dropwise a suspension of $LiAlH_4$ in dry THF (1 M, 0.5 mL, 0.5 mmol) at 0 °C under an

atmosphere of nitrogen. The mixture was stirred at 0 °C for 40 minutes then slowly quenched with water (1 mL) and diluted with DCM (30 mL). The solution was filtered through celite, washed with saturated aqueous sodium carbonate solution (20 mL), layers were separated and the aqueous layer was extracted with DCM (20 mL). The combined organic layers were washed with water (20 mL), dried over sodium sulphate and evaporated *in vacuo* to leave the title compound as an orange solid (92.8 mg, 0.265 mmol, 71%); m. p. 143–146 °C (from DCM); Rf 0.32 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.88 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H), 8.83 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.29 (d, *J* = 8.3 Hz, 2H, aniline 2-H), 7.22 (d, *J* = 8.3 Hz, 3H, aniline 2-H), 6.99 (s, 1H, naphthyridine 3-H), 6.53 (t, *J* = 55.2 Hz, 1H, CHF_2), 3.72 (t, *J* = 6.9 Hz, 2H, H_2C-O), 2.79 (t, *J* = 6.9 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 157.0 (t, *J* = 23.8 Hz, naphthyridine 2-C), 152.6 (naphthyridine 7-CH), 152.2 (sp^2 C), 150.0 (sp^2 C), 137.4 (sp^2 C), 136.7 (sp^2 C), 130.6 (naphthyridine 5-CH), 130.1 (aniline 2-CH), 128.1 (sp^2 C), 126.4 (sp^2 C), 123.9 (aniline 3-H), 113.9 (t, *J* = 241 Hz, CHF_2), 97.0 (t, *J* = 3.2 Hz, naphthyridine 3-CH), 62.6 (CH_2-O), 38.3 (CH_2); ^{19}F NMR (376 MHz, methanol-d_4) δ −118.1 (d, *J* = 55.2 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3255 (br), 2922, 2852, 1637, 1585, 1559, 1523, 1511, 1465, 1443, 1417, 1383, 1366, 1315, 1246 and 1212; m/z (ES) found MH^+ 350.086279, $C_{17}H_{14}{}^{35}ClF_2N_3O$ requires MH^+ 350.086623; HPLC 1.74 min., 100%.

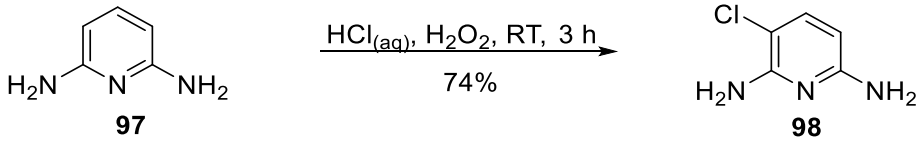
6-chloro-2-(difluoromethyl)-N-[4-(2-ethoxyethyl)phenyl]-1,8-naphthyridin-4-amine (**88**)



87 (40.9 mg, 0.117 mmol) and sodium hydride (60% in mineral oil, 23.4 mg, 0.585 mmol) were dissolved in dry THF (2 mL) under an atmosphere of nitrogen. Iodoethane (10 µL, 0.12 mmol) was added dropwise at 0 °C then the mixture was allowed to warm to room temperature and stirred for 24 h. Saturated aqueous ammonium chloride (5 mL) was added and the solution was extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with water (20 mL), dried over sodium sulphate and evaporated *in vacuo*. The product was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (33–100% ethyl acetate) and the solvent

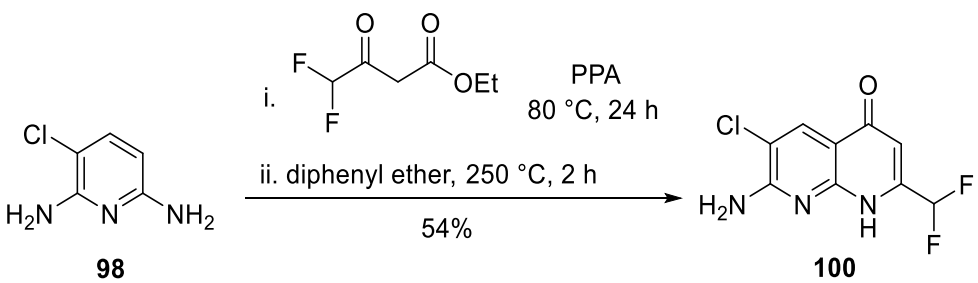
removed *in vacuo* to leave the title compound as an orange solid (6.2 mg, 0.016 mmol, 14%); Rf 0.23 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 8.90 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H), 8.85 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.31 (d, *J* = 8.4 Hz, 2H, aniline 3-H), 7.23 (d, *J* = 8.4 Hz, 2H, aniline 2-H), 7.00 (s, 1H, naphthyridine 3-H), 6.55 (t, *J* = 55.2 Hz, 1H, CHF_2), 3.63 (t, *J* = 6.8 Hz, 2H, H_2C-O), 3.45 (q, *J* = 7.0 Hz, 2H, H_2C-CH_3), 2.84 (t, *J* = 6.8 Hz, 2H, H_2C-phenyl), 1.10 (t, *J* = 7.0 Hz, 3H, CH_3); ^{13}C NMR (101 MHz, methanol-d_4) δ 152.6 (sp^2 C), 152.6 (naphthyridine 7-CH), 152.2 (sp^2 C), 137.4 (sp^2 C), 136.9 (sp^2 C), 130.6 (naphthyridine 5-CH), 130.0 (aniline 3-CH), 128.2 (sp^2 C), 123.8 (aniline 2-CH), 114.8 (sp^2 C), 113.9 (t, *J* = 241 Hz, CHF_2), 100.0 (sp^2 C), 97.0 (t, *J* = 3.3 Hz, naphthyridine 3-CH), 71.0 (CH_2-O), 65.9 (CH_2-CH_3), 35.3 (CH_2-phenyl), 14.0 (CH_3); ^{19}F NMR (282 MHz, methanol-d_4) δ −118.3 (d, *J* = 55.2 Hz, CHF_2).

3-Chloropyridine-2,6-diamine (**98**)



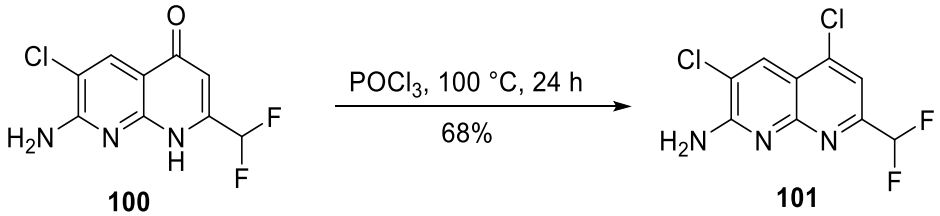
To a stirred mixture of 2,6-diaminopyridine (5.482 g, 50.234 mmol) in concentrated aqueous hydrochloric acid (40 mL) was added dropwise 30% aqueous hydrogen peroxide solution (5 mL, 49.8 mmol). The reaction was stirred for 3 h at room temperature then cooled to 0 °C and neutralised with 50% aqueous sodium hydroxide. The resultant precipitate was isolated using vacuum filtration to give the title compound as a black solid (5.319 g, 37.033 mmol, 74%); m. p. >270 °C (from water); Rf 0.27 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.11 (d, *J* = 8.3 Hz, 1H, 4-H), 5.69 (d, *J* = 8.3 Hz, 1H, 5-H), 5.58 (br s, 4H, NH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.0 (sp^2 C), 154.3 (sp^2 C), 138.1 (4-CH), 99.4 (sp^2 C), 97.7 (5-CH); ν_{max}/cm^{-1} (solid); 3410, 3300, 3169, 1621, 1586, 1567, 1455, 1437, 1352 and 1292; m/z (ES) found MH^+ 144.031719, $C_5H_6{}^{35}ClN_3$ requires MH^+ 144.032301; HPLC 0.89 min., 91%.

7-Amino-6-chloro-2-(difluoromethyl)-1,8-naphthyridin-4(1H)-one (**100**)



A mixture of **98** (2.023 g, 14.087 mmol), ethyl difluoroacetate (1.84 mL, 14.1 mmol) and polyphosphoric acid (20 mL) was stirred for 24 h at 80 °C then allowed to cool and ammonium hydroxide was added until the pH increased to 9. The resultant precipitate was isolated by vacuum filtration to give 6-amino-7-chloro-2-(difluoromethyl)-4H-pyrido[1,2-a]pyrimidin-4-one (**99)** as a brown solid. The crude product was dissolved in diphenyl ether (25 mL) and heated at 250 °C under reflux for 2 h then allowed to cool to room temperature. The resultant precipitate was isolated by vacuum filtration and washed with pet. ether 40–60 to give the title compound as a brown solid (1.883 g, 7.666 mmol, 54%) m. p. 250–5 °C (from diphenyl ether); Rf 0.33 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 8.13 (s, 1H, 5-H), 6.67 (t, *J* = 54.0 Hz, 1H, CHF_2), 6.27 (s, 1H, 3-H); ^{13}C NMR (75 MHz, methanol-d_4) δ 134.3 (5-CH), 111.4 (3-CH), other peaks not resolved; ^{19}F NMR (282 MHz, methanol-d_4) δ −120.7 (d, *J* = 53.8 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3526, 3415, 3384, 3152, 2662, 1614, 1523, 1453, 1423, 1369, 1350, 1276 and 1225; m/z (ES) found MH^+ 246.023423, $C_9H_7{}^{35}ClF_2N_3O$ requires MH^+ 246.024022; HPLC 1.29 min., 100%.

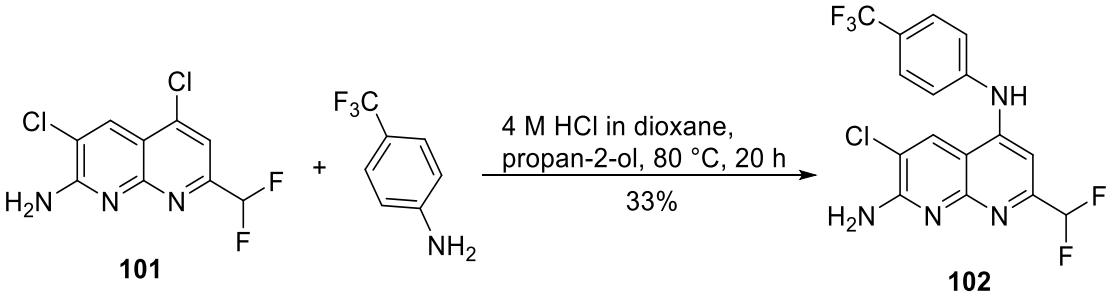
3,5-Dichloro-7-(difluoromethyl)-1,8-naphthyridin-2-amine (**101**)



A mixture of **100** (0.863 g, 3.515 mmol) and phosphoryl chloride (8 mL) was stirred for 24 h at 100 °C then allowed to cool to room temperature. The solution was poured onto ice and neutralised with saturated aqueous sodium hydrogen carbonate. The resultant precipitate was isolated using vacuum filtration to give the title compound as a brown solid and the filtrate extracted with DCM (3 × 100 mL). The combined organic layers were washed with brine (100 mL) and water (100 mL), dried over magnesium sulphate and evaporated to yield further product as a yellow solid (0.630 g, 2.386 mmol, 68%) m. p. 220–2 °C (from ethyl acetate-pet. ether 40–60); Rf 0.53 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.35 (s, 1H, 5-H), 7.52 (s, 1H, 3-H), 6.65 (t, *J* = 55.0 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 157.9 (sp^2 C), 155.0 (t, *J* = 13.9 Hz, 2-C), 154.8 (sp^2 C), 143.1 (sp^2 C), 132.0 (7-CH), 121.0 (sp^2 C), 116.9 (sp^2 C) , 114.0 (t, *J* = 3.2 Hz, 3-CH), 113.4 (t, *J* = 241 Hz, CHF_2); ^{19}F NMR (376 MHz, methanol-d_4) δ −117.9 (d, *J* = 54.8 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3483, 3108, 2568, 2316, 1638, 1608, 1584, 1545,

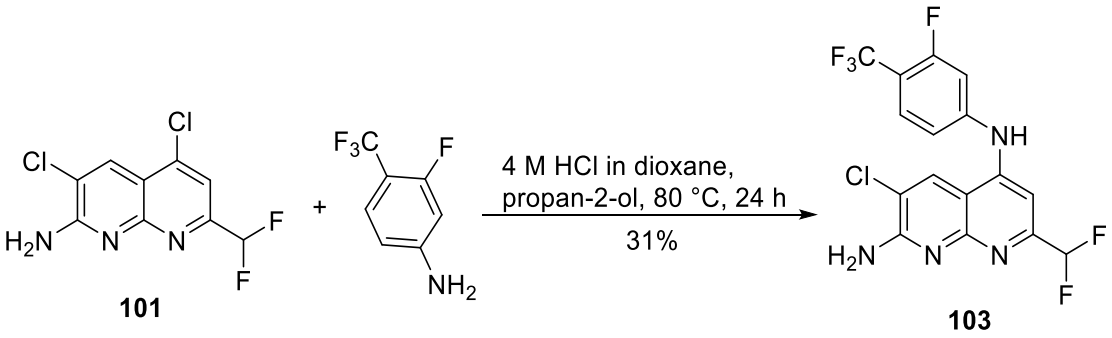
1509, 1469, 1422, 1352, 1315, 1298 and 1279; m/z (ES) found MH^+ 263.989795, $C_9H_6{}^{35}Cl_2F_2N_3$ requires MH^+ 263.990135; HPLC 2.27 min., 93%.

3-Chloro-7-(difluoromethyl)-N^5-(4-(trifluoromethyl)phenyl)-1,8-naphthyridine-2,5-diamine (**102**)



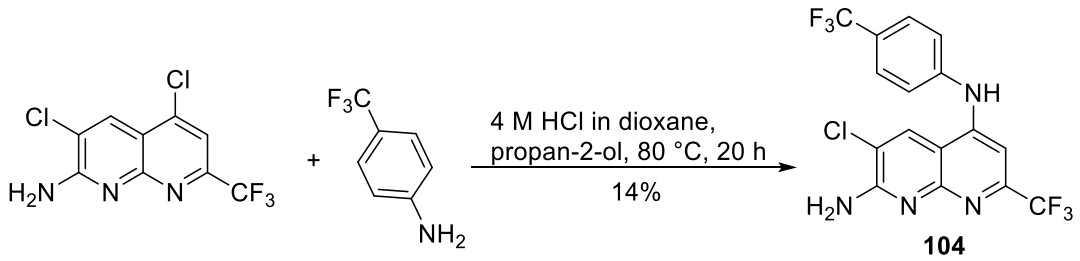
101 (53.3 mg, 0.202 mmol) and 4-(trifluoromethyl)aniline (23 µL, 0.18 mmol) were dissolved in propan-2-ol (2 mL). 4 M HCl in 1,4-dioxane (50 µL, 0.20 mmol) was added and the mixture was stirred for 20 h at 80 °C under reflux then allowed to cool to room temperature. The solvent was evaporated *in vacuo* and the resultant brown residue neutralised with saturated aqueous sodium hydrogen carbonate (40 mL) and extracted with ethyl acetate (2 × 40 mL). The combined organic layers were washed with water (40 mL) and dried over magnesium sulphate. The solvent was removed *in vacuo* to leave a brown solid which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (33%–100% ethyl acetate). The solvent was removed *in vacuo* to give the title compound as a yellow solid (23.5 mg, 0.0605 mmol, 33%) m. p. >270 °C (from ethyl acetate); Rf 0.53 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.47 (s, 1H, naphthyridine 4-H), 7.61 (d, *J* = 8.4 Hz, 2H, aniline 3-H), 7.39 (d, *J* = 8.4 Hz, 2H, aniline 2-H), 7.05 (s, 1H, naphthyridine 6-H), 6.50 (t, *J* = 55.4 Hz, 1H, CHF_2); ^{13}C NMR (75 MHz, methanol-d_4) δ 158.4 (sp^2 C), 156.7 (sp^2 C), 156.4 (t, *J* = 24.5 Hz, naphthyridine 7-C), 150.8 (sp^2 C), 145.1 (sp^2 C), 132.2 (naphthyridine 4-CH), 127.8 (q, *J* = 3.9 Hz, aniline 3-CH), 126.7 (q, *J* = 32.8 Hz), 125.6 (q, *J* = 271 Hz, CF_3), 122.5 (aniline 2-CH), 118.4 (sp^2 C), 115.2 (t, J = 241 Hz, CHF_2), 110.3 (sp^2 C), 98.2 (t, *J* = 3.5 Hz, naphthyridine 6-CH); ν_{max}/cm^{-1} (solid); ^{19}F NMR (376 MHz, methanol-d_4) δ −63.6 (s, CF_3), −118.0 (d, *J* = 55.6 Hz, CHF_2); 3488, 3292, 3179, 2923, 2854, 1603, 1558, 1520, 1492, 1463, 1432, 1400, 1376, 1322 and 1279; m/z (ES) found MH^+ 389.059638, $C_{16}H_{10}{}^{35}ClF_5N_4$ requires MH^+ 389.058692; HPLC 2.39 min., 100%.

3-Chloro-7-(difluoromethyl)-N^5-(3-fluoro-4-(trifluoromethyl)phenyl)-1,8-naphthyridine-2,5-diamine (**103**)



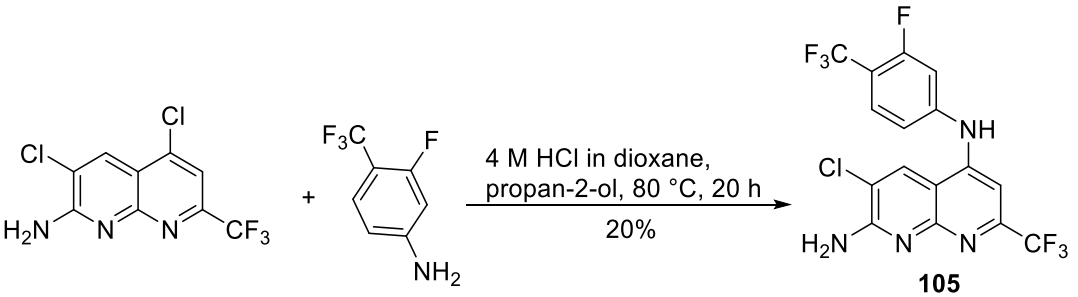
101 (0.109 g, 0.413 mmol) and 4-amino-2-fluorobenzotrifluoride (71.4 mg, 0.413 mmol) were dissolved in propan-2-ol (2 mL). 4 M HCl in 1,4-dioxane (0.11 mL, 0.44 mmol) was added and the mixture was stirred for 24 h at 80 °C under reflux then allowed to cool to room temperature. The solvent was evaporated *in vacuo* and the resultant brown residue neutralised with saturated aqueous sodium hydrogen carbonate (30 mL) and extracted with ethyl acetate (2 × 30 mL). The combined organic layers were washed with brine (20 mL) and water (30 mL) and dried over magnesium sulphate. The solvent was removed *in vacuo* to leave a brown solid which was purified using column chromatography; elution with DCM-methanol (50:1–1:1). The solvent was removed *in vacuo* to give the title compound as an orange solid (49.5 mg, 0.122 mmol, 31%) m. p. >270 °C (from methanol); Rf 0.43 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d$_4$) δ 8.43 (s, 1H, naphthyridine 4-H), 7.58 (t, *J* = 8.3 Hz, 1H, aniline 5-H), 7.17 (s, 1H, naphthyridine 6-H), 7.18 (dd, *J* = 8.3, 2.1 Hz, 1H, aniline 6-H), 7.13 (dd, *J* = 12.4, 2.1 Hz, 1H, aniline 2-H), 6.54 (t, *J* = 55.4 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, methanol-d$_4$) δ 157.1 (sp^2 C), 156.7 (sp^2 C), 155.1 (t, *J* = 33.2 Hz, naphthyridine 2-C), 148.5 (sp^2 C), 147.8 (d, *J* = 264 Hz, C-F), 146.6 (sp^2 C), 130.9 (naphthyridine 4-CH), 128.0 (q, *J* = 4.3 Hz, aniline 5-CH), 124.0 (q, *J* = 269 Hz, CF_3), 121.0 (q, *J* = 37.1 Hz, aniline 4-C), 117.6 (sp^2 C), 115.3 (d, *J* = 3.4 Hz, aniline 6-CH), 113.8 (t, *J* = 240 Hz, CHF_2), 109.6 (sp^2 C), 107.8 (d, *J* = 23.9 Hz, aniline 2-CH), 98.3 (t, *J* = 3.2 Hz, naphthyridine 6-CH); ^{19}F NMR (376 MHz, methanol-d$_4$) δ −62.0 (d, *J* = 13.4 Hz, CF_3), −114.9 (dq, *J* = 23.9 Hz, 12.2 Hz, C-F), −118.0 (d, *J* = 55.6 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3489, 3299, 1622, 1590, 1556, 1515, 1489, 1468, 1424, 1393, 1373, 1344, 1319, 1292 and 1249; m/z (ES) found MH$^+$ 407.049298, $C_{16}H_9{}^{35}ClF_6N_4$ requires MH$^+$ 407.049270; HPLC 2.56 min., 92%.

3-Chloro-7-(trifluoromethyl)-N^5-[4-(trifluoromethyl)phenyl]-1,8-naphthyridine-2,5-diamine (**104**) (Synthesised by MChem student Kyle Orritt)



To a solution of 3,5-dichloro-7-(trifluoromethyl)-1,8-naphthyridin-2-amine (0.105 g, 0.372 mmol) and 4-(trifluoromethyl)aniline (46 µL, 0.36 mmol) in propan-2-ol (2 mL) was added dropwise 4 M HCl in 1,4-dioxane (90 µL, 0.36 mmol). The solution was stirred at 80 °C under reflux for 20 h. The resulting mixture was partitioned between ethyl acetate (30 mL) and saturated aqueous sodium hydrogen carbonate (20 mL). The organic phase was washed with brine (20 mL), dried over magnesium sulphate, and the solvent evaporated *in vacuo*. The resultant yellow solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 ˚C (1:1). The solvent was removed *in vacuo* and the title compound was collected as off-white needles (21.0 mg, 0.052 mmol, 14%); m. p. 260–265 °C decomposed (from ethyl acetate-pet. ether 40–60); Rf 0.22 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d$_4$) δ 8.50 (s, 1H, naphthyridine 4-H), 7.63 (d, *J* = 8.0 Hz, 2H, aniline 3-H), 7.41 (d, *J* = 8.0 Hz, 2H, aniline 2-H), 7.07 (s, 1H, naphthyridine 6-H); ^{13}C NMR (101 MHz, methanol-d$_4$) δ 157.4 (sp^2 C), 155.5 (sp^2 C), 149.9 (sp^2 C), 143.4 (sp^2 C), 130.8 (naphthyridine 4-CH), 126.5 (q, *J* = 3.7 Hz, aniline 3-CH), 121.6 (aniline 2-CH), 109.0 (sp^2 C), 100.0 (sp^2 C), 96.8 (q, *J* = 4.1 Hz, naphthyridine 6-CH), other peaks not resolved; ^{19}F NMR (376 MHz, methanol-*d*$_4$) δ −63.6 (s, CF$_3$), −70.0 (s, CF$_3$); ν_{max}/cm^{-1} (solid); 3498, 3304, 1624, 1600, 1587, 1559, 1521, 1485, 1467, 1427, 1409, 1399, 1377, 1322, 1285 and 1268; m/z (ES) found MH$^+$ 407.040963, $C_{16}H_9{}^{35}ClF_6N_4$ requires MH$^+$ 407.041992; HPLC 3.14 min.; 100%.

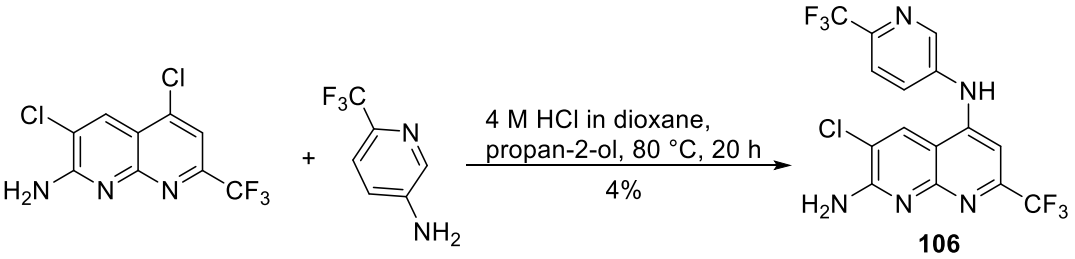
3-Chloro-N^5-[3-fluoro-4-(trifluoromethyl)phenyl]-7-(trifluoromethyl)-1,8-naphthyridine-2,5-diamine (**105**) (Synthesised by MChem student Kyle Orritt)



105

To a solution of 3,5-dichloro-7-(trifluoromethyl)-1,8-naphthyridin-2-amine (0.102 g, 0.362 mmol) and 4-amino-2-fluorobenzotrifluoride (70 mg, 0.391 mmol) in propan-2-ol (2 mL) was added dropwise 4M HCl in 1,4-dioxane (90 µL, 0.36 mmol). The solution was stirred at 80 °C under reflux for 20 h. The resulting mixture was partitioned between ethyl acetate (30 mL) and saturated aqueous sodium hydrogen carbonate (20 mL). The organic phase was washed with brine (20 mL), dried over magnesium sulphate, and the solvent evaporated *in vacuo*. The resultant yellow solid was purified using column chromatography; elution with DCM-methanol (50:1). The solvent was removed *in vacuo* and the title compound was collected as pale yellow needles (31.0 mg, 0.073 mmol, 20%); m. p. 245–249 ˚C decomposed (from DCM-methanol). Rf 0.16 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d$_4$) δ 8.51 (s, 1H, naphthyridine 4-H), 7.61 (dd, *J* = 14.2, 5.8 Hz, 1H, aniline 5-H), 7.20 (s, 1H, naphthyridine 6-H), 7.17 (m, 2H, aniline 2-H, 6-H); ^{13}C NMR (101 MHz, methanol-d4) δ 157.5 (sp^2 C), 153.6 (sp^2 C), 148.9 (sp^2 C), 147.4 (d, *J* = 265 Hz, C-F), 130.8 (naphthyridine 4-CH), 128.2 (q, *J* = 3.1 Hz, aniline 5-CH), 122.8 (sp^2 C), 118.3 (sp^2 C), 115.6 (d, *J* = 3.0 Hz, aniline 6-CH), 109.7 (sp^2 C), 108.2 (d, *J* = 24.2 Hz, aniline 2-CH), 98.2 (naphthyridine 6-CH), other peaks not resolved; ^{19}F NMR (376 MHz, methanol-d$_4$); δ −62.1 (s, C-F), −62.1 (s, CF$_3$), −69.9 (s, CF$_3$); ν_{max}/cm^{-1} (solid); 3490, 3294, 1625, 1589, 1559, 1517, 1494, 1469, 1429, 1409, 1378, 1320, 1269 and 1251; m/z (ES) found MH$^+$ 425.031563, C$_{16}$H$_8$^{35}ClF$_7$N$_4$ requires MH$^+$ 425.032569; HPLC 3.28 min; 100%.

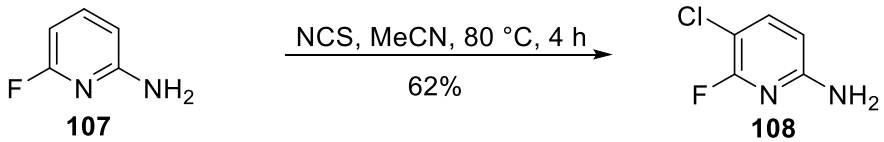
3-Chloro-7-(trifluoromethyl)-N⁵-[6-(trifluoromethyl)pyridin-3-yl]-1,8-naphthyridine-2,5-diamine (**106**) (Synthesised by MChem student Kyle Orritt)



106

To a solution of 3,5-dichloro-7-(trifluoromethyl)-1,8-naphthyridin-2-amine (0.100 g, 0.354 mmol) and 5-amino-2-(trifluoromethyl)pyridine (60 mg, 0.370 mmol) in propan-2-ol (2 mL) was added dropwise 4 M HCl in 1,4-dioxane (90 µL, 0.36 mmol). The solution was stirred at 80 °C under reflux for 20 h. The resulting mixture was partitioned between ethyl acetate (30 mL) and saturated aqueous sodium hydrogen carbonate (20 mL). The organic phase was washed with brine (20 mL), dried over $MgSO_4$, and the solvent evaporated *in vacuo*. The resultant yellow solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:1). The solvent was removed *in vacuo* and the title compound was collected as white needles (5.0 mg, 0.012 mmol; 4%); m. p. >270 °C (from ethyl acetate-pet. ether 40–60); Rf 0.21 (1:1 ethyl acetate-petrol. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.59 (d, *J* = 2.5 Hz, 1H, pyridine 2-H), 8.50 (s, 1H, naphthyridine 4-H), 7.88 (dd, *J* = 8.5, 2.5 Hz, 1H, pyridine 4-H), 7.75 (d, *J* = 8.5 Hz, 1H, pyridine 5-H), 7.14 (s, 1H, naphthyridine 6-H); ^{13}C NMR (101 MHz, methanol-d_4) δ 142.7 (pyridine 2-CH), 130.7 (naphthyridine 4-CH), 128.2 (pyridine 4-CH), 121.3 (pyridine 5-CH), 97.4 (naphthyridine 6-CH), other peaks not resolved. ^{19}F NMR (376 MHz, methanol-d_4) δ −68.3 (s, CF_3), −69.9 (s, CF_3); ν_{max}/cm^{-1} (solid); 3501, 3312, 3183, 1624, 1599, 1586, 1560, 1496, 1469, 1430, 1405, 1337, 1271 and 1231; m/z (ES) found MH^+ 408.037183, $C_{15}H_8{}^{35}ClF_6N_5$ requires MH^+ 408.037241; HPLC 2.77 min.; 100%.

5-Chloro-6-fluoropyridin-2-amine (**108**)

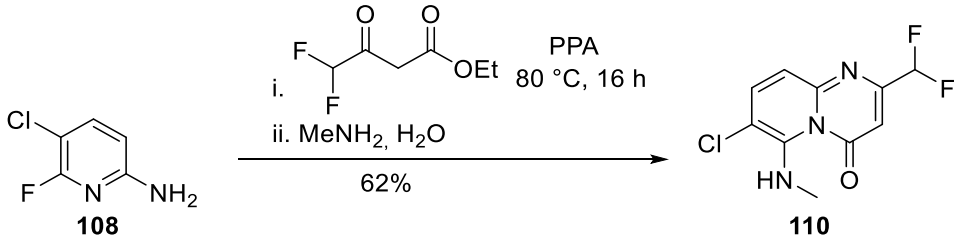


107 **108**

To a stirred mixture of 2-amino-6-fluoropyridine (0.123 g, 1.095 mmol) in acetonitrile (3 mL) was added *N*-chlorosuccinimide (0.155 g, 1.159 mmol). The reaction mixture was stirred for 4 h at 80 °C then the solvent was evaporated *in vacuo*. The resultant residue was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3). The solvent

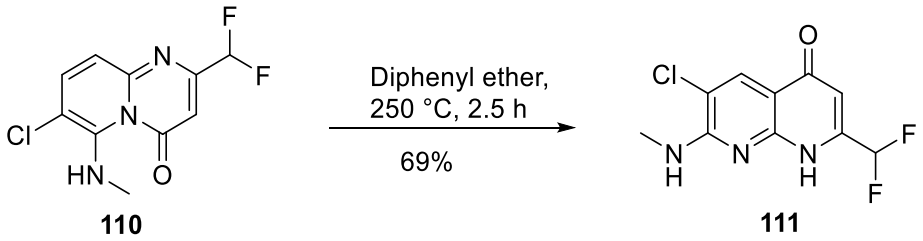
was removed *in vacuo* to give the title compound as off-white needles (86.2 mg, 0.588 mmol, 54%); m. p. 94–5 °C (from ethyl acetate); Rf 0.45 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (dd, *J* = 9.2, 8.3 Hz, 1H, 4-H), 6.23 (dd, *J* = 8.3, 1.1 Hz, 1H, 3-H), 4.47 (br s, 2H, NH_2); ^{13}C NMR (151 MHz, $CDCl_3$) δ 157.8 (d, *J* = 237 Hz, C-F), 155.7 (d, *J* = 15.5 Hz, 2-C), 142.0 (d, *J* = 1.8 Hz, 4-CH), 105.9 (d, *J* = 4.4 Hz, 3-CH), 103.4 (d, *J* = 33.1 Hz, 5-C); ^{19}F NMR (376 MHz, $CDCl_3$) δ −73.4 (d, *J* = 9.4 Hz, C-F); ν_{max}/cm^{-1} (solid); 3470, 3440, 3318, 3293, 3210, 3182, 1630, 1595, 1521, 1472, 1434, 1318, 1287, 1249 and 1223; m/z (ES) found MH^+ 147.011589, $C_5H_4{}^{35}ClFN_3$ requires MH^+ 147.011980; HPLC 1.90 min., 100%.

7-Chloro-2-(difluoromethyl)-6-(methylamino)-4H-pyrido[1,2-a]pyrimidin-4-one (**110**)



A mixture of **108** (0.956 g, 6.518 mmol), ethyl 4,4-difluoroacetoacetate (0.870 mL, 6.65 mmol) and polyphosphoric acid (20 mL) was stirred for 16 h at 80 °C. The solution was allowed to cool then poured onto ice and 40% methylamine in water was added until the pH increased to 9. A yellow precipitate formed which was isolated using vacuum filtration (1.056 g, 4.066 mmol, 62%); m. p. 138–143 °C decomposed (from ethyl acetate); Rf 0.45 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 7.60 (d, *J* = 9.3 Hz, 1H, 8-H), 6.78 (d, *J* = 9.3 Hz, 1H, 9-H), 6.37 (t, *J* = 54.9 Hz, 1H, CHF_2), 6.19 (s, 1H, 3H), 3.10 (s, 3H, CH_3); ^{13}C NMR (101 MHz, methanol-d_4) δ 164.6 (C=O), 156.6 (t, *J* = 25.6 Hz, 2-C), 152.3 (sp^2 C), 150.3 (sp^2 C), 141.5 (8-CH), 112.3 (9-CH) , 112.3 (t, *J* = 241 Hz, CHF_2), 105.3 (sp^2 C), 98.9 (t, *J* = 3.9 Hz, 3-CH), 33.1 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −122.8 (d, *J* = 54.9 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3068, 2943, 1673, 1619, 1580, 1564, 1532, 1493, 1454, 1413, 1385, 1335, 1285, 1258 and 1203; m/z (ES) found MH^+ 260.039622, $C_{10}H_8{}^{35}ClF_2N_3O$ requires MH^+ 260.039672; HPLC 2.50 min., 75%.

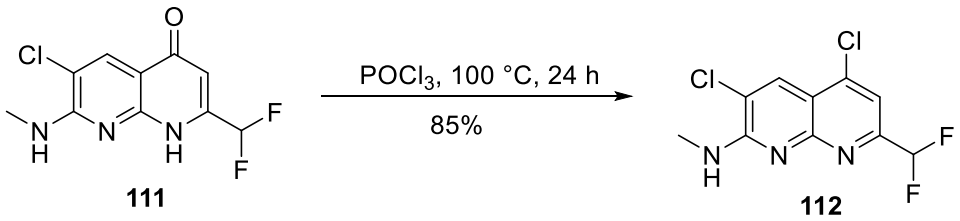
6-Chloro-2-(difluoromethyl)-7-(methylamino)-1,8-naphthyridin-4(1H)-one (**111**)



110 **111**

A solution of **110** (0.206 g, 0.792 mmol), in diphenyl ether (4 mL) was stirred under reflux at 250 °C for 2.5 h. The solution was allowed to cool to room temperature and the precipitate collected using vacuum filtration and washed with pet. ether 40–60 to leave the product as a brown solid (0.141 g, 0.543 mmol, 69%); m. p. 214–8 °C (from diphenyl ether); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.04 (s, 1H, 5-H), 6.66 (t, *J* = 54.0 Hz, 1H, CHF_2), 6.28 (s, 1H, 3-H), 2.97 (s, 3H, CH_3); ^{13}C NMR (101 MHz, methanol-d_4) δ 177.7 (C=O), 156.6 (sp^2 C), 150.1 (sp^2 C), 143.3 (t, *J* = 24.5 Hz, 2-C), 131.7 (5-CH), 115.3 (sp^2 C), 111.7 (sp^2 C), 111.0 (t, *J* = 241 Hz), 106.8 (t, *J* = 5.3 Hz, 3-CH), 27.5 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −120.4 (d, *J* = 54.1 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3438, 2714 (br), 1616, 1542, 1492, 1425, 1405, 1373, 1346, 1257 and 1223; m/z (ES) found MH^+ 260.039674, $C_{10}H_8{}^{35}ClF_2N_3O$ requires MH^+ 260.039672; HPLC 1.47 min., 100%.

3,5-Dichloro-7-(difluoromethyl)-N-methyl-1,8-naphthyridin-2-amine (**112**)

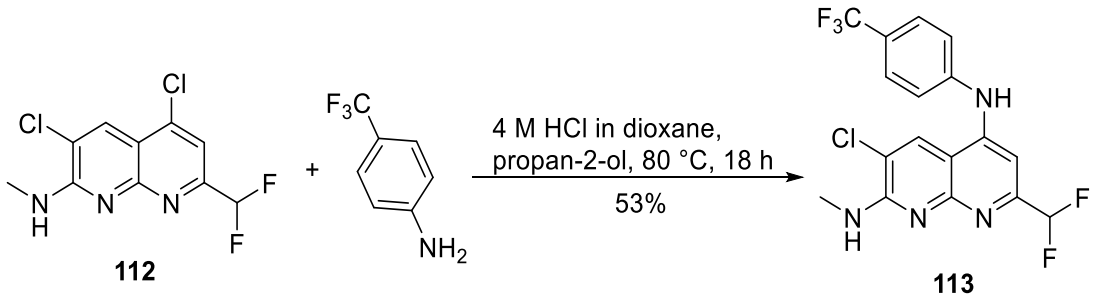


111 **112**

A mixture of **111** (0.441 g, 1.700 mmol) and phosphoryl chloride (4 mL) was stirred under reflux at 100 °C for 24 h. The solution was allowed to cool to room temperature then poured onto ice and saturated aqueous sodium hydrogen carbonate was added until pH increased to 9. The mixture was filtered under reduced pressure to isolate a brown precipitate which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:2–1:1). The solvent was removed *in vacuo* to yield the title compound as a brown solid (0.404 g, 1.451 mmol, 85%); m. p. >270 °C (from ethyl acetate); Rf 0.20 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (s, 1H, 5-H), 7.50 (s, 1H, 3-H), 6.66 (t, *J* = 55.1 Hz, 1H, CHF_2), 5.83 (br s, 1H, N-H), 3.19 (d, *J* = 5.0 Hz, 3H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$)

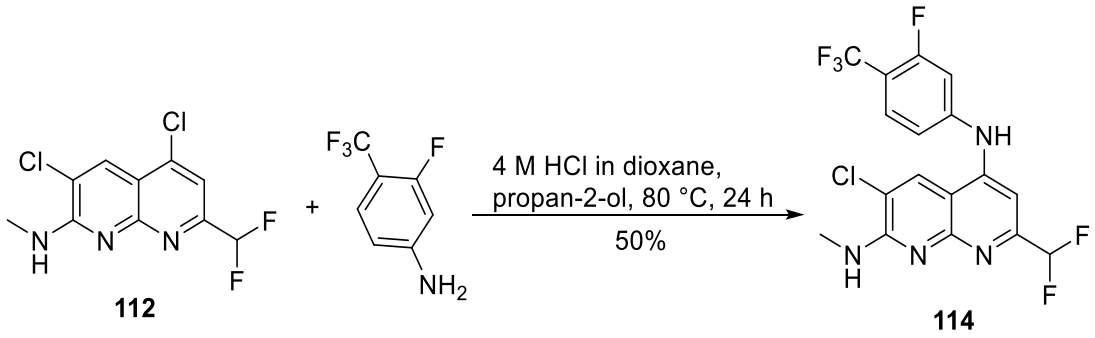
δ 155.8 (sp^2 C), 155.0 (sp^2 C), 154.9 (t, *J* = 27.2 Hz, 2-C), 143.1 (sp^2 C), 131.2 (5-CH), 121.5 (sp^2 C), 117.0 (sp^2 C), 114.4 (t, *J* = 2.6 Hz, 3-CH), 113.8 (t, *J* = 242 Hz, CHF_2), 29.0 (CH_3); ^{19}F NMR (376 MHz, $CDCl_3$) δ −115.0 (d, *J* = 54.5 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3291, 1607, 1551, 1534, 1480, 1448, 1400, 1372, 1295, 1257, 1234 and 1217; m/z (ES) found MH^+ 278.005518, $C_{10}H_7{}^{35}Cl_2F_2N_3$ requires MH^+ 278.005785; HPLC 2.84 min., 100%.

3-Chloro-7-(difluoromethyl)-N2-methyl-N5-(4-(trifluoromethyl)phenyl)-1,8-naphthyridine-2,5-diamine (**113**)



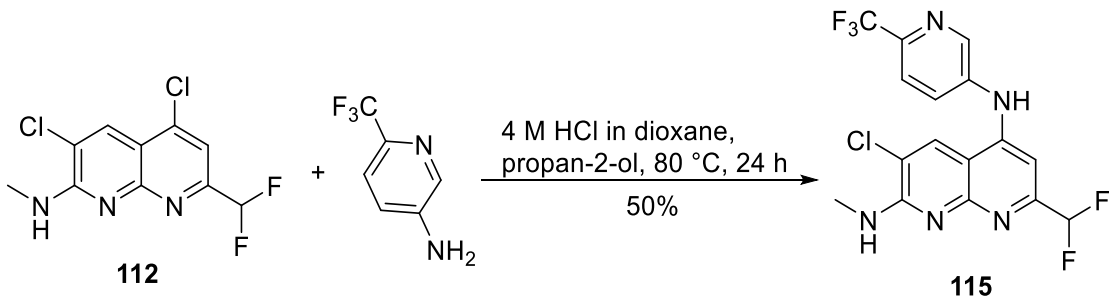
To a stirred mixture of **112** (28.0 mg, 0.101 mmol) in propan-2-ol (1 mL) was added 4-(trifluoromethyl)aniline (14 µL, 0.11 mmol). The reaction mixture was stirred under reflux at 80 °C for 18 h. A pale yellow precipitate formed and was isolated using vacuum filtration then neutralised with saturated aqueous sodium hydrogen carbonate solution (30 mL) and extracted with DCM (2 × 30 mL). The combined organic layers were dried over sodium sulphate then the solvent was removed *in vacuo* to give the title compound as a colourless solid (21.6 mg, 0.0537 mmol, 53%); m. p. 261–4 °C (from DCM); ^1H NMR (501 MHz, DMSO-d_6) δ 9.45 (br s, 1H, N-H), 8.65 (s, 1H, naphthyridine 4-H), 7.77 (d, *J* = 8.9 Hz, 2H, aniline 3-H), 7.50 (d, *J* = 8.9 Hz, 2H, aniline 2-H), 7.44 (q, *J* = 4.5 Hz, 1H, NHMe), 7.12 (s, 1H, naphthyridine 6-H), 6.83 (t, *J* = 55.2 Hz, 1H, CHF_2), 3.00 (d, *J* = 4.5 Hz, 3H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.2 (sp^2 C), 155.5 (sp^2 C), 154.8 (t, *J* = 24.3 Hz, naphthyridine 7-C), 148.7 (sp^2 C), 144.8 (sp^2 C), 130.8 (naphthyridine 4-CH), 127.2 (q, *J* = 3.6 Hz, aniline 3-CH), 124.9 (q, *J* = 271 Hz, CF_3), 123.6 (q, *J* = 32.1 Hz, aniline 4-C), 121.1 (aniline 2-CH), 117.7 (sp^2 C), 114.6 (t, *J* = 240 Hz, CHF_2), 108.9 (sp^2 C), 97.6 (t, *J* = 3.1 Hz, naphthyridine 6-CH), 28.8 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −63.5 (s, CF_3), −117.8 (d, *J* = 55.6 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3457, 3294, 1602, 1558, 1525, 1453, 1396, 1365, 1320 and 1277; m/z (ES) found MH^+ 403.073966, $C_{17}H_{12}{}^{35}ClF_5N_4$ requires MH^+ 403.074342; HPLC 2.62 min., 100%.

3-Chloro-7-(difluoromethyl)-N⁵-[3-fluoro-4-(trifluoromethyl)phenyl]-N²-methyl-1,8-naphthyridine-2,5-diamine (**114**)



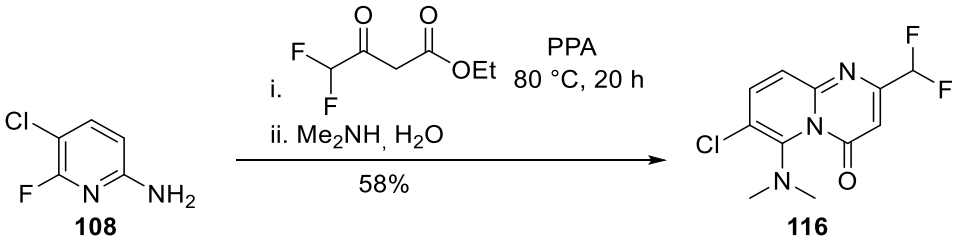
112 (0.132 g, 0.476 mmol) and 4-amino-2-fluorobenzotrifluoride (90.6 mg, 0.506 mmol) were dissolved in propan-2-ol (3 mL). 4 M HCl in 1,4-dioxane (0.13 mL, 0.52 mmol) was added and the mixture was stirred at 80 °C under reflux for 24 h. The reaction mixture was allowed to cool to room temperature then filtered to obtain a brown precipitate which was neutralised with saturated aqueous sodium hydrogen carbonate (50 mL) and extracted with DCM (3 × 50 mL). The combined organic layers were dried over sodium sulphate and the solvent removed *in vacuo*. The product was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:1). The solvent was removed *in vacuo* to give the title compound as a pale yellow solid (0.100 g, 0.238 mmol, 50%); m. p. >270 °C (from ethyl acetate); Rf 0.26 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (501 MHz, DMSO-d_6) δ 9.60 (s, 1H, naphthyridine 4-NH), 8.58 (s, 1H, naphthyridine 4-H), 7.76 (t, *J* = 8.6 Hz, 1H, aniline 5-H), 7.51 (q, *J* = 4.6 Hz, 1H, naphthyridine 2-NH), 7.33 (d, *J* = 13.0 Hz, 1H, aniline 2-H), 7.28 (d, *J* = 8.6 Hz, 1H, aniline 6-H), 7.24 (s, 1H, naphthyridine 6-H), 6.88 (t, *J* = 55.2 Hz, 1H, CHF_2), 3.00 (d, *J* = 4.6 Hz, 3H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.2 (d, *J* = 253 Hz, C-F), 156.2 (sp^2 C) , 155.6 (sp^2 C) , 154.8 (t, *J* = 24.4 Hz, naphthyridine 7-C), 147.7 (sp^2 C) , 147.5 (d, *J* = 11.3 Hz, aniline 1-C), 130.8 (naphthyridine 4-CH) , 128.9 (q, *J* = 6.9 Hz, aniline 5-CH) , 123.4 (q, *J* = 271 Hz, CF_3), 118.1 (sp^2 C) , 115.6 (d, *J* = 2.5 Hz, aniline 6-CH), 114.4 (t, *J* = 240 Hz, CHF_2), 110.3 (d, *J* = 12.0 Hz, aniline 4-C), 109.5 (sp^2 C), 107.6 (d, *J* = 23.6 Hz, aniline 2-CH), 99.3 (naphthyridine 6-CH), 28.9 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −62.0 (d, *J* = 12.2 Hz, CF_3), −114.9 (qd, *J* = 12.6, 8.5 Hz, C-F), −117.8 (d, *J* = 55.4 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3456, 3282, 2957, 1742, 1633, 1606, 1547, 1494, 1457, 1434, 1396, 1365, 1318, 1266 and 1214; m/z (ES) found MH^+ 421.064788, $C_{17}H_{11}{}^{35}ClF_6N_4$ requires MH^+ 421.064920; HPLC 2.65 min., 100%.

3-Chloro-7-(difluoromethyl)-N2-methyl-N5-[6-(trifluoromethyl)pyridin-3-yl]-1,8-naphthyridine-2,5-diamine (**115**)



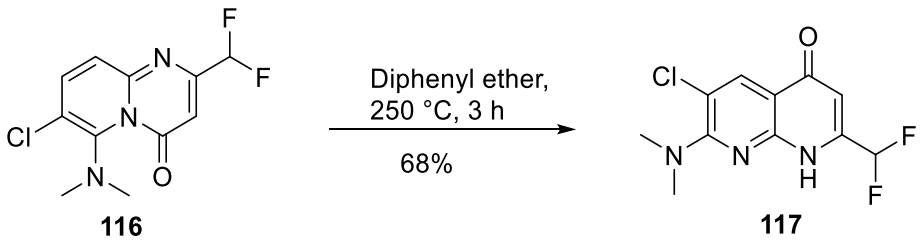
112 (0.205 g, 0.735 mmol) and 5-amino-2-(trifluoromethyl)pyridine (0.131 g, 0.808 mmol) were dissolved in propan-2-ol (4 mL). 4 M HCl in 1,4-dioxane (0.20 mL, 0.80 mmol) was added and the mixture was stirred at 80 °C for 24 h under reflux. The reaction mixture was allowed to cool to room temperature then filtered to obtain a brown precipitate which was neutralised with saturated aqueous sodium hydrogen carbonate (50 mL) and extracted with DCM (3 × 50 mL). The combined organic layers were dried over sodium sulphate and the solvent removed *in vacuo*. The product was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:2–1:1). The solvent was removed *in vacuo* to give the title compound as an orange solid (0.148 g, 0.368 mmol, 50%); m. p. >270 °C; Rf 0.10 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.57 (d, *J* = 2.6 Hz, 1H, pyridine 2-H), 8.38 (s, 1H, naphthyridine 4-H), 7.84 (dd, *J* = 8.6, 2.6 Hz, 1H, pyridine 4-H), 7.73 (d, *J* = 8.6 Hz, 1H, pyridine 5-H), 7.10 (s, 1H, naphthyridine 6-H), 6.54 (t, *J* = 55.3 Hz, 1H, CHF_2), 3.03 (s, 3H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.1 (sp^2 C), 155.6 (sp^2 C), 154.8 (t, *J* = 24.9 Hz, naphthyridine 7-C), 148.1 (sp^2 C), 142.8 (pyridine 2-CH), 141.0 (sp^2 C), 140.4 (q, *J* = 33.7 Hz, pyridine 6-C), 130.8 (naphthyridine 4-CH), 127.8 (pyridine 4-CH), 122.4 (q, *J* = 273 Hz, CF_3), 122.1 (q, *J* = 3.2 Hz, pyridine 5-CH), 118.0 (sp^2 C), 114.4 (t, *J* = 240 Hz, CHF_2), 109.2 (sp^2 C), 98.2 (naphthyridine 6-CH), 28.9 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −68.6 (s, CF_3), −117.8 (d, *J* = 55.3 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3465, 3278, 2972, 1613, 1590, 1552, 1451, 1426, 1397, 1365, 1335, 1299, 1278 and 1232; m/z (ES) found MH^+ 404.069984, $C_{16}H_{11}{}^{35}ClF_5N_5$ requires MH^+ 404.069591; HPLC 2.18 min., 100%.

7-Chloro-2-(difluoromethyl)-6-(dimethylamino)-4H-pyrido[1,2-a]pyrimidin-4-one (**116**)



A stirred mixture of **108** (0.619 g, 4.22 mmol), ethyl 4,4-difluoroacetoacetate (0.56 mL, 4.28 mmol) and polyphosphoric acid (15 mL) was heated at 80 °C for 20 h. The solution was allowed to cool to room temperature then poured onto ice and dimethylamine in water was added until the pH increased to 9. A yellow precipitate formed which was isolated using vacuum filtration. The title compound was obtained as a yellow solid (0.670 g, 2.448 mmol, 58%); m. p. 107–110 °C (from water); ^{1}H NMR (400 MHz, methanol-d_4) δ 7.82 (d, J = 9.3 Hz, 1H, 8-H), 7.24 (d, J = 9.3 Hz, 1H, 9-H), 6.56 (t, J = 54.9 Hz, 1H, CHF_2), 6.40 (s, 1H, 3-H), 2.98 (s, 6H. CH_3); ^{13}C NMR (101 MHz, methanol-d_4) δ 161.7 (C=O), 156.4 (t, J = 25.0 Hz, 2-C), 153.7 (sp^2 C), 145.5 (sp^2 C), 139.6 (8-CH), 119.3 (9-CH), 112.4 (t, J = 241 Hz, CHF_2), 104.1 (sp^2 C), 99.3 (t, J = 4.3 Hz, 3-CH), 40.5 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −122.3 (d, J = 54.7 Hz, CHF2); ν_{max}/cm^{-1} (solid); 2993, 2801, 1762, 1708, 1651, 1611, 1576, 1536, 1499, 1456, 1410, 1374, 1322, 1302, 1283 and 1217; m/z (ES) found MH^+ 274.055613, $C_{11}H_{10}{}^{35}ClF_2N_3O$ requires MH^+ 274.055323; HPLC 2.67 min., 100%.

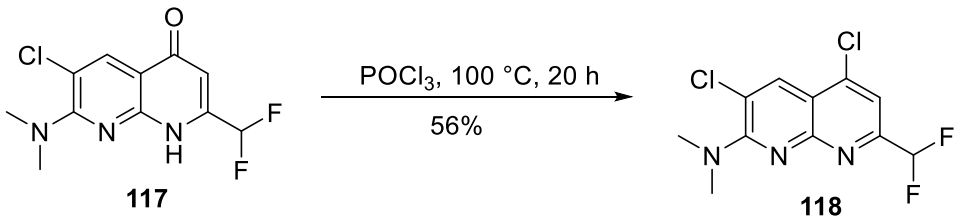
6-Chloro-2-(difluoromethyl)-7-(dimethylamino)-1,4-dihydro-1,8-naphthyridin-4-one (**117**)



A solution of **116** (0.323 g, 1.181 mmol) in diphenyl ether (7 mL) was heated under reflux at 250 °C for 3 h. After cooling to room temperature, the solid was collected using vacuum filtration and washed with pet. ether 40–60 to give the title compound as a brown solid (0.219 g, 0.799 mmol, 68%); m. p. >270 °C (from diphenyl ether); Rf 0.49 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (s, 1H, 5-CH), 6.93 (t, J = 53.8 Hz, 1H, CHF_2), 6.29 (s, 1H, 3-CH), 3.17 (s, 6H, NMe_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.8

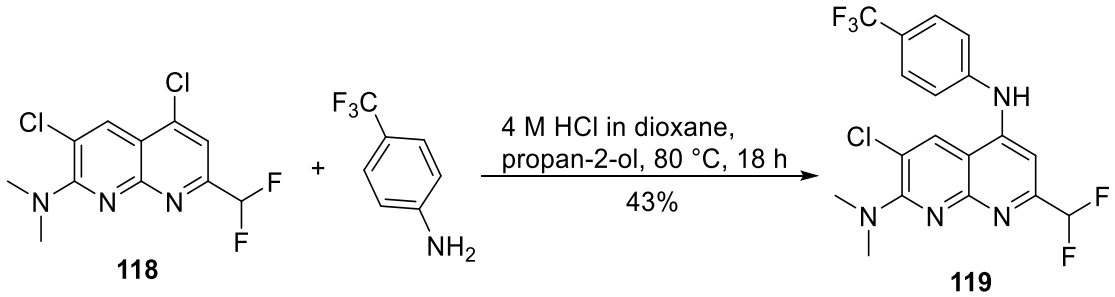
(sp^2 C), 157.1 (sp^2 C) , 136.6 (sp^2 C), 130.5 (5-CH), 123.9 (sp^2 C), 119.1 (3-CH), 115.8 (sp^2 C), 114.5 (t, *J* = 246 Hz, CHF_2), 112.0 (sp^2 C), 41.5 (CH_3); ^{19}F NMR (376 MHz, DMSO-d_6) δ −118.2 (s, CHF_2); ν_{max}/cm^{-1} (solid); 2789 (br), 1611, 1549, 1486, 1398 and 1231; m/z (ES) found MH^+ 274.055009, $C_{11}H_{10}{}^{35}ClF_2N_3O$ requires MH^+ 274.055323; HPLC 2.67 min., 91%.

3,5-Dichloro-7-(difluoromethyl)-N,N-dimethyl-1,8-naphthyridin-2-amine (**118**)



A mixture of phosphoryl chloride (3 mL) and **117** (0.202 g, 0.739 mmol), was stirred at 100 °C for 20 h. After cooling, the solution was poured onto ice and ammonium hydroxide was added until the pH increased to 9. The solution was filtered to obtain a brown solid and the filtrate extracted with chloroform (2 × 50 mL) and evaporated to obtain further product. The title compound was obtained as a brown solid (0.121 g, 0.415 mmol, 56%); Rf 0.49 (1:2 ethyl acetate-pet. ether 40–60); m. p. >270 °C (from chloroform); ^1H NMR (400 MHz, methanol-d_4) δ 8.45 (s, 1H, 4-H), 7.68 (s, 1H, 6-H), 6.79 (t, *J* = 54.9 Hz, 1H, CHF_2), 3.33 (s, 6H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 159.0 (sp^2 C), 153.6 (sp^2 C), 142.4 (sp^2 C), 135.1 (4-CH), 123.0 (sp^2 C), 119.1 (sp^2 C), 117.8 (sp^2 C), 115.4 (t, *J* = 3.2 Hz, 6-CH), 113.7 (t, *J* = 241 Hz, CHF_2), 41.6 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −117.7 (d, *J* = 54.9 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3380, 1698, 1589, 1550, 1518, 1455, 1415, 1403, 1378, 1350, 1331, 1284, 1232 and 1208; m/z (ES) found MH^+ 292.020767, $C_{11}H_9{}^{35}Cl_2F_2N_3$ requires MH^+ 292.021436; HPLC 3.58 min., 95%.

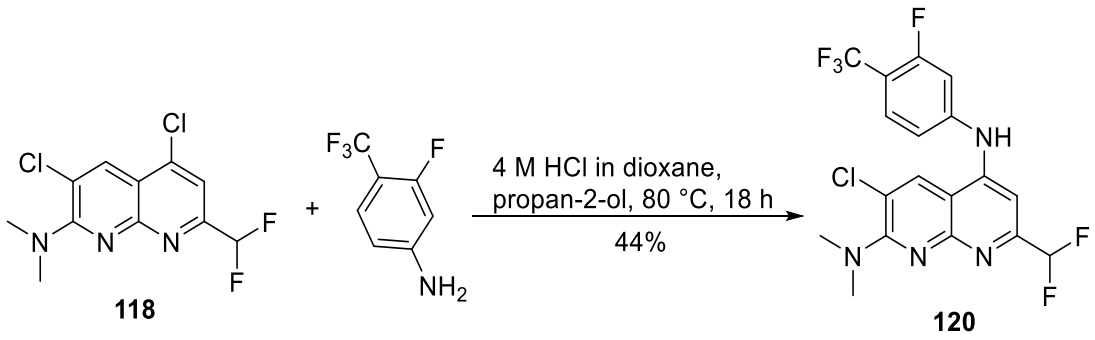
3-Chloro-7-(difluoromethyl)-N^2,N^2-dimethyl-N^5-[4-(trifluoromethyl)phenyl]-1,8-naphthyridine-2,5-diamine (**119**)



118 (63.3 mg, 0.217 mmol) and 4-(trifluoromethyl)aniline (30 µL, 0.24 mmol) were dissolved in propan-2-ol (2 mL). 4 M HCl in 1,4-dioxane (60 µL, 0.24 mmol) was added and the mixture was stirred at 80 °C for 18 h. The

solution was evaporated *in vacuo* then saturated aqueous sodium hydrogen carbonate (50 mL) was added and the product was extracted with DCM (2 × 50 mL) then dried over sodium sulphate. The solvent was removed *in vacuo* and the resultant brown residue purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:2). The solvent was removed *in vacuo* to give the title compound as an orange solid (39.3 mg, 0.0943 mmol, 43%); Rf 0.36 (1:2 ethyl acetate-pet. ether 40–60); m. p. 250–2 °C (from ethyl acetate); ^{1}H NMR (501 MHz, DMSO-d_6) δ 9.60 (br s, 1H, N-H), 8.77 (s, 1H, naphthyridine 4-H), 7.79 (d, *J* = 8.4 Hz, 2H, aniline 3-H), 7.53 (d, *J* = 8.4 Hz, 2H, aniline 2-H), 7.17 (s, 1H, naphthyridine 6-H), 6.88 (t, *J* = 55.1 Hz, 1H, CHF_2), 3.17 (s, 6H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.8 (sp^2 C), 155.4 (t, *J* = 23.4 Hz, naphthyridine 7-C), 154.6 (sp^2 C), 148.9 (sp^2 C), 144.4 (sp^2 C), 134.7 (naphthyridine 4-CH) , 127.3 (q, *J* = 3.6 Hz, aniline 3-CH), 124.9 (q, *J* = 271 Hz, CF_3), 124.0 (q, *J* = 32.3 Hz, aniline 4-C), 121.5 (aniline 2-CH), 119.5 (sp^2 C), 114.5 (t, *J* = 240 Hz, CHF_2), 110.7 (sp^2 C) , 97.8 (t, *J* = 2.9 Hz, naphthyridine 6-CH), 41.5 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −63.5 (CF_3), −117.9 (d, *J* = 55.3 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2933, 2874, 1595, 1508, 1447, 1410, 1394, 1353, 1321 and 1281; m/z (ES) found MH^+ 417.090325, $C_{18}H_{14}{}^{35}ClF_5N_4$ requires MH^+ 417.089992; HPLC 2.73 min., 97%.

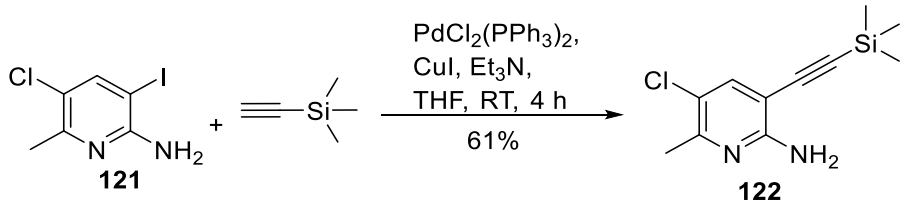
3-Chloro-7-(difluoromethyl)-N^5-[3-fluoro-4-(trifluoromethyl)phenyl]-N^2,N^2-dimethyl-1,8-naphthyridine-2,5-diamine (**120**)



118 (51.8 mg, 0.177 mmol) and 4-amino-2-fluorobenzotrifluoride (36.8 mg, 0.205 mmol) were dissolved in propan-2-ol (2 mL). 4 M HCl in 1,4-dioxane (50 µL, 0.20 mmol) was added and the mixture was stirred at 80 °C for 18 h. The mixture was concentrated *in vacuo*, neutralised with saturated aqueous sodium hydrogen carbonate (50 mL), extracted with DCM (2 × 50 mL), dried over sodium sulphate and purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:2). The solvent was removed *in vacuo* to give the title compound as yellow needles (33.6 mg, 0.0773 mmol, 44%); m. p. 241–3 °C (from ethyl acetate); Rf 0.33 (1:2 ethyl acetate-pet. ether 40–

60); ^{1}H NMR (501 MHz, DMSO-d_6) δ 9.91 (br s, 1H, N-H), 8.73 (s, 1H, naphthyridine 4-H), 7.82 (t, *J* = 8.5 Hz, 1H, aniline 5-H), 7.41 (d, *J* = 13.1 Hz, 1H, aniline 2-H), 7.34 (d, *J* = 8.8 Hz, 1H, aniline 6-H), 7.29 (s, 1H, naphthyridine 6-H), 6.95 (t, *J* = 54.6 Hz, CHF_2), 3.20 (s, 6H, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.7 (d, *J* = 253 Hz, C-F), 158.3 (sp^2 C), 153.3 (sp^2 C), 148.2 (sp^2 C), 146.2 (sp^2 C), 134.3 (naphthyridine 4-CH), 128.5 (aniline 5-CH), 122.8 (q, *J* = 271 Hz, CF_3), 119.3 (aniline 6-CH), 116.1 (aniline 2-CH), 113.4 (t, *J* = 240 Hz, CHF_2), 110.3 (sp^2 C), 108.2 (d, *J* = 23.5 Hz, aniline 4-C), 99.1 (naphthyridine 6-CH) , 83.0 (sp^2 C), 80.5 (sp^2 C), 41.0 (CH_3); ^{19}F NMR (376 MHz, methanol-d_4) δ −62.1 (d, *J* = 1.5 Hz, CF_3), −114.7 – −114.8 (m, C-F), −117.9 (d, *J* = 55.2 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2932, 1636, 1594, 1554, 1514, 1415, 1410, 1391, 1319 and 1250; m/z (ES) found MH^+ 457.062093, $C_{18}H_{13}{}^{35}ClF_6N_4$ requires MNa^+ 457.062514; HPLC 2.82 min., 100%.

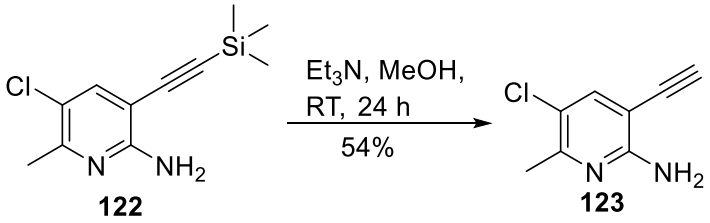
5-Chloro-6-methyl-3-((trimethylsilyl)ethynyl)pyridin-2-amine (**122**)



5-Chloro-6-methyl-3-iodopyridin-2-amine (0.751 g, 2.797 mmol), copper(I) iodide (14.5 mg, 0.0761 mmol) and bis(triphenylphosphine)palladium(II) dichloride (37.7 mg, 0.0537 mmol) were dissolved in THF (20 mL) at room temperature and flushed with nitrogen for 30 minutes. To the resultant dark brown solution was added with stirring triethylamine (2.0 mL, 14 mmol) followed immediately by ethynyltrimethylsilane (1.1 mL, 7.9 mmol). A black solution formed and, after stirring in a water bath at room temperature for 4 h, was partitioned between pet. ether 40–60 (30 mL) and water (10 mL). The layers were separated and the organic layer was washed with water (2 × 10 mL) and brine (10 mL). The organic layer was evaporated to give a black solid which was dissolved in DCM (50 mL). APDTC (59.8 mg, 0.363 mmol) was added followed by water (10 mL). The mixture was stirred at room temperature for 2 h then filtered through celite to remove the solid. The layers were then separated and the organic layer dried over magnesium sulphate. The solvent was removed *in vacuo* to leave a brown solid. Pet. ether 40–60 (5 mL) was added and the suspension stirred at 0 °C for 30 minutes then filtered under reduced pressure and the product obtained as a grey solid (0.405 g, 1.696 mmol, 61%), m. p. 126–129 °C decomposed (from pet. ether 40–60); Rf 0.79 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (s, 1H, 4-H), 4.93 (br s, 2H, NH_2), 2.44 (s, 3H, CH_3), 0.25 (s,

9H, Si-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.0 (pyridine C), 154.6 (pyridine C), 139.9 (pyridine 4-CH), 119.1 (pyridine C), 101.9 (pyridine C), 101.6 (C≡C), 99.2 (C≡C), 22.5 (CH_3), 0.0 (Si-CH_3); ν_{max}/cm^{-1} (solid); 3457, 3293, 3152, 2958, 2150, 1627, 1551, 1445, 1405, 1247 and 1206; m/z (ES) found MH^+ 239.078224, $C_{11}H_{15}{}^{35}ClN_2Si$ requires MH^+ 239.076581; HPLC 3.08 min., 100%.

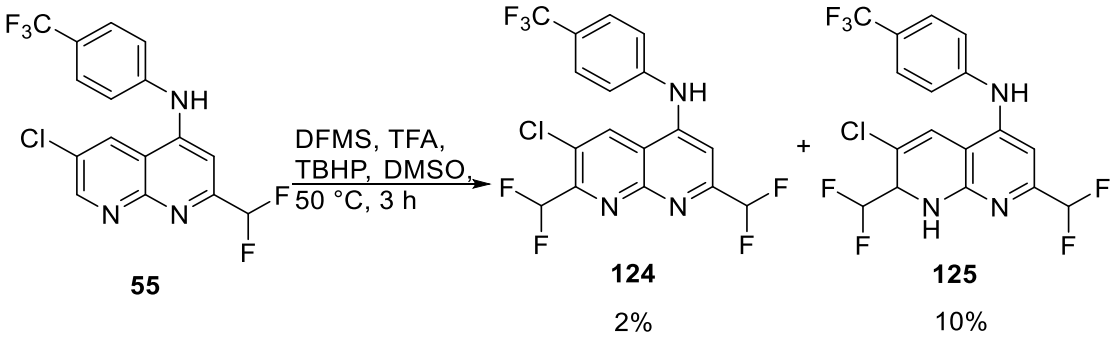
5-Chloro-3-ethynyl-6-methylpyridin-2-amine (**123**)



122 **123**

To a solution of **122** (0.402 g, 1.683 mmol) in methanol (20 mL) was added triethylamine (0.71 mL, 5.1 mmol). The solution was stirred at room temperature for 24 h then the solvent was removed *in vacuo*. The solid residue was dissolved in diethyl ether and filtered through a plug of silica gel to remove polar black impurities. The resultant orange solution was concentrated *in vacuo* and the solid residue dissolved in acetone (3 mL). Pet. ether 40–60 (6 mL) was added and the solution was left at –20 °C for 20 h. The orange solid was collected using vacuum filtration and washed with cold pet. ether 40–60 to give the title compound as pale orange needles (0.152 g, 0.915 mmol, 54%), m. p. 107–109 °C (from DCM); Rf 0.25 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 (s, 1H, 4-H), 4.98 (s, 2H, NH_2), 3.39 (s, 1H, C≡C-H), 2.45 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.2 (pyridine C), 155.0 (pyridine C), 140.3 (pyridine 4-CH), 119.0 (pyridine C), 100.5 (pyridine C), 83.7 (C≡C), 78.3 (C≡C-H), 22.5 (CH_3); ν_{max}/cm^{-1} (solid); 3431, 3287, 3244, 3149, 2850, 2100, 1625, 1582, 1555, 1442, 1265, and 1203; m/z (ES) found MH^+ 167.037304, $C_8H_7{}^{35}ClN_2$ requires MH^+ 167.037052; HPLC 1.40 min., 100%.

6-Chloro-2,7-di(difluoromethyl)-N-(4-(trifluoromethyl)phenyl)-1,8-naphthyridin-4-amine (**124**)

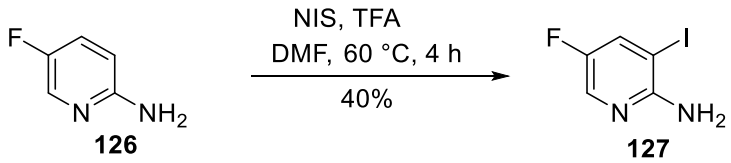


55 **124** 2% **125** 10%

Trifluoroacetic acid (36 µL, 0.47 mmol) was added to a solution of **55** (0.167 g, 0.446 mmol) and bis(((difluoromethyl)sulfinyl)oxy) zinc (0.270 g, 0.914 mmol) in DMSO (3 mL). The reaction was cooled in ice as ^{t}butyl hydroperoxide (70% aq. solution, 0.186 mL, 1.34 mmol) was added slowly to the stirred solution. The mixture was stirred to 50 °C for 3 h. The reaction mixture was allowed to cool to room temperature, diluted with EDTA/sodium bicarbonate solution (1.03 g EDTA in 30 mL sat. $NaHCO_{3(aq)}$) and extracted with ethyl acetate (2 × 40 mL). The combined organic extracts were washed with brine (40 mL) and water (40 mL), dried over magnesium sulphate and concentrated *in vacuo*. The yellow residue was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (25–100% ethyl acetate) and the solvent removed *in vacuo* to give the title compound as a yellow solid (4.0 mg, 0.0094 mmol, 2%); Rf 0.27 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 9.13 (s, 1H, naphthyridine 5-H), 7.81 (d, *J* = 8.4 Hz, 2H, aniline 3-H), 7.63 (d, *J* = 8.4 Hz, 2H, aniline 2-H), 7.43 (s, 1H, naphthyridine 3-H), 7.16 (t, *J* = 53.4 Hz, 1H, CHF_2), 6.76 (t, *J* = 55.0 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 134.4 (naphthyridine 5-CH), 126.7 (aniline 3-CH), 122.4 (aniline 2-CH), 99.2 (naphthyridine 3-CH), other peaks not resolved; ^{19}F NMR (376 MHz, methanol-d_4) δ −63.7 (s, CF_3), −118.5 (d, *J* = 55.2 Hz, CHF_2), −120.7 (d, *J* = 53.1 Hz, CHF_2); LCMS 0.7 min., MH^+ 424.34. **125** was also obtained as an orange solid (19.1 mg, 0.0449 mmol, 10%); m. p. 68–70 °C (from ethyl acetate-pet. ether 40–60); Rf 0.46 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 7.56 (d, *J* = 8.4 Hz, 2H, aniline 3-H), 7.24 (d, *J* = 8.4 Hz, 2H, aniline 2-H), 6.84 (s, 1H, naphthyridine 3-H), 6.64 (s, 1H, naphthyridine 5-H), 6.34 (t, *J* = 55.4 Hz, 1H, 2-CHF_2), 5.83 (td, *J* = 55.9, 2.8 Hz, 1H, 7-CHF_2), 4.55 (td, *J* = 12.9, 2.7 Hz, 1H, H-C-CHF_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 127.4 (naphthyridine 5-CH), 126.3 (q, *J* = 3.6 Hz, aniline 3-CH), 121.1 (aniline 2-CH), 115.3 (t, *J* = 247 Hz, 7-CHF_2), 113.3 (t, *J*

= 240 Hz, 2-CHF_2), 100.7 (t, *J* = 4.3 Hz, naphthyridine 3-CH), 43.6 (t, *J* = 22.1 Hz, H-C-CHF_2), other peaks not resolved; ^{19}F NMR (282 MHz, methanol-d_4) δ −63.6 (s, CF_3), −118.9 (dd, *J* = 55.4, 8.6 Hz, 2-CHF_2), −125.4 (ddd, J = 55.9, 17.1, 12.8 Hz, 7-CHF_2); ν_{max}/cm^{-1} (solid); 3250, 3069, 2961, 1752, 1639, 1598, 1524, 1494, 1445, 1372, 1322, 1270 and 1213; m/z (ES) found MH^+ 426.059588, $C_{17}H_{11}{}^{35}ClF_7N_3$ requires MH^+ 426.060249; HPLC 3.71 min., 18%, 3.73 min., 71%.

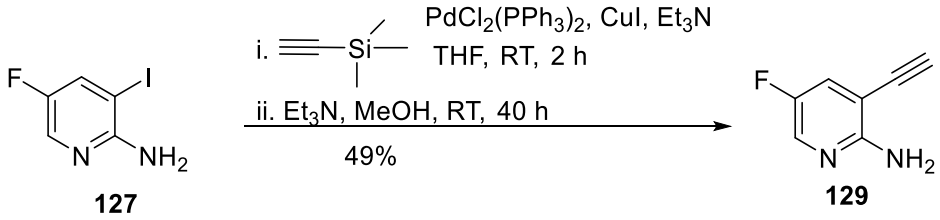
5-Fluoro-3-iodopyridin-2-amine (**127**)



To a stirred suspension of 5-fluoropyridin-2-amine (2.319 g, 20.683 mmol) in dry DMF (20 mL) cooled to 0 °C was added dropwise trifluoroacetic acid (1.9 mL, 25 mmol) at a rate sufficient to maintain the temperature below 10 °C. To the resultant suspension was added *N*-iodosuccinimide (5.059 g, 22.486 mmol). After 1 h at room temperature the mixture was stirred at 60 °C under an atmosphere of nitrogen for 4 h. The dark brown reaction mixture was allowed to cool to room temperature then poured into water (350 mL). Ethyl acetate (30 mL) was added and the mixture stirred rapidly while excess *N*-iodosuccinimide was destroyed by adding solid sodium bisulphite (0.6 g). After 2 minutes, solid sodium bicarbonate was added until effervescence ceased. Layers were separated and the aqueous layer was extracted with ethyl acetate (3 × 20 mL). Combined organic extracts were washed with water (20 mL) and brine (20 mL), dried over sodium sulphate and evaporated *in vacuo* to leave a dark viscous oil which solidified on standing. The residue was dissolved in DCM (20 mL) and filtered through a column of silica, washing with DCM until no further product eluted. Concentration of the filtrate gave a dark orange solid which was heated in hexane (25 mL). Most of the solid dissolved giving an orange solution and a tarry residue which was washed with hexane (5 mL). The combined hexane solutions were cooled to give the title compound (1.994 g, 8.379 mmol, 40%) as pale orange plates, m. p. 76–77 °C (from hexane) (lit. 76 °C[158]); Rf 0.38 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (d, *J* = 2.7 Hz, 1H, 6-H), 7.67 (dd, *J* = 7.2, 2.7 Hz, 1H, 4-H), 4.85 (s, 2H, NH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 154.5 (2-C), 152.2 (d, *J* = 249 Hz, C-F), 134.7 (d, *J* = 24.1 Hz, 6-CH), 134.3 (d, *J* = 21.7 Hz, 4-CH), 75.6 (d, *J* = 3.4 Hz, C-I) (consistent with lit.[159]); ^{19}F NMR (282 MHz, $CDCl_3$) δ −139.7 (d, *J* = 7.2 Hz, C-F); ν_{max}/cm^{-1} (solid); 3455,

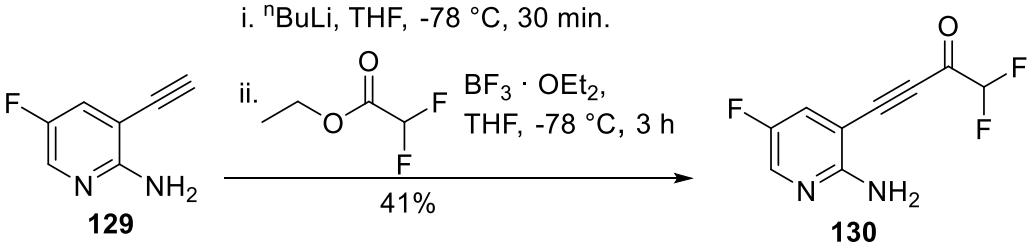
3280, 3144, 1623, 1592, 1566, 1549, 1466, 1397, 1319, 1276 and 1237; m/z (ES) found MH^+ 238.946964, $C_5H_4FIN_2$ requires MH^+ 238.947601; HPLC 1.15 min., 94%.

3-Ethynyl-5-fluoropyridin-2-amine (**129**)



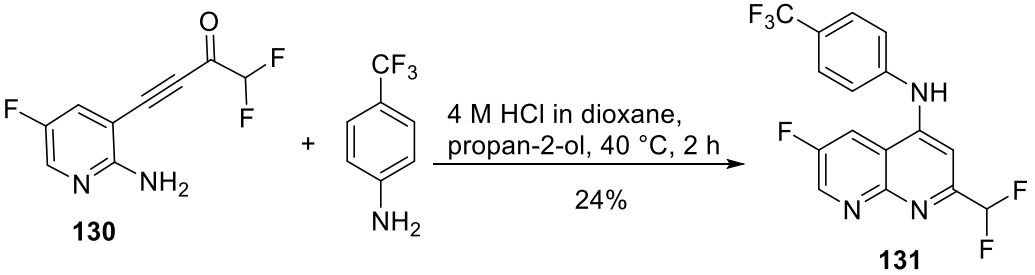
127 (1.881 g, 7.903 mmol) was flushed with nitrogen then ethynyltrimethylsilane (3.2 mL, 23 mmol), triethylamine (5.3 mL, 38 mmol) and dry THF (30 mL) were added. The reaction mixture was cooled using a water bath at room temperature as copper(I) iodide (41.3 mg, 0.217 mmol) and bis(triphenylphosphine)palladium(II) dichloride (0.110 g, 0.156 mmol) were added. The mixture was stirred at room temperature under an atmosphere of nitrogen for 2 h and became red-orange, with a grey precipitate forming. The mixture was poured into diethyl ether (80 mL) and washed with water (3 × 50 mL). The organic layer was dried over sodium sulphate and evaporated *in vacuo* to leave the crude intermediate as a brown solid, which was dissolved in methanol (20 mL). Triethylamine (0.3 mL, 2.1 mmol) was added and the mixture stirred at room temperature for 40 h. The solvent was removed *in vacuo* at 20 °C and the resultant black residue purified using column chromatography; elution with DCM then diethyl ether-DCM (1:9) to give the title compound (0.531 g, 3.908 mmol, 49%) as a pale yellow solid, m. p. 70–72 °C (from DCM); Rf 0.21 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, *J* = 3.0 Hz, 1H, 6-H), 7.33 (dd, *J* = 8.2, 3.0 Hz, 1H, 4-H), 4.93 (s, 2H, NH_2), 3.44 (s, 1H, C≡C-H) (consistent with lit.[154]); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.2 (2-C), 152.6 (d, *J* = 243 Hz, C-F), 135.9 (d, *J* = 24.9 Hz, 6-CH), 127.5 (d, *J* = 21.3 Hz, 4-CH), 102.1 (d, *J* = 5.3 Hz, 3-C), 84.2 (C≡C-H), 78.3 (d, *J* = 2.0 Hz, C≡C); ^{19}F NMR (376 MHz, $CDCl_3$) δ −142.3 (d, *J* = 8.5 Hz, C-F); ν_{max}/cm^{-1} (solid); 3469, 3440, 3276, 3229, 3167, 2097, 1622, 1592, 1580, 1454, 1419, 1319, 1260, 1237 and 1205; m/z (ES) found MH^+ 137.050470, $C_7H_5FN_2$ requires MH^+ 137.050953. HPLC 0.85 min., 100%.

4-(2-Amino-5-fluoropyridin-3-yl)-1,1-difluorobut-3-yn-2-one (**130**)



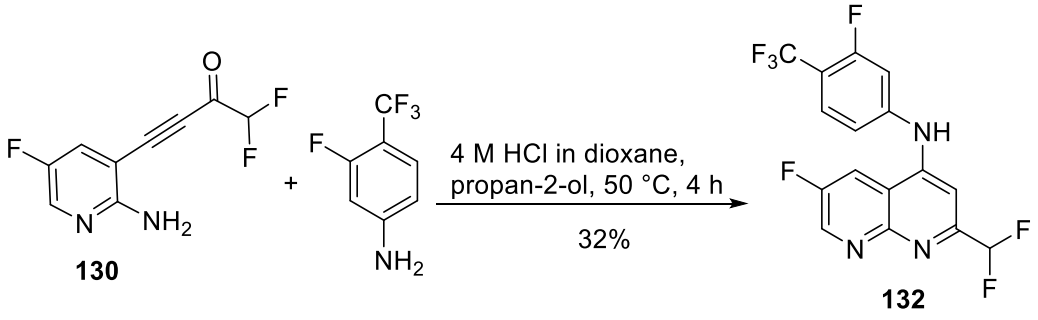
129 (0.430 g, 1.948 mmol) was dissolved in dry THF (25 mL) under an atmosphere of nitrogen. A 1.6 M solution of ^{n}butyllithium in hexanes (4.8 ml, 7.7 mmol) was added and the solution was stirred at −78 °C for 30 minutes. A solution of ethyl difluoroacetate (0.37 mL, 3.5 mmol) and boron trifluoride diethyl etherate (0.47 mL, 3.8 mmol) in dry THF (30 mL) was added and the mixture was stirred at −78 °C for 3 h then quenched with saturated aqueous ammonium chloride solution (10 mL). The slurry was allowed to warm to room temperature then the THF was removed *in vacuo*. The residue was diluted with diethyl ether (70 mL), washed with brine (2 × 30 mL) and dried over magnesium sulphate. The solvent was removed *in vacuo* to leave a dark brown residue which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:1). The solvent was removed *in vacuo* to leave the title compound as an orange solid (0.275 g, 1.286 mmol, 41%); m. p. 129-132 °C (from ethyl acetate); Rf 0.28 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.21 (d, *J* = 3.0 Hz, 1H, 6-H), 7.83 (dd, *J* = 8.5, 3.0 Hz, 1H, 4-H), 6.73 (br s, 2H, NH_2), 6.58 (t, *J* = 53.5 Hz, 1H, CHF_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.6 (C=O), 158.4 (2-C), 151.0 (d, *J* = 240 Hz, C-F), 140.7 (d, *J* = 24.9 Hz, 6-CH), 128.7 (d, *J* = 21.2 Hz, 4-CH), 108.9 (t, *J* = 249 Hz, CHF_2), 95.3 (d, *J* = 5.7 Hz, 3-C), 94.1 (alkyne C), 90.4 (alkyne C); ^{19}F NMR (282 MHz, DMSO-d_6) δ −127.0 (d, *J* = 53.6 Hz, CHF_2), −143.6 (d, *J* = 8.4 Hz, C-F); ν_{max}/cm^{-1} (solid); 3434, 3168, 2183, 1682, 1637, 1561, 1480, 1433, 1332, 1302, 1263 and 1212; $(M - H)^-$ 213.027547 $C_9H_5F_3N_2O$ requires $(M - H)^-$ 213.028121; HPLC 0.98 min., 77%.

2-(Difluoromethyl)-6-fluoro-N-(4-(trifluoromethyl)phenyl)-1,8-naphthyridin-4-amine (**131**)



To a solution of **130** (87.2 mg, 0.407 mmol) in propan-2-ol (2 mL) was added 4-(trifluoromethyl)aniline (50 µL, 0.34 mmol) followed by 4 M HCl in 1,4-dioxane (0.11 mL, 0.44 mmol). The reaction was stirred at room temperature under an atmosphere of nitrogen for 1 h then heated to 40 °C for 2 h. The reaction mixture was allowed to cool to room temperature then filtered to obtain an off-white precipitate. The precipitate was neutralised with 7 M ammonia in methanol and the solvent removed *in vacuo*. The product was purified by recrystallisation from propan-2-ol to give the title compound as a yellow solid (34.2 mg, 0.0958 mmol, 24%); m. p. 173–175 °C (from propan-2-ol); Rf 0.28 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 9.07 (d, *J* = 2.8 Hz, 1H, naphthyridine 7-H), 8.83 (dd, *J* = 8.8 Hz, 2.8 Hz, 1H, naphthyridine 5-H), 7.81 (d, *J* = 8.4 Hz, 2H, aniline 3-H), 7.64 (d, *J* = 8.4 Hz, 2H, aniline 2-H), 7.18 (s, 1H, naphthyridine 3-H), 6.93 (t, *J* = 53.5 Hz, 1H, CHF_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.3 (d, *J* = 255 Hz, C-F), 152.3 (sp^2 C), 151.7 (d, *J* = 3.4 Hz, sp^2 C), 145.9 (d, *J* = 27.3 Hz, naphthyridine 7-CH), 143.6 (sp^2 C), 127.3 (d, *J* = 3.9 Hz, sp^2 C), 126.9 (q, *J* = 3.5 Hz, aniline 3-CH), 126.5 (q, *J* = 270 Hz, CF_3), 125.1 (q, *J* = 32.3 Hz, aniline 4-C), 122.8 (aniline 2-CH), 117.7 (d, *J* = 20.6 Hz, naphthyridine 5-CH), 115.8 (d, *J* = 5.9 Hz, sp^2 C), 113.6 (t, *J* = 240 Hz, CHF_2), 99.3 (naphthyridine 3-CH); ^{19}F NMR (282 MHz, methanol-d_4) δ −64.1 (s, CF_3), −120.5 (d, *J* = 53.6 Hz, CHF_2), −125.3 (d, *J* = 9.0 Hz, C-F); ν_{max}/cm^{-1} (solid); 2844, 2588, 1594, 1562, 1509, 1460, 1406, 1320 and 1218; m/z (ES) found MH^+ 358.077161, $C_{16}H_9F_6N_3$ requires MH^+ 358.077343; HPLC 2.96 min.,100%.

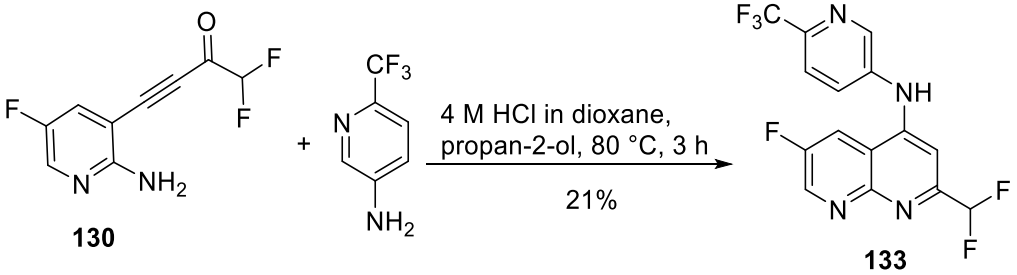
2-(Difluoromethyl)-6-fluoro-N-(3-fluoro-4-(trifluoromethyl)phenyl)-1,8-naphthyridin-4-amine (**132**)



To a solution of **130** (0.269 g, 1.258 mmol) and 4-amino-2-fluorobenzotrifluoride (0.220 g, 1.226 mmol) in propan-2-ol (8 mL) was added 4 M HCl in 1,4-dioxane (0.33 mL, 1.3 mmol). The reaction was stirred at 50 °C under an atmosphere of nitrogen for 4 h. The reaction mixture was allowed to cool to room temperature then concentrated *in vacuo*. The

resultant brown residue was neutralised with saturated aqueous sodium hydrogen carbonate (50 mL) and extracted with DCM (2 × 50 mL). The combined organic layers were evaporated *in vacuo* then purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:1). The solvent was removed *in vacuo* to give the title compound as a yellow solid (0.147 g, 0.392 mmol, 32%); m. p. 169–172 °C (from ethyl acetate); Rf 0.27 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 8.95 (d, *J* = 2.9 Hz, 1H, naphthyridine 7-H), 8.46 (dd, J = 9.0, 2.9 Hz, 1H, naphthyridine 5-H), 7.63 (t, *J* = 8.3 Hz, 1H, aniline 5-H), 7.44 (s, 1H, naphthyridine 3-H), 7.24 (m, 2H, aniline 2-H, 6-H), 6.66 (t, *J* = 55.1 Hz, 1H, CHF_2); ^{13}C NMR (75 MHz, methanol-d_4) δ 163.6 (d, *J* = 1.7 Hz, sp^2 C), 160.2 (t, *J* = 25.3 Hz, naphthyridine 2-C), 157.9 (d, *J* = 257 Hz, C-F), 155.7 (d, *J* = 204 Hz, C-F), 151.2 (d, *J* = 4.6 Hz, sp^2 C), 147.3 (d, *J* = 11.0 Hz, sp^2 C), 146.1 (d, *J* = 28.1 Hz, naphthyridine 7-CH), 129.5 (dq, *J* = 7.8, 3.8 Hz, aniline 5-CH), 124.1 (dq, J = 270, 2.2 Hz, CF_3), 121.3 (dq, *J* = 43.6, 4.5 Hz, aniline 4-C), 117.4 (d, *J* = 5.5 Hz, sp^2 C), 117.3 (d, *J* = 3.3 Hz, aniline 6-CH), 116.8 (d, *J* = 20.6 Hz, naphthyridine 5-CH), 115.1 (t, *J* = 243 Hz, CHF_2), 109.9 (d, *J* = 24.1 Hz, aniline 2-CH), 101.5 (t, *J* = 2.8 Hz, naphthyridine 3-CH); ^{19}F NMR (282 MHz, methanol-d_4) δ −62.2 (d, *J* = 12.2 Hz, CF_3), −114.5 – −114.7 (m, aniline C-F), −118.0 (d, *J* = 55.1 Hz, CHF_2), −128.0 (d, *J* = 9.0 Hz, naphthyridine C-F); ν_{max}/cm^{-1} (solid); 2844, 2588, 1594, 1562, 1509, 1460, 1406, 1320 and 1218; m/z (ES) found MH^+ 376.067709, $C_{16}H_8F_7N_3$ requires MH^+ 376.067921; HPLC 3.23 min., 100%.

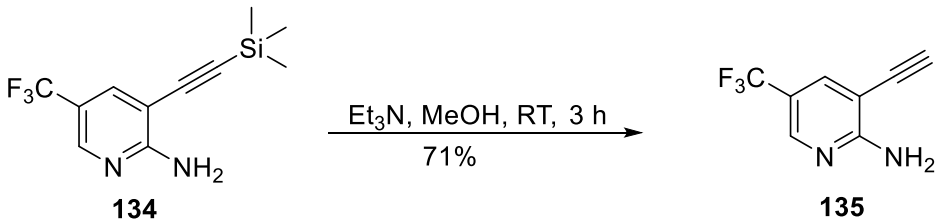
2-(Difluoromethyl)-6-fluoro-N-(6-(trifluoromethyl)pyridin-3-yl)-1,8-naphthyridin-4-amine (**133**)



To a solution of **130** (84.7 mg, 0.396 mmol) and 5-amino-2-(trifluoromethyl)pyridine (57.9 mg, 0.357 mmol) in propan-2-ol (2 mL) was added 4 M HCl in 1,4-dioxane (0.10 mL, 0.40 mmol). The reaction mixture was stirred at room temperature under an atmosphere of nitrogen for 66 h. The reaction mixture was concentrated *in vacuo*, neutralised with saturated aqueous sodium hydrogen carbonate (20 mL) and extracted with chloroform (2 × 30 mL). The combined organic layers were evaporated *in*

vacuo then purified by column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:1). The solvent was removed *in vacuo* to give the title compound as an orange solid (10.1 mg, 0.0282 mmol, 8%) and the intermediate 2-(difluoromethyl)-4-chloro-6-fluoro-1,8-naphthyridine (50.9 mg, 0.219 mmol, 61%). This intermediate was dissolved in propan-2-ol (1.5 mL), and 5-amino-2-(trifluoromethyl)pyridine (44.6 mg, 0.275 mmol) and 4 M HCl in 1,4-dioxane (60 µl, 0.24 mmol) were added. The mixture was stirred under reflux at 80 °C for 3 h then allowed to cool to room temperature. The resultant suspension was filtered to obtain an off-white precipitate which was neutralised with saturated aqueous sodium hydrogen carbonate solution (20 mL) and extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with brine (20 mL) then water (20 mL) and dried over magnesium sulphate. The solvent was removed *in vacuo* to leave the title compound as an orange solid. Total yield 26.7 mg, 0.0746 mmol, 21%; m. p. 180–190 °C decomposed (from ethyl acetate); Rf 0.22 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.97 (d, *J* = 2.9 Hz, 1H, naphthyridine 7-H), 8.65 (d, *J* = 2.6 Hz, 1H, pyridine 2-H), 8.50 (dd, *J* = 9.0, 2.9 Hz, 1H, naphthyridine 5-H), 7.95 (dd, *J* = 8.5, 2.6 Hz, 1H, pyridine 6-H), 7.79 (d, *J* = 8.5 Hz, 1H, pyridine 5-H), 7.37 (s, 1H, naphthyridine 3-H), 6.66 (t, *J* = 55.1 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 156.7 (d, *J* = 257 Hz, C-F), 155.8 (t, *J* = 26.6 Hz, naphthyridine 2-C), 153.0 (sp^2 C), 150.2 (d, *J* = 4.4 Hz, sp^2 C), 144.9 (d, *J* = 28.1 Hz, naphthyridine 7-CH), 143.0 (pyridine 2-CH), 142.3 (q, *J* = 35.1 Hz, pyridine 4-C), 139.9 (sp^2 C), 128.7 (pyridine 6-CH), 121.7 (q, *J* = 273 Hz, CF_3), 121.4 (q, *J* = 2.7 Hz, pyridine 5-CH), 116.1 (d, *J* = 6.1 Hz, sp^2 C), 115.5 (d, *J* = 20.7 Hz, naphthyridine 5-CH), 113.7 (t, *J* = 241 Hz, CHF_2), 99.5 (naphthyridine 3-CH); ^{19}F NMR (376 MHz, methanol-d_4) δ −68.7 (CF_3), −117.9 (d, *J* = 55.2 Hz, CHF_2), −127.8 (d, *J* = 9.1 Hz, C-F); ν_{max}/cm^{-1} (solid); 2925, 1561, 1540, 1336, 1315 and 1238; m/z (ES) found MH^+ 359.073046, $C_{15}H_8F_6N_4$ requires MH^+ 359.072592; HPLC 2.76 min., 100%.

3-Ethynyl-5-(trifluoromethyl)pyridin-2-amine (**135**)

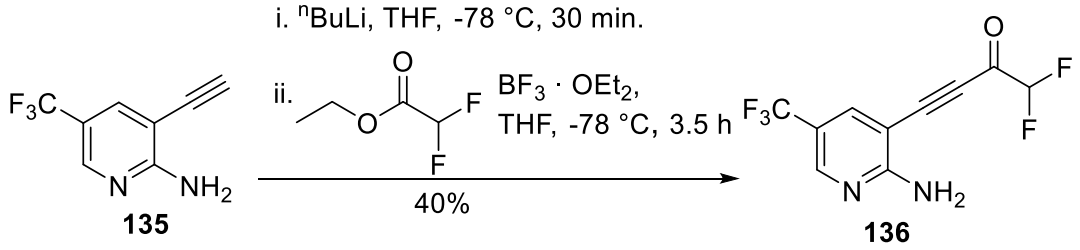


134 **135**

To a stirred suspension of **134** (1.071 g, 4.145 mmol) in methanol (10 mL) was added triethylamine (0.59 mL, 4.2 mmol). The reaction mixture was

stirred at room temperature for 3 h then concentrated *in vacuo* while maintaining the temperature below 30 °C. The resultant residue was dissolved in diethyl ether (2 mL) and cooled to 0 °C. Hexane (16 mL) was added in 2 mL aliquots with stirring and the resultant solid collected using vacuum filtration. The title compound was obtained as colourless needles (0.546 g, 2.931 mmol, 71%); m. p. 103–105 °C (from diethyl ether-hexane); Rf 0.25 (1:5 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (s, 1H, 6-H), 7.77 (d, *J* = 2.3 Hz, 1H, 4-H), 5.43 (s, 2H, NH_2), 3.46 (s, 1H, C≡C-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.0 (2-C), 146.0 (q, *J* = 4.2 Hz, 6-CH), 137.7 (q, *J* = 3.3 Hz, 4-CH), 123.8 (q, *J* = 271 Hz, CF_3), 116.5 (q, *J* = 33.3 Hz, 5-C), 101.4 (3-C), 84.6 (C≡C-H), 77.8 (C≡C); ^{19}F NMR (282 MHz, $CDCl_3$) δ −61.4 (s, CF_3); ν_{max}/cm^{-1} (solid); 3478, 3307, 3151, 3120, 1637, 1598, 1565, 1496, 1425, 1355, 1331, 1299 and 1266; m/z (ES) found MH^+ 187.047443, $C_8H_5F_3N_2$ requires MH^+ 187.047759; HPLC 1.99 min., 100%.

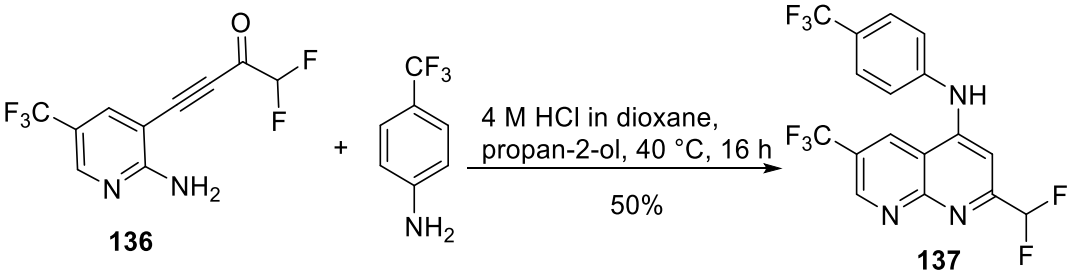
4-(2-Amino-5-(trifluoromethyl)pyridin-3-yl)-1,1-difluorobut-3-yn-2-one (**136**)



135 (0.298 g, 1.604 mmol) was dissolved in dry THF (20 mL) under an atmosphere of nitrogen and cooled to −78 °C. To the stirred solution was added ^{n}butyllithium (2.6 mL, 4.2 mmol) and stirred for 30 minutes, then a solution of boron trifluoride etherate (0.24 mL, 2.0 mmol) and ethyl difluoroacetate (0.19 mL, 1.8 mmol) in dry THF (20 mL) was added dropwise. The reaction mixture was stirred under nitrogen at −78 °C for 3.5 h then saturated aqueous ammonium chloride (8 mL) was added. The slurry was allowed to warm to room temperature then the solvent removed *in vacuo*. Diethyl ether (50 mL) was added and the solution washed with brine (2 × 20 mL) and dried over anhydrous magnesium sulphate. The solvent was removed *in vacuo* to leave a brown solid which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:1) afforded the title compound as a brown solid (0.167 g, 0.634 mmol, 40%); m. p. 117–121 °C decomposed (from ethyl acetate); Rf 0.35 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (d, *J* = 1.8 Hz, 1H, 6-H), 7.94 (d, *J* = 1.8 Hz, 1H, 4-H), 5.89 (t, *J* = 54.2 Hz, 1H, CHF_2), 5.66 (s, 2H, NH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.6 (C=O), 161.8 (2-C), 149.8 (q, *J* =

3.9 Hz, 6-CH), 140.2 (q, *J* = 3.7 Hz, 4-CH), 123.3 (q, *J* = 271 Hz, CF_3), 117.3 (q, *J* = 34.3 Hz, 5-C), 108.8 (t, *J* = 253 Hz, CHF_2), 97.0 (3-C), 93.1 (C≡C), 77.2 (C≡C); ^{19}F NMR (282 MHz, $CDCl_3$) δ −61.6 (s, CF_3) , −124.9 (d, *J* = 54.2 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3411, 3308, 3163, 2172, 1689, 1646, 1603, 1557, 1505, 1433, 1340, 1293 and 1248; m/z (ES) found MH^+ 265.039178, $C_{10}H_5F_5N_2O$ requires MH^+ 265.039480; HPLC 2.02 min., 97%.

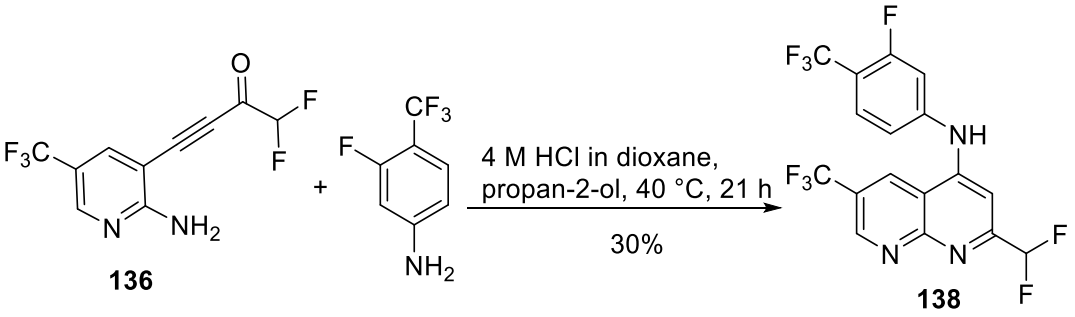
2-(Difluoromethyl)-6-(trifluoromethyl)-N-(4-(trifluoromethyl)phenyl)-1,8-naphthyridin-4-amine (**137**)



136 (0.144 g, 0.546 mmol) and 4-(trifluoromethyl)aniline (60 µL, 0.48 mmol) were dissolved in propan-2-ol (5 mL). To the stirred solution was added 4 M HCl in 1,4-dioxane (0.14 mL, 0.56 mmol) and the mixture was stirred at 40 °C for 16 h under an atmosphere of nitrogen. The reaction mixture was concentrated *in vacuo* then neutralised with saturated aqueous sodium hydrogen carbonate solution (20 mL) and extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with water (20 mL) and dried over anhydrous magnesium sulphate. The solvent was removed *in vacuo* to leave a brown solid which was dissolved in ethyl acetate (4 mL) and cooled to 0 °C. Pet. ether 40–60 was added until a precipitate formed which was isolated using vacuum filtration which afforded the title compound as an orange solid (96.6 mg, 0.237 mmol, 50%); m. p. 198–203 °C decomposed (from ethyl acetate-pet. ether 40–60); Rf 0.56 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 9.23 (s, 1H, naphthyridine 5-H), 9.21 (s, 1H, naphthyridine 7-H), 7.70 (d, *J* = 8.2 Hz, 2H, aniline 3-H), 7.52 (d, *J* = 8.2 Hz, 2H, aniline 2-H), 7.31 (s, 1H, naphthyridine 3-H), 6.64 (t, *J* = 55.0 Hz, 1H, CHF_2); ^{13}C NMR (75 MHz, methanol-d_4) δ 161.2 (sp^2 C), 158.4 (sp^2 C), 153.5 (sp^2 C), 150.9 (q, *J* = 4.1 Hz, naphthyridine 7-CH), 144.0 (t, *J* = 1.4 Hz, naphthyridine 2-C), 132.5 (q, *J* = 4.4 Hz, naphthyridine 5-CH), 128.6 (q, *J* = 32.6 Hz, C-CF_3), 127.9 (q, *J* = 4.4 Hz, aniline 3-CH), 125.5 (q, *J* = 271 Hz, CF_3), 124.9 (q, *J* = 272 Hz, CF_3), 124.8 (q, *J* = 28.2 Hz, C-CF_3), 123.9 (aniline 2-CH), 115.0 (sp^2 C), 114.9 (t, *J* = 242 Hz, CHF_2), 100.3 (naphthyridine 3-CH); ^{19}F NMR (282 MHz, methanol-d_4) δ −63.2 (s, CF_3), −63.8 (s, CF_3), −118.9 (d, *J* = 55.1 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2962, 1740, 1623, 1595, 1562, 1520,

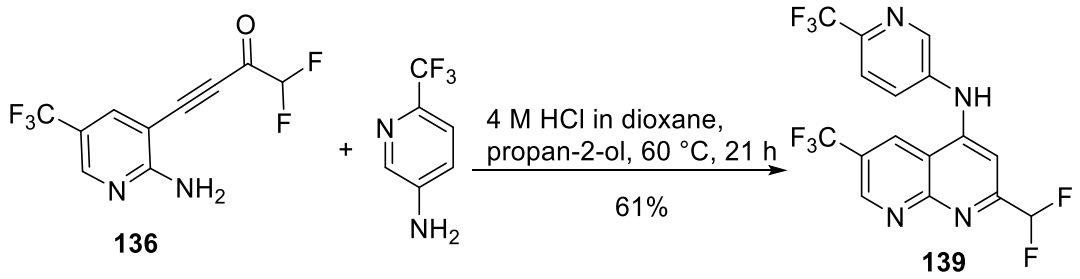
1412, 1369, 1316, 1295, 1257 and 1221; m/z (ES) found MH^+ 408.073493, $C_{17}H_9F_8N_3$ requires MH^+ 408.074150; HPLC 3.54 min., 100%.

2-(Difluoromethyl)-N-(3-fluoro-4-(trifluoromethyl)phenyl)-6-(trifluoromethyl)-1,8-naphthyridin-4-amine (**138**)



136 (87.0 mg, 0.330 mmol) and 4-amino-2-fluorobenzotrifluoride (56.1 mg, 0.313 mmol) were dissolved in propan-2-ol (2 mL). To the stirred solution was added 4 M HCl in 1,4-dioxane (90 µL, 0.36 mmol) and the mixture was stirred at 40 °C for 21 h under an atmosphere of nitrogen. The reaction mixture was allowed to cool to room temperature then filtered to obtain a yellow precipitate which was neutralised with saturated aqueous sodium hydrogen carbonate solution (20 mL) and extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with water (20 mL) and dried over anhydrous magnesium sulphate. The solvent was removed *in vacuo* to leave the title compound as a yellow solid (40.2 mg, 0.0946 mmol, 30%); m. p. 170–180 °C decomposed (from ethyl acetate-pet. ether 40–60); Rf 0.33 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 9.22 (d, *J* = 2.4 Hz, 1H, naphthyridine 5-H), 9.20 (s, 1H, naphthyridine 7-H), 7.67 (t, *J* = 8.3 Hz, 1H, aniline 5-H), 7.44 (s, 1H, naphthyridine, 3-H), 7.33 – 7.27 (m, 2H, aniline 2-H, 6-H), 6.68 (t, *J* = 55.0 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.2 (d, *J* = 253 Hz, C-F), 158.2 (t, *J* = 24.5 Hz, naphthyridine 2-C), 157.7 (sp^2 C), 150.8 (sp^2 C), 150.4 (q, *J* = 3.0 Hz, naphthyridine 7-CH), 146.3 (d, *J* = 11.0 Hz, aniline 1-C), 132.3 (q, *J* = 3.3 Hz, naphthyridine 5-CH), 129.5 – 128.3 (m, aniline 4-C, 5-CH), 124.1 (q, *J* = 273 Hz, CF_3), 123.3 (q, *J* = 271 Hz, CF_3), 122.6 (q, J = 32 Hz, naphthyridine 6-C), 117.5 (d, *J* = 2.9 Hz, aniline 6-H), 114.3 (sp^2 C), 113.9 (t, *J* = 241 Hz, CHF_2), 109.6 (d, *J* = 23.5 Hz, aniline 2-CH), 101.6 (t, J = 3.0 Hz, naphthyridine 3-CH); ^{19}F NMR (376 MHz, methanol-d_4) δ −62.2 (d, *J* = 12.5 Hz, aniline CF_3), −63.1 (s, naphthyridine CF_3), −114.4 (m, C-F), −118.8 (d, *J* = 55.0 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3221, 2933, 1628, 1598, 1562, 1515, 1452, 1412, 1369 and 1309; m/z (ES) found MNa^+ 448.046610, $C_{17}H_8F_9N_3$ requires MNa^+ 448.046672; HPLC 3.77 min., 93%.

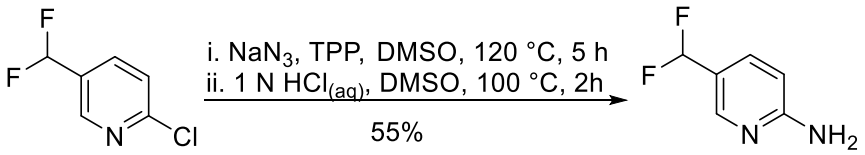
2-(Difluoromethyl)-6-(trifluoromethyl)-N-(6-(trifluoromethyl)pyridin-3-yl)-1,8-naphthyridin-4-amine (**139**)



136 (89.4 mg, 0.339 mmol) and 5-amino-2-trifluoropyridine (52.3 mg, 0.323 mmol) were dissolved in propan-2-ol (2 mL). To the stirred solution was added 4 M HCl in 1,4-dioxane (90 µL, 0.36 mmol) and the mixture was stirred at 60 °C for 21 h under an atmosphere of nitrogen. The reaction mixture was allowed to cool to room temperature then filtered to obtain a white precipitate which was neutralised with saturated aqueous sodium hydrogen carbonate solution (20 mL) and extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with water (20 mL) and dried over anhydrous magnesium sulphate. The solvent was *removed in vacuo* to leave the title compound as a pale orange solid (80.9 mg, 0.198 mmol, 61%); m. p. 204–206 °C (from ethyl acetate); Rf 0.34 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (300 MHz, methanol-d_4) δ 9.24 (s, 2H, naphthyridine 7-H, 5-H), 8.70 (d, *J* = 2.5 Hz, 1H, pyridine 2-H), 8.01 (dd, *J* = 8.5, 2.5 Hz, 1H, pyridine 6-H), 7.82 (d, *J* = 8.5 Hz, 1H, pyridine 5-H), 7.39 (s, 1H, naphthyridine 3-H), 6.68 (t, *J* = 55.0 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 158.4 (t, *J* = 26.0 Hz, naphthyridine 2-C), 157.1 (sp^2 C), 151.5 (sp^2 C), 149.8 (q, *J* = 3.4 Hz, naphthyridine 7-CH), 143.7 (pyridine 2-CH), 143.1 (q, *J* = 35.1 Hz, pyridine 4-C), 139.4 (sp^2 C), 131.2 (q, *J* = 4.2 Hz, naphthyridine 5-CH), 129.7 (pyridine 6-CH), 123.7 (q, *J* = 33.5 Hz, naphthyridine 6-C)), 123.5 (q, *J* = 272 Hz, CF_3), 121.6 (q, *J* = 273 Hz, CF_3), 121.4 (q, *J* = 2.5 Hz, pyridine 5-CH), 114.1 (sp^2 C), 113.5 (t, *J* = 242 Hz, CHF_2), 99.6 (naphthyridine 3-CH); ^{19}F NMR (282 MHz, methanol-d_4) δ −63.2 (CF_3), −68.9 (CF_3), −118.9 (d, *J* = 55.1 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3251, 1629, 1586, 1560, 1498, 1405, 1368, 1336, 1311, 1251 and 1201; m/z (ES) found MH^+ 409.069141, $C_{16}H_8F_8N_4$ requires MH^+ 409.069398; HPLC 3.20 min., 100%.

5-(Difluoromethyl)pyridin-2-amine (**140**)

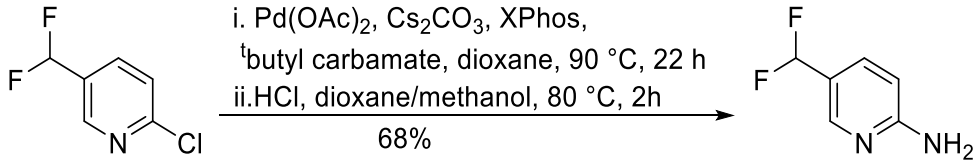
Method 1:



150 **140**

150 (0.902 g, 5.512 mmol), sodium azide (0.721 g, 11.10 mmol) and triphenylphosphine (2.892 g, 11.024 mmol) were dissolved in DMSO (30 mL). The reaction mixture was stirred at 120 °C for 5 h. To this solution was added hydrochloric acid (1 M, 10 mL) and the mixture was stirred at 100 °C for 2 h. The reaction mixture was allowed to cool to room temperature and diluted with hydrochloric acid (1 M, 10 mL) then poured into water (50 mL) and washed with ethyl acetate (2 × 30 mL) to remove triphenylphosphine oxide. The aqueous layer was slowly neutralised with saturated aqueous sodium hydrogen carbonate solution and extracted with ethyl acetate (2 × 30 mL). The combined organic layer was washed with water (30 mL) and brine (30 mL), dried over sodium sulphate, filtered and concentrated *in vacuo*. The residue was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:1). The solvent was removed *in vacuo* to leave the title compound as a colourless solid (0.324 g, 2.247 mmol, 55%);

Method 2:

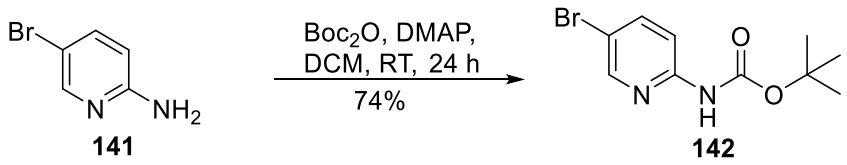


150 **140**

Anhydrous 1,4-dioxane (20 mL) was added to XPhos (1.086 g, 2.277 mmol) and palladium acetate (0.264 g, 1.175 mmol) and the mixture was stirred at room temperature for 45 minutes. The solution was added to a mixture of **150** (3.482 g, 21.282 mmol), ^{t}butyl carbamate (3.935 g, 33.592 mmol) and caesium carbonate (13.760 g, 42.231 mmol) in 1,4-dioxane (20 mL) and the reaction mixture was stirred at 90 °C for 22 h under an atmosphere of nitrogen. The mixture was allowed to cool to room temperature then diluted with ethyl acetate (100 mL) and washed with saturated aqueous ammonium chloride (2 × 100 mL) and water (100 mL). The organic layer was dried over sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The resultant orange residue was dissolved in methanol (40 mL) and 4 M HCl in 1,4-dioxane (40 mL) was added. The mixture was stirred at 80 °C for 2 h

then allowed to cool to room temperature. Water (100 mL) was added and the aqueous layer was washed with ethyl acetate (2 × 100 mL). The aqueous layer was basified to pH 9 with solid sodium hydroxide then extracted with ethyl acetate (3 × 100 mL). The combined organic layer was dried over sodium sulphate, filtered and evaporated under reduced pressure to give the title compound as a colourless solid (2.074 g, 14.402 mmol, 68%); m. p. 100–110 °C decomposed (from ethyl acetate); Rf 0.11 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, *J* = 2.4 Hz, 1H, 6-H), 7.58 (dd, *J* = 8.6, 2.4 Hz, 1H, 4-H), 6.56 (t, *J* = 56.3 Hz, 1H, CHF_2), 6.53 (d, *J* = 8.6 Hz, 1H, 3-H), 4.81 (br s, 2H, NH_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.1 (2-C), 146.5 (t, *J* = 7.3 Hz, 6-CH), 135.1 (t, *J* = 4.5 Hz, 4-CH), 120.3 (t, *J* = 23.6 Hz, 5-C), 114.2 (t, *J* = 237 Hz, CHF_2), 108.3 (3-C); ^{19}F NMR (376 MHz, $CDCl_3$) δ −108.7 (d, *J* = 56.1 Hz, CHF_2).; ν_{max}/cm^{-1} (solid); 3483, 3310, 3149, 1634, 1608, 1565, 1515, 1455, 1369, 1336, 1286 and 1234; m/z (ES) found MH^+ 145.056650, $C_6H_5{}^{35}ClF_2N$ requires MH^+ 145.057181; HPLC 0.57 min., 93%.

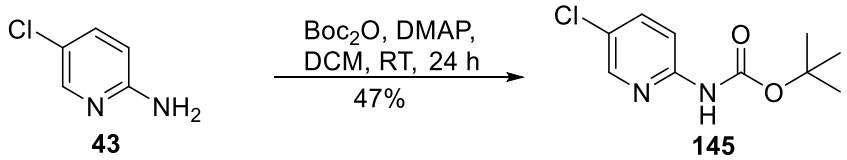
^{t}Butyl (5-bromopyridin-2-yl)carbamate (**142**)



141 **142**

To a solution of 5-bromopyridin-2-amine (0.920 g, 5.320 mmol) in DCM (10 mL) was added DMAP (1.067 g, 8.395 mmol) and di-*tert*-butyl dicarbonate (1.5 mL, 6.5 mmol). The reaction mixture was stirred at room temperature for 24 h. The solvent was removed *in vacuo* and the resultant residue purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:2). The solvent was removed *in vacuo* to give the title compound as a colourless solid (1.045 g, 3.828 mmol, 74%); m. p. 156–159 °C (from ethyl acetate-pet. ether 40–60) (lit. 173–175 °C[160]); Rf 0.58 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.06 (br s, 1H, N-H), 8.39 (dd, *J* = 2.5, 0.7 Hz, 1H, 6-H), 7.95 (d, *J* = 9.0 Hz, 1H, 3-H), 7.76 (ddd, *J* = 9.0, 2.5, 0.6 Hz, 1H, 4-H), 1.56 (s, 9H, CH_3) consistent with lit.;[161] ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.5 (C=O), 151.3 (2-C), 148.5 (6-CH), 140.7 (4-CH), 113.8 (3-CH), 112.9 (5-C), 81.4 (C-O), 28.4 (CH_3); ν_{max}/cm^{-1} (solid); 2981, 1752, 1716, 1580, 1525, 1464, 1365, 1302, 1248 and 1223; m/z (ES) found MNa^+ 295.004833, $C_{10}H_{13}{}^{79}BrN_2O_2$ requires MNa^+ 295.005260; HPLC 1.18 min., 91%.

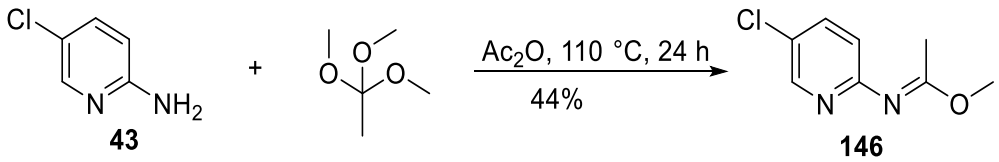
*t*Butyl (5-chloropyridin-2-yl)carbamate (**145**)



43 **145**

To a solution of 5-chloropyridin-2-amine (0.403 g, 3.134 mmol) in DCM (6 mL) was added DMAP (0.614 g, 5.025 mmol) and di-*tert*-butyl dicarbonate (0.870 mL, 3.79 mmol). The reaction mixture was stirred at room temperature for 24 h. The solvent was removed *in vacuo* and the resultant residue purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:5–1:2). The solvent was removed *in vacuo* to give the title compound as a colourless solid (0.339 g, 1.481 mmol, 47%); m. p. 154–6 °C (from ethyl acetate-pet. ether 40–60); Rf 0.59 (1:4 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (br s, 1H, N-H), 8.26 (d, *J* = 2.6 Hz, 1H, 6-H), 7.97 (d, *J* = 9.0 Hz, 1H, 3-H), 7.63 (dd, *J* = 9.0, 2.6 Hz, 1H, 4-H), 1.55 (s, 9H, CH_3) consistent with lit.;[162] ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.4 (C=O), 150.7 (2-C), 146.3 (6-CH), 137.9 (4-CH), 125.3 (5-C), 113.1 (3-CH), 81.4 (C-O), 28.3 (CH_3); ν_{max}/cm^{-1} (solid); 2975, 2933, 1718, 1584, 1519, 1466, 1380, 1366, 1305, 1286, 1250 and 1225; m/z (ES) found MH^+ 251.055247, $C_{10}H_{13}{}^{35}ClN_2O_2$ requires MH^+ 251.055776; HPLC 3.31 min., 92%.

(E)-(Methyl N-(5-chloropyridin-2-yl)ethanimidate) (**146**)

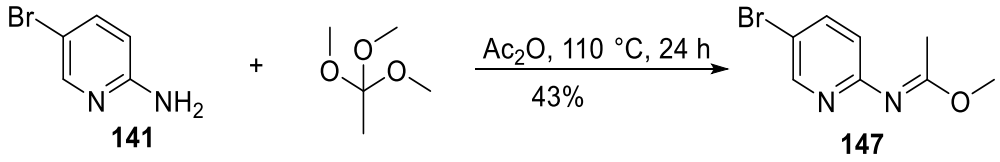


43 **146**

5-Chloro-pyridin-2-amine (0.172 g, 0.932 mmol) was heated to 110 °C under reflux for 24 h in a mixture of trimethyl orthoacetate (2.5 mL) and acetic anhydride (1 mL). The solution was concentrated in vacuo to leave a brown residue which was dissolved in diethyl ether (20 mL) and washed with 2 M aqueous sodium carbonate (2 × 10 mL), water (10 mL) and brine (2 × 10 mL) then dried over sodium sulphate. The solvent was removed *in vacuo* and the resultant residue purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:5–1:3). The solvent was removed *in vacuo* to give the title compound as a colourless oil (75.9 mg, 0.411 mmol, 44%); Rf 0.49 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (dd, *J* = 2.6, 0.7 Hz, 1H, 6-H), 7.59 (dd, *J* = 8.5, 2.6 Hz, 1H, 4-H), 6.75 (dd, *J* = 8.5, 0.7 Hz, 1H, 2-H), 3.82 (s, 3H, O-CH_3), 1.92 (s, 3H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.5 (sp^2 C), 159.5 (sp^2 C), 147.2 (6-CH), 137.6 (4-CH), 126.4 (sp^2

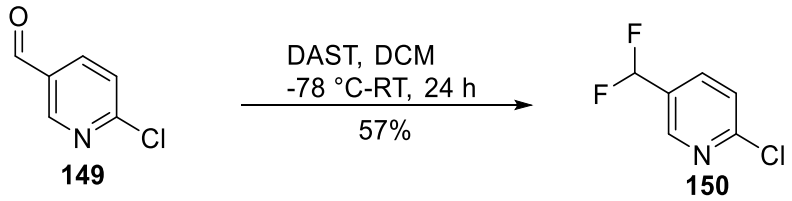
C), 117.6 (3-CH), 53.7 (O-CH_3), 16.8 (CH_3); ν_{max}/cm^{-1} (solid); 2947, 1664, 1579, 1552, 1456, 1438, 1369, 1291, 1256, 1221; m/z (ES) found MH^+ 129.020974, $C_5H_5{}^{35}ClN_2$ requires MH^+ 129.021402; HPLC 0.52 min., 79%.

(E)-(Methyl N-(5-bromopyridin-2-yl)ethanimidate) (**147**)



5-Bromo-pyridin-2-amine (0.178 g, 1.030 mmol) was heated to 110 °C under reflux for 24 h in a mixture of trimethyl orthoacetate (2.5 mL) and acetic anhydride (1 mL). The solution was concentrated *in vacuo* to leave a brown residue which was dissolved in diethyl ether (20 mL) and washed with 2 M aqueous sodium carbonate (2 × 20 mL), water (20 mL) and brine (20 mL) then dried over sodium sulphate. The solvent was removed *in vacuo* and the resultant residue was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:5–1:3). The solvent was removed *in vacuo* to give the title compound as a colourless oil (0.102 g, 0.444 mmol, 43%); Rf 0.33 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (501 MHz, $CDCl_3$) δ 8.42 (dd, *J* = 2.5, 0.7 Hz, 1H, 6-H), 7.72 (dd, *J* = 8.5, 2.5 Hz, 1H, 4-H), 6.71 (dd, *J* = 8.5, 0.7 Hz, 1H, 3-H), 3.82 (s, 3H, O-CH_3), 1.92 (s, 3H, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.4 (sp^2 C), 159.9 (sp^2 C), 149.6 (6-CH), 140.3 (4-CH), 118.2 (3-CH), 114.5 (sp^2 C), 53.7 (O-CH_3), 16.8 (CH_3); ν_{max}/cm^{-1} (solid); 2945, 1661, 1571, 1547, 1527, 1456, 1436, 1365, 1290, 1254 and 1220; m/z (ES) found MH^+ 172.970664, $C_5H_5{}^{79}BrN_2$ requires MH^+ 172.970887; HPLC 0.68 min., 90%.

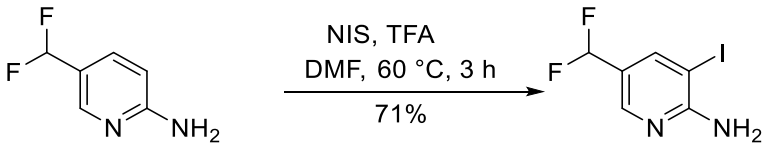
2-Chloro-5-(difluoromethyl)pyridine (**150**)



To a solution of 2-chloropyridine-5-carboxaldehyde (5.264 g, 37.184 mmol) in dry DCM (50 mL) at −78 °C under an atmosphere of nitrogen was added dropwise (diethylamino)sulphurtrifluoride (10 mL, 76 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 24 h. The reaction mixture was diluted with DCM (20 mL), cooled in ice, then aqueous sodium carbonate solution (2 M, 40 mL) was added dropwise. The resultant mixture was stirred for 10 minutes then the layers were separated. The

organic layer was washed with water (40 mL) and brine (40 mL), dried over magnesium sulphate, filtered and concentrated *in vacuo*. The residue was purified using column chromatography; elution with diethyl ether-hexane (0–10% ether). The solvent was removed *in vacuo* to leave the title compound as a colourless liquid (3.489 g, 21.325 mmol, 57%); Rf 0.59 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (s, 1H, 6-H), 7.82 (dd, *J* = 7.9, 2.1 Hz, 1H, 4-H), 7.45 (d, *J* = 8.3 Hz, 1H, 3-H), 6.71 (t, *J* = 55.6 Hz, 1H, CHF_2) consistent with lit.;[163] ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.0 (2-C), 147.1 (t, *J* = 6.9 Hz, 6-CH), 136.0 (t, *J* = 5.1 Hz, 4-CH), 129.0 (t, *J* = 23.5 Hz, 5-C), 124.6 (3-CH), 112.7 (t, *J* = 240 Hz, CHF_2); ^{19}F NMR (376 MHz, $CDCl_3$) δ −112.4 (d, *J* = 55.9 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2981, 1597, 1573, 1464, 1406, 1348, 1290, 1243 and 1223; m/z (ES) found MH^+ 164.006715, $C_6H_5{}^{35}ClF_2N$ requires MH^+ 164.007310; HPLC 2.10 min., 93%.

5-(Difluoromethyl)-3-iodopyridin-2-amine (**153**)

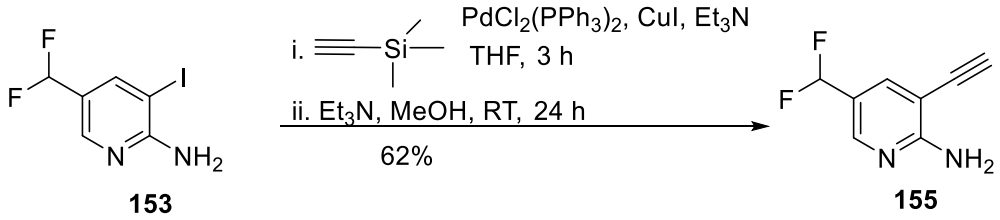


140 **153**

N-Iodosuccinimide (0.596 g, 2.650 mmol) was added to DMF (3 mL) portionwise at room temperature with stirring. To the resultant solution was added **140** (0.318 g, 2.207 mmol) and the mixture was stirred under an atmosphere of nitrogen for 30 minutes. To the resultant suspension was added trifluoroacetic acid (0.21 mL, 2.7 mmol). After 10 minutes at room temperature the mixture was stirred at 60 °C under nitrogen for 3 h. The reaction mixture was poured into water (30 mL) overlaid with diethyl ether (20 mL) with rapid stirring. Excess *N*-iodosuccinimide was destroyed by adding solid sodium bisulphite (0.255 g) followed by solid sodium bicarbonate until effervescence ceased. Layers were separated and the aqueous layer was extracted with diethyl ether (2 × 20 mL). Combined organic extracts were washed with water (20 mL) and brine (20 mL), dried over sodium sulphate and evaporated *in vacuo*. The residue was purified using column chromatography; elution with diethyl ether-pet. ether 40–60 (1:2–1:1) to give the title compound as a colourless solid (0.424 g, 1.571 mmol, 71%), m. p. 102–104 °C (from diethyl ether); Rf 0.24 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, *J* = 1.9 Hz, 1H, 6-H), 8.01 (s, 1H, 4-H), 6.53 (t, *J* = 56.1 Hz, 1H, CHF_2), 5.32 (s, 2H, NH_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.8 (2-C), 146.5 (t, *J* = 6.8 Hz, 6-CH), 144.5 (t, *J* = 4.9 Hz, 4-CH), 120.2 (t, *J* = 23.4 Hz, 5-C), 114.2 (t, *J* = 234 Hz, CHF_2), 77.1 (3-C); ^{19}F NMR (376 MHz, $CDCl_3$) δ

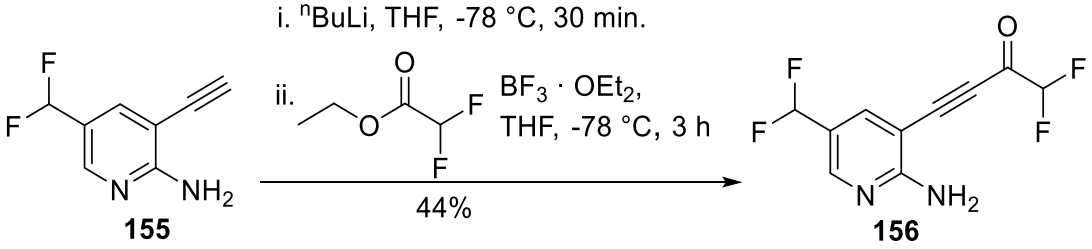
−109.1 (d, *J* = 56.2 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3445, 3289, 3130 (br), 1632, 1569, 1454, 1384 and 1236; m/z (ES) found MH^+ 270.953982, $C_6H_5F_2IN_2$ requires MH^+ 270.953829; HPLC 2.10 min., 100%.

5-(Difluoromethyl)-3-ethynylpyridin-2-amine (**155**)



153 (0.484 g, 1.793 mmol) was flushed with nitrogen then ethynyltrimethylsilane (0.75 mL, 5.4 mmol), triethylamine (1.2 mL, 8.5 mmol) and dry THF (10 mL) were added. The reaction mixture was cooled using a water bath at room temperature as copper(I) iodide (20.0 mg, 0.105 mmol) and bis(triphenylphosphine)palladium(II) dichloride (37.3 mg, 0.0531 mmol) were added. The mixture was stirred at room temperature under an atmosphere of nitrogen for 3 h then poured into diethyl ether (20 mL) and washed with water (3 × 20 mL). The organic layer was dried over sodium sulphate and evaporated *in vacuo* to leave the crude intermediate as a brown solid, which was dissolved in methanol (10 mL). Triethylamine (0.20 mL, 1.4 mmol) was added and the mixture stirred at room temperature for 24 h. The solvent was removed *in vacuo* at room temperature and the black residue purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:2–1:1) to give the title compound (0.187 g, 1.111 mmol, 62%) as brown needles, m. p. 69–71 °C (from ethyl acetate), Rf 0.25 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (q, *J* = 1.9 Hz, 1H, 6-H), 7.72 (d, *J* = 2.2 Hz, 1H, 4-H), 6.55 (t, *J* = 56.2 Hz, 1H, CHF_2), 5.31 (s, 2H, NH_2), 3.45 (s, 1H, C≡C-H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.6 (2-C), 146.5 (t, *J* = 6.9 Hz, 6-CH), 138.0 (t, *J* = 4.8 Hz, 4-CH), 120.1 (t, *J* = 23.8 Hz, 5-C), 113.4 (t, *J* = 237.7 Hz, CHF_2), 101.7 (3-C), 84.3 (C≡C-H), 78.3 (C≡C); ^{19}F NMR (376 MHz, $CDCl_3$) δ −109.4 (d, *J* = 57.1 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3465, 3298, 3154, 1628, 1572, 1487, 1437, 1366, 1301, and 1226; m/z (ES) found MH+ 169.057041, $C_8H_6F_2N_2$ requires MH^+ 169.057181. HPLC 1.07 min., 86%, 1.13 min., 14%.

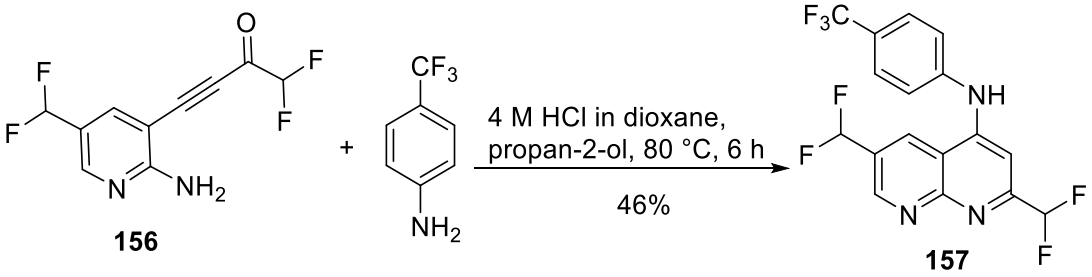
4-[2-amino-5-(difluoromethyl)pyridin-3-yl]-1,1-difluorobut-3-yn-2-one (**156**)



155 **156**

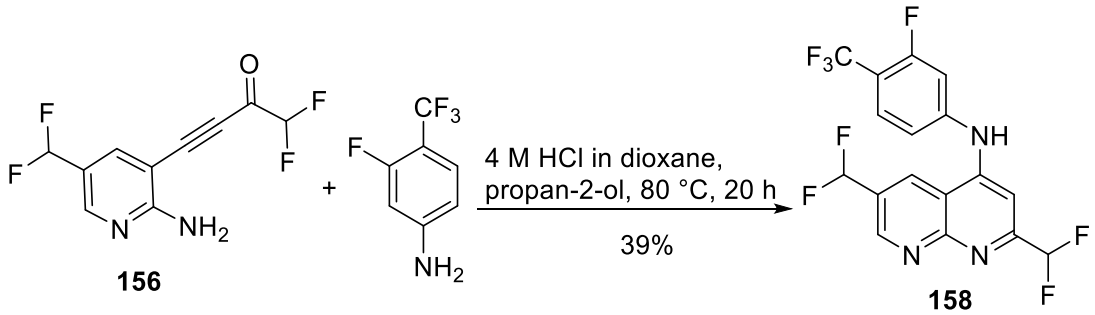
155 (0.289 g, 1.718 mmol) was dissolved in dry THF (20 mL) under an atmosphere of nitrogen. A 1.6 M solution of ^{n}butyllithium in hexanes (2.8 ml, 4.5 mmol) was added and the solution was stirred at −78 °C for 30 minutes. A solution of ethyl difluoroacetate (0.21 mL, 2.0 mmol) and boron trifluoride diethyl etherate (0.26 mL, 2.1 mmol) in dry THF (10 mL) was added and the mixture was stirred at −78 °C for 3 h then quenched with saturated aqueous ammonium chloride solution (10 mL). The slurry was allowed to warm to room temperature then the THF was removed *in vacuo*. The residue was diluted with diethyl ether (60 mL), washed with brine (2 × 50 mL) and dried over magnesium sulphate. The solvent was removed *in vacuo* to leave a brown residue which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:2–1:1). The solvent was removed *in vacuo* to leave the title compound as a brown solid (0.185 g, 0.752 mmol, 44%); m. p. 107–109 °C (from ethyl acetate); Rf 0.23 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36 (d, *J* = 2.2 Hz, 1H, 6-H), 7.99 (d, *J* = 2.2 Hz, 1H, 4-H), 7.29 (br s, 2H, NH_2), 6.93 (t, *J* = 55.6 Hz, 1H, CHF_2), 6.59 (t, *J* = 53.5 Hz, 1H, CHF_2); ^{19}F NMR (376 MHz, DMSO-d_6) δ −108.3 (d, *J* = 55.5 Hz, CHF_2), −127.0 (d, *J* = 54.0 Hz, CHF_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.9 (C=O), 162.4 (2-C), 151.1 (t, *J* = 5.7 Hz, 6-CH), 141.3 (4-CH), 119.0 (t, *J* = 23.7 Hz, 5-C), 114.3 (t, *J* = 234 Hz, CHF_2), 109.4 (t, *J* = 249 Hz, CHF_2), 95.7 (3-C), 94.9 (C≡C), 90.9 (C≡C); ν_{max}/cm^{-1} (solid); 3421, 3161, 2173, 2153, 1684, 1643, 1603, 1558, 1499, 1371, 1349, 1311, 1248 and 1205; m/z (ES) found MH^+ 247.048173, $C_{10}H_6F_4N_2O$ requires MH^+ 247.048902; HPLC 1.04 min., 94%.

2,6-Bis(difluoromethyl)-N-[4-(trifluoromethyl)phenyl]-1,8-naphthyridin-4-amine (**157**)



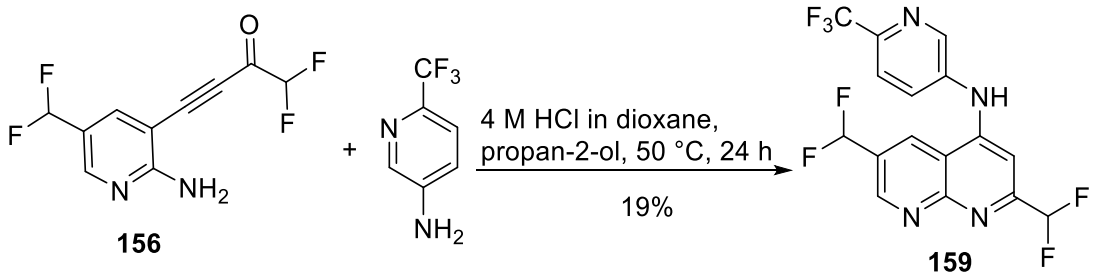
To a brown solution of **156** (0.101 g, 0.411 mmol) in propan-2-ol (10 mL) was added 4-(trifluoromethyl)aniline (51 μL, 0.41 mmol) followed by 4 M HCl in 1,4-dioxane (115 μL, 0.460 mmol). The reaction was stirred at 80 °C for 6 h then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave a brown solid which was neutralised with saturated aqueous sodium hydrogen carbonate (30 mL) then extracted with chloroform (2 × 50 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:2–1:1). The solvent was removed *in vacuo* to leave the title compound as a yellow solid (72.8 mg, 0.187 mmol, 46%), m. p. 200–202 °C (from ethyl acetate-pet. ether 40–60); Rf 0.49 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 9.10 (d, *J* = 2.2 Hz, 1H, naphthyridine 7-H), 9.01 (d, *J* = 2.2 Hz, 1H, naphthyridine 5-H), 7.68 (d, *J* = 8.3 Hz, 2H, aniline 3-H), 7.51 (d, *J* = 8.3 Hz, 2H, aniline 2-H), 7.30 (s, 1H, naphthyridine 3-H), 7.05 (t, *J* = 55.5 Hz, 1H, CHF_2), 6.63 (t, *J* = 55.1 Hz, 1H, CHF_2); ^{13}C NMR (126 MHz, methanol-d_4) δ 157.5 (t, *J* = 25.3 Hz, naphthyridine 2-C), 156.7 (sp^2 C), 151.7 (sp^2 C), 150.9 (t, *J* = 5.4 Hz, naphthyridine 7-CH), 143.0 (sp^2 C), 130.5 (t, *J* = 6.4 Hz, naphthyridine 5-CH), 128.1 (t, *J* = 23.3 Hz, naphthyridine 6-C), 126.6 (q, *J* = 3.9 Hz, aniline 3-CH), 126.2 (q, *J* = 32.8 Hz, aniline 4-C), 124.2 (q, *J* = 271 Hz, CF_3), 122.3 (aniline 3-CH), 114.2 (sp^2 C), 113.7 (t, *J* = 241 Hz, CHF_2), 113.5 (t, *J* = 238 Hz, CHF_2), 98.8 (t, *J* = 3.1 Hz, naphthyridine 3-CH); ^{19}F NMR (376 MHz, methanol-d_4) δ −63.7 (s, CF_3), −113.5 (d, *J* = 55.3 Hz, CHF_2), −118.4 (d, *J* = 55.5 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2973, 1621, 1595, 1563, 1413, 1355, 1319 and 1256; m/z (ES) found MH^+ 390.084474, $C_{16}H_9{}^{35}ClF_5N_3$ requires MH^+ 390.083571; HPLC 3.34 min., 100%.

2,6-Bis(difluoromethyl)-N-[3-fluoro-4-(trifluoromethyl)phenyl]-1,8-naphthyridin-4-amine (**158**)



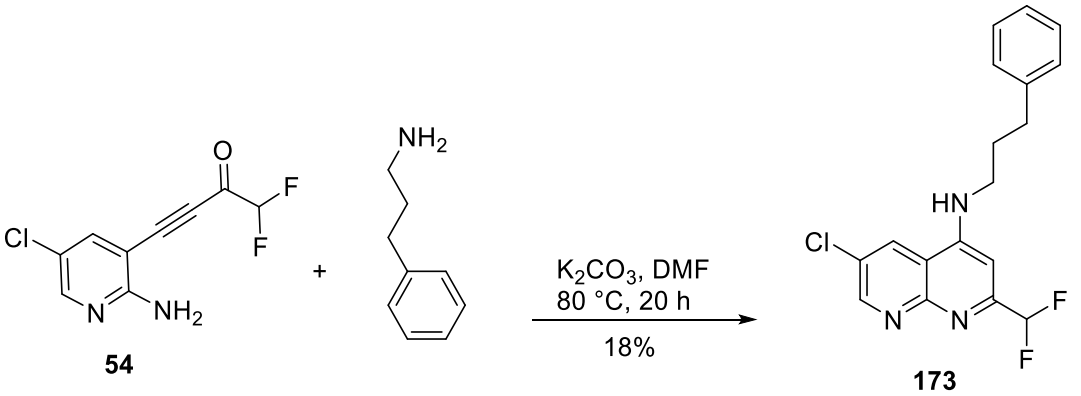
To a brown solution of **156** (0.105 g, 0.426 mmol) and 4-amino-2-fluorobenzotrifluoride (68.6 mg, 0.383 mmol) in propan-2-ol (5 mL) was added 4 M HCl in 1,4-dioxane (0.13 mL, 0.52 mmol). The reaction was stirred at 80 °C for 20 h under an atmosphere of nitrogen then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave a brown solid which was neutralised with saturated aqueous sodium hydrogen carbonate (30 mL) then extracted with chloroform (2 × 30 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:2–1:1). The solvent was removed *in vacuo* to leave the title compound as a yellow solid (61.3 mg, 0.151 mmol, 39%), m. p. 104–105 °C (from chloroform-pet. ether 40–60); Rf 0.23 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 9.12 (s, 1H, naphthyridine 7-H), 8.97 (d, *J* = 2.0 Hz, 1H, naphthyridine 5-H), 7.66 (t, *J* = 8.3 Hz, 1H, naphthyridine 3-H), 7.45 (s, 1H, aniline 2-H), 7.31–7.25 (m, 2H, aniline 5-H, 6-H), 7.05 (t, *J* = 55.4 Hz, 1H, CHF_2), 6.68 (t, *J* = 55.0 Hz, 1H, CHF_2); ^{13}C NMR (126 MHz, methanol-d_4) δ 160.6 (d, *J* = 254 Hz, C-F), 157.6 (t, *J* = 25.4 Hz, naphthyridine 2-C), 156.6 (sp^2 C), 151.1 (t, *J* = 5.4 Hz, naphthyridine 7-CH), 150.8 (sp^2 C), 145.7 (d, *J* = 10.3 Hz, aniline 1-C), 130.5 (t, *J* = 6.4 Hz, naphthyridine 6-C), 128.6 – 128.1 (m, aniline 4-C, 5-CH), 128.4 (sp^2 C), 122.8 (q, *J* = 270 Hz, CF_3), 116.5 (d, *J* = 3.3 Hz, aniline 6-CH), 114.7 (sp^2 C), 113.7 (t, *J* = 241 Hz, CHF_2), 113.5 (t, *J* = 238 Hz, CHF_2), 109.1 (d, *J* = 23.9 Hz, aniline 2-CH), 100.2 (t, *J* = 2.5 Hz, naphthyridine 3-CH); ^{19}F NMR (376 MHz, methanol-d_4) δ −62.2 (d, *J* = 12.4 Hz, CF_3), −113.7 (d, *J* = 55.4 Hz, CHF_2), −114.4 (m, C-F), −118.4 (d, *J* = 55.1 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 2966, 2928, 1628, 1595, 1564, 1515, 1416, 1365, 1317, 1271 and 1246; m/z (ES) found MH^+ 408.075117, $C_{17}H_9F_8N_3$ requires MH^+ 408.074150; HPLC 3.02 min., 98%.

2,6-Bis(difluoromethyl)-N-[6-(trifluoromethyl)pyridin-3-yl]-1,8-naphthyridin-4-amine (**159**)



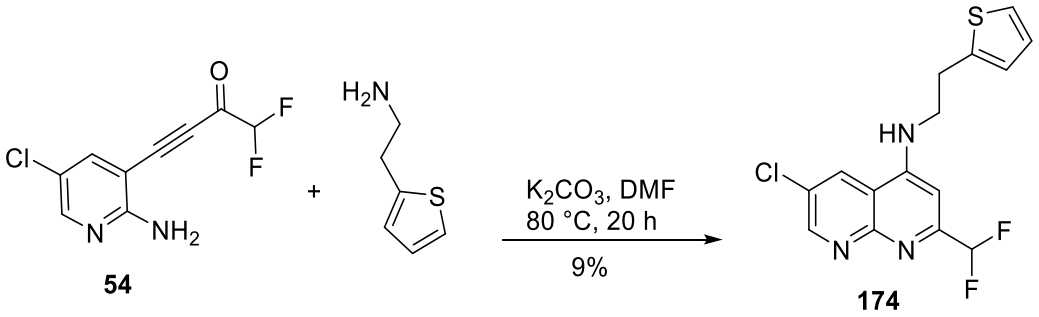
To a brown solution of **156** (0.115 g, 0.468 mmol) and 5-amino-2-(trifluoromethyl)pyridine (72.9 mg, 0.450 mmol) in propan-2-ol (5 mL) was added 4 M hydrochloric acid in 1,4-dioxane (0.13 mL, 0.52 mmol). The reaction was stirred at 50 °C for 24 h under an atmosphere of nitrogen then allowed to cool to room temperature. The solvent was removed *in vacuo* to leave a brown solid which was neutralised with saturated aqueous sodium hydrogen carbonate (20 mL) then extracted with chloroform (2 × 20 mL). The organic layer was dried over magnesium sulphate and the solvent removed *in vacuo*. The resulting brown solid was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:2–1:1). The solvent was removed *in vacuo* to leave the title compound as a solid (33.5 mg, 0.0858 mmol, 19%), m. p. 237–240 °C (from ethyl acetate-pet. ether 40–60); Rf 0.19 (1:1 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 9.23 (d, *J* = 1.9 Hz, 1H, naphthyridine 7-H), 9.12 (d, *J* = 1.9 Hz, 1H, naphthyridine 5-H), 8.80 (d, *J* = 2.6 Hz, 1H, pyridine 2-H), 8.11 (dd, *J* = 8.5, 2.6 Hz, 1H, pyridine 4-H), 7.92 (d, *J* = 8.5 Hz, 1H, pyridine 5-H), 7.48 (s, 1H, naphthyridine 3-H), 7.18 (t, *J* = 55.4 Hz, 1H, CHF_2), 6.78 (t, *J* = 55.0 Hz, 1H, CHF_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 157.6 (t, *J* = 23.8 Hz, naphthyridine 2-C), 156.6 (sp^2 C), 151.2 (sp^2 C), 151.1 (t, *J* = 6.0 Hz, naphthyridine 7-CH), 143.4 (pyridine 2-CH), 142.8 (q, *J* = 35.3 Hz, pyridine 6-C), 139.6 (sp^2 C), 130.5 (t, *J* = 6.4 Hz, naphthyridine 5-CH), 129.3 (pyridine 4-CH), 128.4 (t, *J* = 23.5 Hz, naphthyridine 6-C), 121.7 (q, *J* = 274 Hz, CF_3), 121.4 (q, *J* = 3.2 Hz, pyridine 5-CH), 114.6 (sp^2 C), 113.6 (t, *J* = 241 Hz, CHF_2), 113.5 (t, *J* = 238 Hz, CHF_2), 99.4 (t, *J* = 3.3 Hz, naphthyridine 3-CH); ν_{max}/cm^{-1} (solid); 2960, 1562, 1497, 1405, 1335 and 1235; m/z (ES) found MH^+ 391.079084, $C_{16}H_9F_7N_4$ requires MH^+ 391.078820; HPLC 2.75 min., 100%.

6-Chloro-2-(difluoromethyl)-N-(3-phenylpropyl)-1,8-naphthyridin-4-amine (**173**)



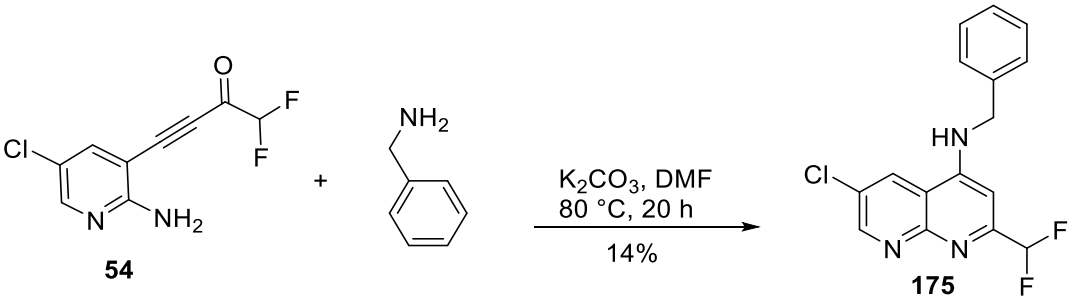
To a solution of **54** (69.8 mg, 0.303 mmol) and potassium carbonate (99.2 mg, 0.718 mmol) in anhydrous DMF (2 mL) was added 3-phenylpropylamine (50 µL, 0.35 mmol). The reaction mixture was stirred at 80 °C for 20 h under an atmosphere of nitrogen then allowed to cool to room temperature. Water (20 mL) was added and the product was extracted with ethyl acetate (3 × 20 mL). The combined organic layer was washed with water (20 mL) and aqueous lithium chloride solution (2 × 20 mL) then dried over magnesium sulphate and the solvent removed *in vacuo*. The product was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3–1:1) to give the title compound as an orange solid (18.6 mg, 0.0535 mmol, 18%); m. p. 159–161 °C (from ethyl acetate); Rf 0.54 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.88 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H), 8.69 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.29–7.20 (m, 2H, phenyl 3-H), 7.24 (d, *J* = 3.0 Hz, 2H, phenyl 2-H), 7.16 (tt, *J* = 6.8, 1.6 Hz, 1H, phenyl 4-H), 6.70 (s, 1H, naphthyridine 3-H), 6.65 (t, *J* = 55.3 Hz, 1H, CHF_2), 3.41 (t, *J* = 7.2 Hz, 2H, propyl 1-CH_2), 2.79 (t, *J* = 7.4 Hz, 2H, propyl 3-CH_2), 2.10 (p, *J* = 7.4 Hz, 2H, propyl 2-CH_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 156.3 (t, *J* = 25.0 Hz, naphthyridine 2-C), 153.8 (sp^2 C), 153.1 (sp^2 C), 152.0 (naphthyridine 7-CH), 141.3 (sp^2 C), 130.3 (naphthyridine 5-CH), 128.1 (phenyl 3-CH), 128.0 (phenyl 2-CH), 127.5 (sp^2 C), 125.6 (phenyl 4-CH), 114.1 (sp^2 C), 114.0 (t, *J* = 241 Hz, CHF_2), 94.5 (naphthyridine 3-CH), 42.5 (propyl 1-CH_2), 32.9 (propyl 3-CH_2), 29.3 (propyl 2-CH_2); ^{19}F NMR (376 MHz, methanol-d_4) δ −117.9 (d, *J* = 55.5 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3327, 2927, 1671, 1589, 1553, 1443, 1391, 1316 and 1203; m/z (ES) found MH^+ 348.106922, $C_{18}H_{16}{}^{35}ClF_2N_3$ requires MH^+ 348.107358; HPLC 2.38 min., 100%.

6-Chloro-2-(difluoromethyl)-N-[2-(thiophen-2-yl)ethyl]-1,8-naphthyridin-4-amine (**174**)



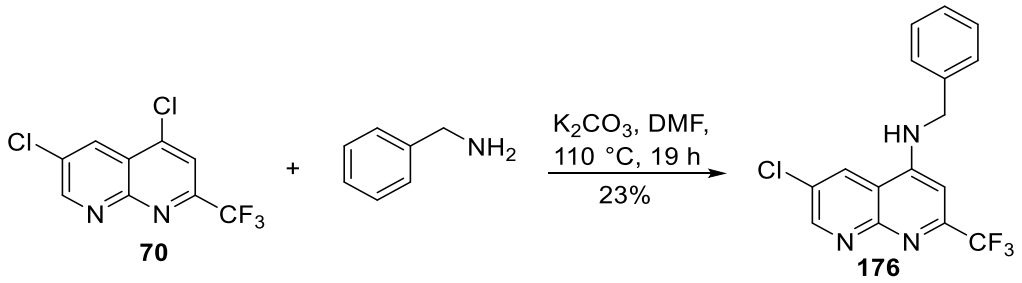
To a solution of **54** (82.6 mg, 0.358 mmol) and potassium carbonate (0.121 g, 0.872 mmol) in anhydrous DMF (3 mL) was added 2-thiopheneethylamine (50 µL, 0.43 mmol). The reaction mixture was stirred at 80 °C for 20 h under an atmosphere of nitrogen then allowed to cool to room temperature. Water (20 mL) was added and the product extracted with ethyl acetate (3 × 20 mL). The combined organic layer was washed with water (20 mL) and aqueous lithium chloride solution (2 × 20 mL) then dried over magnesium sulphate and the solvent removed *in vacuo*. The product was purified using column chromatography; elution with ethyl acetate-pet .ether 40–60 (1:3–1:1) to give the title compound as an orange solid (10.8 mg, 0.0318 mmol, 9%); m. p. 181–4 °C (from ethyl acetate); Rf 0.52 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.80 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H), 8.63 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.11 (dd, *J* = 4.8, 1.5 Hz, 1H, thiophene 4-H), 6.85–6.79 (m, 2H, thiophene 3-H, 5-H), 6.70 (s, 1H, naphthyridine 3-H), 6.55 (t, *J* = 55.3 Hz, 1H, CHF_2), 3.60 (t, *J* = 6.9 Hz, 2H, ethyl 1-CH_2), 3.16 (t, *J* = 6.9 Hz, 2H, ethyl 2-CH_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 156.4 (t, *J* = 24.9 Hz, naphthyridine 2-C), 153.9 (sp^2 C) , 153.0 (sp^2 C), 152.1 (naphthyridine 7-CH), 140.7 (sp^2 C), 130.3 (naphthyridine 5-CH), 127.7 (sp^2 C), 126.6 (thiophene 5-CH), 125.3 (thiophene 3-CH), 123.7 (thiophene 4-CH), 114.1 (sp^2 C), 114.0 (t, *J* = 241 Hz, CHF_2), 94.7 (t, *J* = 2.8 Hz, naphthyridine 3-CH), 44.5 (ethyl 1-CH_2), 28.1 (ethyl 2-CH_2); ^{19}F NMR (376 MHz, methanol-d_4) δ −118.0 (d, *J* = 55.3 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3271, 3062, 2904, 1591, 1567, 1509, 1475, 1441, 1397, 1353, 1314, 1279, 1236 and 1218; m/z (ES) found MH^+ 362.030081, $C_{15}H_{12}{}^{35}ClF_2N_3S$ requires MH^+ 362.030073; HPLC 2.07 min., 96%.

6-Chloro-2-difluromethyl-N-(1-phenylmethyl)-1,8-naphthyridine-4-amine (**175**) (Synthesised by MChem student Omar Allam)



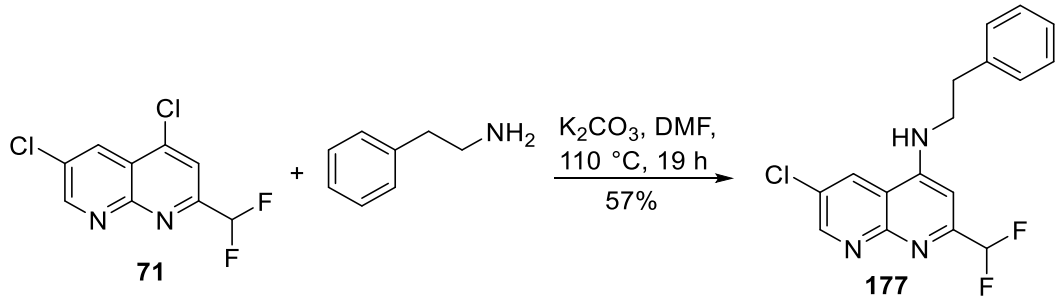
54 (48.3 mg, 0.209 mmol), benzylamine (23 µL, 0.21 mmol) and potassium carbonate (57.7 mg, 0.418 mmol) were dissolved in anhydrous DMF (2 mL) under an atmosphere of nitrogen. The mixture was stirred at 80 °C for 20 h then allowed to cool to room temperature. Water (10 mL) was added and the product was extracted with ethyl acetate (3 × 10 mL). The organic layer was washed with water (5 mL) and brine (3 × 15 mL) then dried over magnesium sulphate and the solvent was removed *in vacuo*. The product was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3) and the solvent removed *in vacuo* to give the title compound as an off-white solid (9.1 mg, 0.028 mmol, 14%); m. p. 212–215 °C (from ethyl acetate-pet. ether 40–60); Rf 0.38 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, methanol-d_4) δ 8.82 (d, *J* = 2.5 Hz, 1H, naphthyridine 7-H), 8.74 (d, *J* = 2.5 Hz, 1H, naphthyridine 5-H), 7.30 (d, *J* = 6.9 Hz, 2H, phenyl 2-H), 7.25 (t, *J* = 6.9 Hz, 2H, phenyl 3-H) 7.17 (t, *J* = 7.2 Hz, 1H, phenyl 4-H), 6.64 (s, 1H, naphthyridine 3-H), 6.49 (t, *J* = 55.3 Hz, 1H, CHF_2), 4.56 (s, 2H, aniline CH_2); ^{13}C NMR (101 MHz, methanol-d_4) δ 156.3 (sp^2 C), 153.8 (sp^2 C), 153.1 (t, *J* = 23.7 Hz, naphthyridine 2-C), 152.2 (naphthyridine 7-CH), 137.1 (sp^2 C), 130.4 (naphthyridine 5-CH), 128.4 (phenyl 3-CH), 127.8 (sp^2 C), 127.2 (phenyl 4-CH), 126.8 (phenyl 2-CH), 114.2 (sp^2 C), 113.9 (t, *J* = 241 Hz, CHF_2), 100.0 (sp^2 C), 95.5 (naphthyridine 3-CH); 47.7 (CH_2); ^{19}F NMR (376 MHz, methanol-d_4) δ −118.2 (d, *J* = 52.6 Hz, CHF_2); ν_{max}/cm^{-1} (solid) 3270, 3053, 1590, 1561, 1446, 1391, 1359, 1319 and 1206; m/z (ES) found MH^+ 320.076152, $C_{16}H_{13}{}^{35}ClN_3F_2$ requires MH^+ 320.076058; HPLC 2.06 min., 100%.

6-Chloro-N-(1-phenylmethyl)-2-trifluromethyl-1,8-naphthyridine-4-amine (**176**) (Synthesised by MChem student Omar Allam)



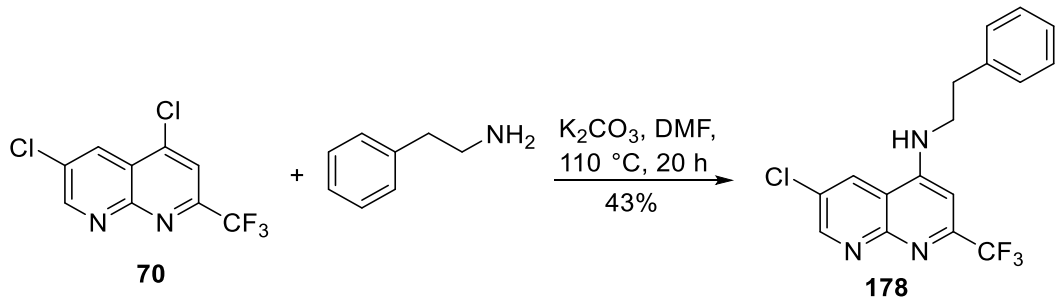
4,6-Dichloro-2-(trifluoromethyl)-1,8-naphthyridine (0.308 g, 1.154 mmol), benzylamine (0.125 mL, 1.15 mmol) and potassium carbonate (0.108 g, 0.782 mmol) were dissolved in anhydrous DMF (6 mL) under an atmosphere of nitrogen. The reaction mixture was stirred at 110 °C for 19 h then allowed to cool to room temperature. Water (30 mL) was added and the product was extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with water (20 mL) and brine (2 × 30 mL) then dried over magnesium sulphate. The solvent was removed *in vacuo* and the crude product was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3). The solvent was removed *in vacuo* to give the title compound as a yellow solid (86.3 mg, 0.256 mmol, 23%); m. p. >270 °C (from ethyl acetate-pet. ether 40–60); Rf. 0.22 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.09 (d, *J* = 2.6 Hz, 1H, naphthyridine 5-H), 9.04 (d, *J* = 2.6 Hz, 1H, naphthyridine 7-H), 8.77 (t, *J* = 5.8 Hz, 1H, N-H), 7.43 (d, *J* = 7.0 Hz, 2H, phenyl 2-H), 7.36 (t, *J* = 7.1 Hz, 2H, phenyl 3-H), 7.30 (t, *J* = 6.6 Hz, 1H, phenyl 4-H), 6.83 (s, 1H, naphthyridine 3-H), 4.67 (d, *J* = 5.8 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6), δ 153.8 (sp^2 C), 153.1 (sp^2 C), 152.9 (naphthyridine 7-CH), 149.9 (q, *J* = 33 Hz, naphthyridine 2-C), 140.9 (sp^2 C), 137.4 (sp^2 C), 130.8 (naphthyridine 5-CH), 128.6 (phenyl 2-CH), 128.2 (phenyl 4-CH), 127.3 (phenyl 3-CH), 118.7 (q, *J* = 275 Hz, CF_3), 114.2 (sp^2 C), 95.4 (naphthyridine 3-CH), 46.0 (CH_2); ^{19}F NMR (376 MHz, DMSO-d_6), δ −67.2 (s, CF_3); ν_{max}/cm^{-1} (solid); 3284, 3055, 2922, 2853, 1590, 1496, 1447, 1289, 1246 and 1228; m/z (ES) found MNa^+ 360.048659, $C_{16}H_{11}{}^{35}ClF_3N_3$ requires MNa^+ 360.048580; HPLC 3.11 min., 97%.

6-Chloro-2-(difluoromethyl)-N-(2-phenylethyl)-1,8-naphthyridine-4-amine (**177**) (Synthesised by MChem student Omar Allam)



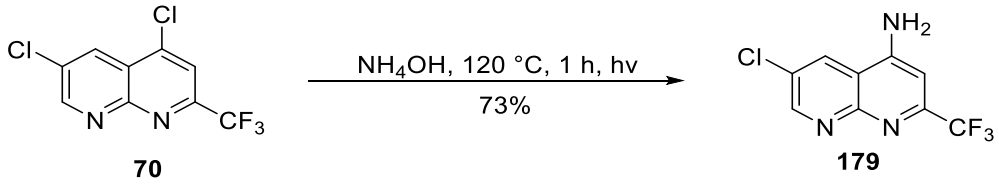
4,6-Dichloro-2-difluoromethyl-1,8-naphthyridine (58.2 mg, 0.234 mmol), phenethylamine (30 μL, 0.24 mmol) and potassium carbonate (67.5 mg, 0.488 mmol) were dissolved in anhydrous DMF (2 mL) under an atmosphere of nitrogen. The reaction mixture was stirred at 110 °C for 19 h then allowed to cool to room temperature. Water (15 mL) was added and the product was extracted with ethyl acetate (3 × 15 mL). The combined organic extracts were washed with water (10 mL) and brine (2 × 15 mL) then dried over magnesium sulphate. The solvent was removed *in vacuo* leaving the crude product which was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3). The solvent was removed *in vacuo* to give the title compound as an off-white solid (44.3 mg, 0.133 mmol, 57%); m. p. 222.5–223.0 °C (from ethyl acetate-pet. ether 40–60); Rf. 0.20 (1:2 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.98 (s, 2H, naphthyridine 5-H, 7-H), 7.98 (t, *J* = 5.3 Hz, 1H, N-H), 7.33-7.30 (m, 4H, phenyl 2-H, 3-H), 7.22 (m, 1H, phenyl 4-H), 6.88 (t, *J* = 55.0 Hz, 1H, CHF_2), 6.80 (s, 1H, naphthyridine 3-H), 3.59 (q, *J* = 7.4 Hz, 2H, phenethyl 2-CH_2), 2.99 (t, *J* = 7.4 Hz, 2H, phenethyl 1-CH_2); ^{13}C NMR (101 MHz, DMSO-d_6), δ 155.8 (sp^2 C), 154.1 (sp^2 C), 152.5 (sp^2 C), 152.2 (naphthyridine 7-CH), 139.1 (sp^2 C), 130.6 (naphthyridine 5-CH), 128.8 (phenyl 2/3-CH), 128.4 (phenyl 2/3-CH), 126.6 (sp^2 C), 126.3 (phenyl 4-CH), 114.2 (t, *J* = 240 Hz, CHF_2), 113.9 (sp^2 C), 95.0 (naphthyridine 3-CH), 44.4 (phenethyl 2-CH_2), 33.8 (phenethyl 1-CH_2); ^{19}F NMR (376 MHz, DMSO-d_6), δ −115.8 (d, *J* = 52.6 Hz, CHF_2); ν_{max}/cm^{-1} (solid); 3289, 3055, 2929, 2854, 1591, 1496, 1234, and 1206; m/z (ES) found MH^+ 334.091654, $C_{17}H_{14}{}^{35}ClF_2N_3$ requires MH^+ 334.091708; HPLC 2.16 min., 98%.

6-Chloro-N-(2-phenylethyl)-2-trifluromethyl-1,8-naphthyridine-4-amine (**178**) (Synthesised by MChem student Omar Allam)



4,6-Dichloro-(2-trifluoromethyl)-1,8-naphthyridine (0.101 g, 0.377 mmol), phenethylamine (50 μL, 0.40 mmol) and potassium carbonate (0.105 g, 0.756 mmol) were dissolved in anhydrous DMF (3 mL) under an atmosphere of nitrogen. The reaction mixture was stirred at 110 °C for 20 h then allowed to cool to room temperature. Water (20 mL) was added and the product was extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were washed with water (10 mL) and brine (2 × 30 mL) then dried over magnesium sulphate. The solvent was removed *in vacuo* and the crude product was purified using column chromatography; elution with ethyl acetate-pet. ether 40–60 (1:3). The solvent was removed *in vacuo* to give the title compound as a yellow solid (56.7 mg, 0.161 mmol, 43%); m. p. >270 °C (from ethyl acetate-pet. ether 40–60); Rf. 0.16 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (d, *J* = 2.7 Hz, 1H, naphthyridine 7-H), 9.01 (d, *J* = 2.7 Hz, 1H, naphthyridine 5-H), 8.16 (t, *J* = 5.4 Hz, 1H, N-H), 7.31 (d, *J* = 2.1 Hz, 2H, phenyl 2-H), 7.29 (dd, *J* = 8.0, 1.0 Hz, 2H, phenyl 3-H), 7.22 (t, *J* = 6.3 Hz, 1H, phenyl 4-H), 6.86 (s, 1H, naphthyridine 3-H), 3.68–3.61 (m, 2H, phenethyl 2-CH_2), 3.00 (t, *J* = 7.5 Hz, 2H, phenethyl 1-CH_2); ^{13}C NMR (101 MHz, DMSO-d_6), δ 153.8 (sp^2 C), 153.0 (sp^2 C), 152.8 (naphthyridine 7-CH), 150.1 (q, *J* = 33 Hz, naphthyridine 2-C), 139.0 (sp^2 C), 130.6 (naphthyridine 5-CH), 128.8 (phenyl 2-CH), 128.4 (phenyl 3-CH), 127.1 (sp^2 C), 126.3 (phenyl 4-CH), 121.6 (q, *J* = 274 Hz, CF_3), 113.9 (sp^2 C), 95.0 (naphthyridine 3-CH), 44.3 (phenethyl 2-CH_2), 33.9 (phenethyl 1-CH_2); ^{19}F NMR (376 MHz, DMSO-d_6), δ −67.1 (s, CF_3); ν_{max}/cm^{-1} (solid); 3289, 3058, 2925, 2853, 1590, 1497, 1441, 1327, 1290 and 1226; m/z (ES) found MH^+ 352.082758, $C_{17}H_{13}{}^{35}ClF_3N_3$ requires MH^+ 352.082286; HPLC 3.17 min., 95%.

4-Amino-6-chloro-2-(trifluoromethyl)-1,8-naphthyridine (**179**) (Synthesised by MChem student Omar Allam)



70 **179**

To 4,6-dichloro-2-(trifluoromethyl)-1,8-naphthyridine (0.101 g, 0.376 mmol), was added ammonia (30% in water, 1.5 mL). The suspension was heated to 120 °C for 1 h with microwave irradiation then allowed to cool to room temperature. The title compound was isolated using vacuum filtration as a red solid (0.680 g, 0.275 mmol, 73%); m. p. 252–256 °C (from water); Rf. 0.43 (1:3 ethyl acetate-pet. ether 40–60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.01 (d, *J* = 2.6 Hz, 1H, naphthyridine 7-CH), 8.92 (d, *J* = 2.6 Hz, 1H, naphthyridine 5-CH), 7.80 (s, 2H, NH_2), 6.99 (s, 1H, naphthyridine 3-CH); ^{13}C NMR (101 MHz, DMSO-d_6), δ 155.1 (sp^2 C), 153.2 (naphthyridine 7-CH), 149.6 (q, *J* = 32 Hz, naphthyridine 2-C), 132.1 (sp^2 C), 131.1 (naphthyridine 5-CH), 126.8 (sp^2 C), 121.6 (q, *J* = 274 Hz, CF_3), 113.5 (sp^2 C), 98.7 (naphthyridine 3-CH); ^{19}F NMR (376 MHz, DMSO-d_6), δ −67.3 (s, CF_3); ν_{max}/cm^{-1} (solid); 3333, 3208, 3062, 1596, 1443, 1331, 1283, 1254 and 1219; m/z (ES) found MH^+ 248.019957, $C_9H_5{}^{35}ClF_3N_3$ requires 248.019686; HPLC 1.55 min., 95%.

9 Bibliography

1. P. S. Brachman, *Int. J. Epidemiol.*, 2003, **32**, 684-686.
2. C. Dye, *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, 2014, **369**, DOI: 10.1098/rstb.2013.0426.
3. World Health Organisation, *Global Tuberculosis Report 2018*, 2018.
4. P. Borst and M. Ouellette, *Annual Reviews in Microbiology*, 1995, **49**, 427-460.
5. S. R. Palumbi, *Science*, 2001, **293**, 1786-1790.
6. B. Spellberg, J. H. Powers, E. P. Brass, L. G. Miller and J. E. Edwards, *Clinical Infectious Diseases*, 2004, **38**, 1279-1286.
7. World Health Organisation, *Antimicrobial Resistance: Global Report on Surveillance*, 2014.
8. A. M. Dondorp, S. Yeung, L. White, C. Nguon, N. P. J. Day, D. Socheat and L. von Seidlein, *Nat Rev Micro*, 2010, **8**, 272-280.
9. I. M. Hastings, *Trends in Parasitology*, 2004, **20**, 512-518.
10. M. Vanaerschot, S. Huijben, F. Van den Broeck and J.-C. Dujardin, *FEMS Microbiology Reviews*, 2014, **38**, 41-55.
11. B. Ullman, *Journal of Bioenergetics and Biomembranes*, 1995, **27**, 77-84.
12. V. Jeffers, E. T. Kamau, A. R. Srinivasan, J. Harper, P. Sankaran, S. E. Post, J. M. Varberg, W. J. Sullivan and J. P. Boyle, *mSphere*, 2017, **2**.
13. D. C. McFadden, S. Tomavo, E. A. Berry and J. C. Boothroyd, *Molecular and Biochemical Parasitology*, 2000, **108**, 1-12.
14. C. Doliwa, S. Escotte-Binet, D. Aubert, V. Sauvage, F. Velard, A. Schmid and I. Villena, *Parasite*, 2013, **20**, 19.
15. B. Hanboonkunupakarn and N. J. White, *Tropical Diseases, Travel Medicine and Vaccines*, 2016, **2**, 10.
16. World Health Organisation, *World Malaria Report 2018*, 2018.
17. W. A. Krotoski, *Prog Clin Parasitol*, 1989, **1**, 1-19.
18. World Health Organisation, *World Malaria Report 2015*, 2015.
19. F. Nosten and N. J. White, *Am J Trop Med Hyg*, 2007, **77**, 181-192.
20. N. M. Douglas, N. M. Anstey, B. J. Angus, F. Nosten and R. N. Price, *The Lancet Infectious Diseases*, **10**, 405-416.
21. J. K. Baird and S. L. Hoffman, *Clinical Infectious Diseases*, 2004, **39**, 1336-1345.
22. D. Thomas, H. Tazerouni, K. G. S. Sundararaj and J. C. Cooper, *Acta Tropica*, 2016, **160**, 35-38.
23. J. Flegr, J. Prandota, M. Sovičková and Z. H. Israili, *PLoS One*, 2014, **9**, e90203.
24. F. Robert-Gangneux and M. L. Dardé, *Clin Microbiol Rev*, 2012, **25**, 264-296.
25. R. E. Gilbert, K. Freeman, E. G. Lago, L. M. G. Bahia-Oliveira, H. K. Tan, M. Wallon, W. Buffolano, M. R. Stanford and E. Petersen, *PLoS Negl. Trop. Dis.*, 2008, **2**, e277.
26. G. Pappasa, N. Roussosa and M. E. Falagas, *Int. J. Parasitol.*, 2009, **39**, 1385-1394.

27. E. J. C. Goldstein, J. G. Montoya and J. S. Remington, *Clinical Infectious Diseases*, 2008, **47**, 554-566.
28. D. E. Eyles and N. Coleman, *Antibiot. Chemother.*, 1953, **3**, 483-490.
29. S. K. Halonen and L. M. Weiss, *Handb. Clin. Neurol.*, 2013, **114**, 125–145.
30. C. Leport, F. Raffi, S. Matheron, C. Katlama, B. Regnier, A. G. Saimot, C. Marche, C. Vedrenne and J. L. Vilde, *J. Am. Med.*, 1988, **84**, 94–100.
31. W. M. Ingram, L. M. Goodrich, E. A. Robey and M. B. Eisen, *PLoS ONE*, 2013, **8**, e75246.
32. A. Alipour, S. Shojaee, M. Mohebali, M. Tehrandoost, F. A. Masoleh and H. Keshavarz, *Iran J. Parasitol.*, 2011, **6**, 31-37.
33. B. D. Pearce, D. Kruszon-Moran and J. L. Jones, *Biol. Psychiat.* , 2012, **72**, 290–295.
34. O. Miman, O. Y. Kusbeci, O. C. Aktepe and Z. Cetinkaya, *Neurosci. Lett.*, 2010, **475**, 129-131.
35. O. Y. Kusbeci, O. Miman, M. Yaman, O. C. Aktepe and S. Yazar, *Alzheimer Dis. Assoc. Disord.*, 2011, **25**, 1-3.
36. M. M. Oskouei, F. Hamidi, M. Talebi, M. Farhoudi, A. A. Taheraghdam, T. Kazemi, H. Sadeghi-Bazarghani and E. Fallah, *J. Parasit. Dis.*, 2014, 1-5.
37. M. Mahami-Oskouei, F. Hamidi, M. Talebi, M. Farhoudi, A. A. Taheraghdam, T. Kazemi, H. Sadeghi-Bazargani and E. Fallah, *Parasitol. Res.*, 2016, **115**, 3169-3174.
38. J. J. Kessl, K. H. Ha, A. K. Merritt, S. R. Meshnick and B. L. Trumpower, *Molecular and Biochemical Parasitology*, 2006, **146**, 255-258.
39. C. Doliwa, S. Escotte-Binet, D. Aubert and V. Sauvage, *Parasite*, 2013, **20**, DOI: 10.1051/parasite/2013020.
40. J. Tanwar, S. Das, Z. Fatima and S. Hameed, *Interdisciplinary Perspectives on Infectious Diseases*, 2014, **2014**, 7.
41. H. Munier-Lehmann, P.-O. Vidalain, F. Tangy and Y. L. Janin, *Journal of Medicinal Chemistry*, 2013, **56**, 3148-3167.
42. M. Nagy, F. Lacroute and D. Thomas, *Proceedings of the National Academy of Sciences*, 1992, **89**, 8966-8970.
43. F. S. Nielsen, P. S. Andersen and K. F. Jensen, *Journal of Biological Chemistry*, 1996, **271**, 29359-29365.
44. J.-J. Chen and M. E. Jones, *Archives of Biochemistry and Biophysics*, 1976, **176**, 82-90.
45. O. Björnberg, A.-C. Grüner, P. Roepstorff and K. F. Jensen, *Biochemistry*, 1999, **38**, 2899-2908.
46. R. L. Fagan, K. F. Jensen, O. Björnberg and B. A. Palfey, *Biochemistry*, 2007, **46**, 4028-4036.
47. R. L. Fagan, M. N. Nelson, P. M. Pagano and B. A. Palfey, *Biochemistry*, 2006, **45**, 14926-14932.
48. R. L. Fagan and B. A. Palfey, *Biochemistry*, 2009, **48**, 7169-7178.
49. R. R. Bartlett, G. Campion, P. Musikic, R. Schleyerbach, T. Zielinski and H. Schorlemmer, *Leflunomide: a novel immunomodulating drug*, Marcel Dekker, Inc., New York, 1994.

50. R. I. Fox, M. L. Herrmann, C. G. Frangou, G. M. Wahl, R. E. Morris, V. Strand and B. J. Kirschbaum, *Clinical Immunology*, 1999, **93**, 198-208.
51. M. C. Claussen and T. Korn, *Clinical Immunology*, 2012, **142**, 49-56.
52. B. Chacko and G. T. John, *Transpl Infect Dis*, 2012, **14**, 111-120.
53. S.-F. Chen, F. W. Perrella, D. L. Behrens and L. M. Papp, *Cancer Research*, 1992, **52**, 3521.
54. S. Liu, E. A. Neidhardt, T. H. Grossman, T. Ocain and J. Clardy, *Structure*, 2000, **8**, 25-33.
55. M. Qing, G. Zou, Q. Y. Wang, H. Y. Xu, H. Dong, Z. Yuan and P. Y. Shi, *Antimicrob Agents Chemother*, 2010, **54**, 3686-3695.
56. S. Bhatt, P. W. Gething, O. J. Brady, J. P. Messina, A. W. Farlow, C. L. Moyes, J. M. Drake, J. S. Brownstein, A. G. Hoen, O. Sankoh, M. F. Myers, D. B. George, T. Jaenisch, G. R. W. Wint, C. P. Simmons, T. W. Scott, J. J. Farrar and S. I. Hay, *Nature*, 2013, **496**, 504-507.
57. Q. Y. Wang, S. Bushell, M. Qing, H. Y. Xu, A. Bonavia, S. Nunes, J. Zhou, M. K. Poh, P. Florez de Sessions, P. Niyomrattanakit, H. Dong, K. Hoffmaster, A. Goh, S. Nilar, W. Schul, S. Jones, L. Kramer, T. Compton and P. Y. Shi, *J Virol*, 2011, **85**, 6548-6556.
58. K. L. Yeo, Y. L. Chen, H. Y. Xu, H. Dong, Q. Y. Wang, F. Yokokawa and P. Y. Shi, *Antimicrob Agents Chemother*, 2015, **59**, 2086-2093.
59. R. M. Deans, D. W. Morgens, A. Ökesli, S. Pillay, M. A. Horlbeck, M. Kampmann, L. A. Gilbert, A. Li, R. Mateo, M. Smith, J. S. Glenn, J. E. Carette, C. Khosla and M. C. Bassik, *Nature Chemical Biology*, 2016, **12**, 361.
60. R. Harvey, K. Brown, Q. Zhang, M. Gartland, L. Walton, C. Talarico, W. Lawrence, D. Selleseth, N. Coffield, J. Leary, K. Moniri, S. Singer, J. Strum, K. Gudmundsson, K. Biron, K. R. Romines and P. Sethna, *Antiviral Research*, 2009, **82**, 1-11.
61. M. J. Blaser, *British Medical Journal*, 1998, **316**, 1507.
62. G. L. Mendz, B. M. Jimenez, S. L. Hazell, A. M. Gero and W. J. O'Sullivan, *J Appl Bacteriol*, 1994, **77**, 1-8.
63. R. A. Copeland, J. Marcinkeviciene, T. S. Haque, L. M. Kopcho, W. Jiang, K. Wang, L. D. Ecret, C. Sizemore, K. A. Amsler, L. Foster, S. Tadesse, A. P. Combs, A. M. Stern, G. L. Trainor, A. Slee, M. J. Rogers and F. Hobbs, *J Biol Chem*, 2000, **275**, 33373-33378.
64. H. P. de Koning, D. J. Bridges and R. J. Burchmore, *FEMS Microbiol Rev*, 2005, **29**, 987-1020.
65. M. Berg, L. Kohl, P. Van der Veken, J. Joossens, M. I. Al-Salabi, V. Castagna, F. Giannese, P. Cos, W. Versées, J. Steyaert, P. Grellier, A. Haemers, M. Degano, L. Maes, H. P. de Koning and K. Augustyns, *Antimicrob Agents Chemother*, 2010, **54**, 1900-1908.
66. T. Annoura, T. Nara, T. Makiuchi, T. Hashimoto and T. Aoki, *Journal of Molecular Evolution*, 2005, **60**, 113-127.
67. T. L. Arakaki, F. S. Buckner, J. R. Gillespie, N. A. Malmquist, M. A. Phillips, O. Kalyuzhniy, J. R. Luft, G. T. Detitta, C. L. Verlinde, W. C. Van Voorhis, W. G. Hol and E. A. Merritt, *Mol Microbiol*, 2008, **68**, 37-50.
68. S. Gudin, N. B. Quashie, D. Candlish, M. I. Al-Salabi, S. M. Jarvis, L. C. Ranford-Cartwright and H. P. de Koning, *Experimental Parasitology*, 2006, **114**, 118-125.

69. B. A. Fox and D. J. Bzik, *Nature*, 2002, **415**, 926-929.
70. M. A. Hortua Triana, M.-H. Huynh, M. F. Garavito, B. A. Fox, D. J. Bzik, V. B. Carruthers, M. Löffler and B. H. Zimmermann, *Molecular and Biochemical Parasitology*, 2012, **184**, 71-81.
71. M. A. Hortua Triana, D. Cajiao Herrera, B. H. Zimmermann, B. A. Fox and D. J. Bzik, *Infect Immun*, 2016, **84**, 2974-2981.
72. J. Hegewald, U. Gross and W. Bohne, *Molecular and Biochemical Parasitology*, 2013, **190**, 6-15.
73. M. J. Gardner, N. Hall, E. Fung, O. White, M. Berriman, R. W. Hyman, J. M. Carlton, A. Pain, K. E. Nelson, S. Bowman, I. T. Paulsen, K. James, J. A. Eisen, K. Rutherford, S. L. Salzberg, A. Craig, S. Kyes, M.-S. Chan, V. Nene, S. J. Shallom, B. Suh, J. Peterson, S. Angiuoli, M. Pertea, J. Allen, J. Selengut, D. Haft, M. W. Mather, A. B. Vaidya, D. M. A. Martin, A. H. Fairlamb, M. J. Fraunholz, D. S. Roos, S. A. Ralph, G. I. McFadden, L. M. Cummings, G. M. Subramanian, C. Mungall, J. C. Venter, D. J. Carucci, S. L. Hoffman, C. Newbold, R. W. Davis, C. M. Fraser and B. Barrell, *Nature*, 2002, **419**, 10.1038/nature01097.
74. T. Heikkilä, S. Thirumalairajan, M. Davies, M. R. Parsons, A. G. McConkey, C. W. Fishwick and A. P. Johnson, *Bioorg Med Chem Lett*, 2006, **16**, 88-92.
75. L. McRobert and G. A. McConkey, *Molecular and Biochemical Parasitology*, 2002, **119**, 273-278.
76. H. Tamta and A. K. Mukhopadhyay, *Curr. Res. Inf. Pharm. Sci*, 2003, **4**, 6-9.
77. T. Heikkilä, C. Ramsey, M. Davies, C. Galtier, A. M. W. Stead, A. P. Johnson, C. W. G. Fishwick, A. N. Boa and G. A. McConkey, *Journal of Medicinal Chemistry*, 2007, **50**, 186-191.
78. M. Davies, T. Heikkilä, G. A. McConkey, C. W. G. Fishwick, M. R. Parsons and A. P. Johnson, *Journal of Medicinal Chemistry*, 2009, **52**, 2683-2693.
79. D. Cowen, P. Bedingfield, G. A. McConkey, C. W. G. Fishwick and A. P. Johnson, *Bioorganic & Medicinal Chemistry Letters*, 2010, **20**, 1284-1287.
80. V. Patel, M. Booker, M. Kramer, L. Ross, C. A. Celatka, L. M. Kennedy, J. D. Dvorin, M. T. Duraisingh, P. Sliz, D. F. Wirth and J. Clardy, *Journal of Biological Chemistry*, 2008, **283**, 35078-35085.
81. M. L. Booker, C. M. Bastos, M. L. Kramer, R. H. Barker, R. Skerlj, A. B. Sidhu, X. Deng, C. Celatka, J. F. Cortese, J. E. Guerrero Bravo, K. N. Crespo Llado, A. E. Serrano, I. Angulo-Barturen, M. B. Jiménez-Díaz, S. Viera, H. Garuti, S. Wittlin, P. Papastogiannidis, J.-w. Lin, C. J. Janse, S. M. Khan, M. Duraisingh, B. Coleman, E. J. Goldsmith, M. A. Phillips, B. Munoz, D. F. Wirth, J. D. Klinger, R. Wiegand and E. Sybertz, *Journal of Biological Chemistry*, 2010, **285**, 33054-33064.
82. R. T. Skerlj, C. M. Bastos, M. L. Booker, M. L. Kramer, R. H. Barker, C. A. Celatka, T. J. O'Shea, B. Munoz, A. B. Sidhu, J. F. Cortese, S. Wittlin, P. Papastogiannidis, I. Angulo-Barturen, M. B. Jimenez-Diaz and E. Sybertz, *ACS Medicinal Chemistry Letters*, 2011, **2**, 708-713.
83. M. A. Phillips, J. Lotharius, K. Marsh, J. White, A. Dayan, K. L. White, J. W. Njoroge, F. El Mazouni, Y. Lao, S. Kokkonda, D. R. Tomchick, X. Deng, T. Laird, S. N. Bhatia, S. March, C. L. Ng, D. A. Fidock, S.

Wittlin, M. Lafuente-Monasterio, F. J. G. Benito, L. M. S. Alonso, M. S. Martinez, M. B. Jimenez-Diaz, S. F. Bazaga, I. Angulo-Barturen, J. N. Haselden, J. Louttit, Y. Cui, A. Sridhar, A.-M. Zeeman, C. Kocken, R. Sauerwein, K. Dechering, V. M. Avery, S. Duffy, M. Delves, R. Sinden, A. Ruecker, K. S. Wickham, R. Rochford, J. Gahagen, L. Iyer, E. Riccio, J. Mirsalis, I. Bathhurst, T. Rueckle, X. Ding, B. Campo, D. Leroy, M. J. Rogers, P. K. Rathod, J. N. Burrows and S. A. Charman, *Science Translational Medicine*, 2015, **7**, 296ra111.

84. M. A. Phillips, K. L. White, S. Kokkonda, X. Deng, J. White, F. El Mazouni, K. Marsh, D. R. Tomchick, K. Manjalanagara, K. R. Rudra, G. Wirjanata, R. Noviyanti, R. N. Price, J. Marfurt, D. M. Shackleford, F. C. K. Chiu, M. Campbell, M. B. Jimenez-Diaz, S. F. Bazaga, I. Angulo-Barturen, M. S. Martinez, M. Lafuente-Monasterio, W. Kaminsky, K. Silue, A.-M. Zeeman, C. Kocken, D. Leroy, B. Blasco, E. Rossignol, T. Rueckle, D. Matthews, J. N. Burrows, D. Waterson, M. J. Palmer, P. K. Rathod and S. A. Charman, *ACS Infectious Diseases*, 2016, **2**, 945-957.
85. M. A. Phillips, R. Gujjar, N. A. Malmquist, J. White, F. El Mazouni, J. Baldwin and P. K. Rathod, *J Med Chem*, 2008, **51**, 3649-3653.
86. R. Gujjar, A. Marwaha, F. El Mazouni, J. White, K. L. White, S. Creason, D. M. Shackleford, J. Baldwin, W. N. Charman, F. S. Buckner, S. Charman, P. K. Rathod and M. A. Phillips, *Journal of Medicinal Chemistry*, 2009, **52**, 1864-1872.
87. J. M. Coteron, M. Marco, J. Esquivias, X. Deng, K. L. White, J. White, M. Koltun, F. El Mazouni, S. Kokkonda, K. Katneni, R. Bhamidipati, D. M. Shackleford, I. Angulo-Barturen, S. B. Ferrer, M. B. Jiménez-Díaz, F.-J. Gamo, E. J. Goldsmith, W. N. Charman, I. Bathurst, D. Floyd, D. Matthews, J. N. Burrows, P. K. Rathod, S. A. Charman and M. A. Phillips, *Journal of Medicinal Chemistry*, 2011, **54**, 5540-5561.
88. A. Marwaha, J. White, F. El_Mazouni, S. A. Creason, S. Kokkonda, F. S. Buckner, S. A. Charman, M. A. Phillips and P. K. Rathod, *Journal of Medicinal Chemistry*, 2012, **55**, 7425-7436.
89. X. Deng, S. Kokkonda, F. El Mazouni, J. White, J. N. Burrows, W. Kaminsky, S. A. Charman, D. Matthews, P. K. Rathod and M. A. Phillips, *Journal of Medicinal Chemistry*, 2014, **57**, 5381-5394.
90. J. S. McCarthy, J. Lotharius, T. Rückle, S. Chalon, M. A. Phillips, S. Elliott, S. Sekuloski, P. Griffin, C. L. Ng, D. A. Fidock, L. Marquart, N. S. Williams, N. Gobeau, L. Bebrevska, M. Rosario, K. Marsh and J. J. Möhrle, *Lancet Infect Dis*, 2017, **17**, 626-635.
91. K. A. Collins, T. Rückle, S. Elliott, L. Marquart, E. Ballard, S. Chalon, P. Griffin, J. J. Möhrle and J. S. McCarthy, *Antimicrobial Agents and Chemotherapy*, 2019, **63**, e01837-01818.
92. M. Sulyok, T. Rückle, A. Roth, R. E. Mürbeth, S. Chalon, N. Kerr, S. S. Samec, N. Gobeau, C. L. Calle, J. Ibáñez, Z. Sulyok, J. Held, T. Gebru, P. Granados, S. Brückner, C. Nguetse, J. Mengue, A. Lalremruata, B. K. L. Sim, S. L. Hoffman, J. J. Möhrle, P. G. Kremsner and B. Mordmüller, *Lancet Infect Dis*, 2017, **17**, 636-644.
93. S. C. Murphy, E. R. Duke, K. J. Shipman, R. L. Jensen, Y. Fong, S. Ferguson, H. E. Janes, K. Gillespie, A. M. Seilie, A. E. Hanron, L. Rinn, M. Fishbaugher, T. VonGoedert, E. Fritzen, S. H. Kappe, M. Chang, J. C. Sousa, S. R. Marcsisin, S. Chalon, S. Duparc, N. Kerr, J.

J. Möhrle, N. Andenmatten, T. Rueckle and J. G. Kublin, *J Infect Dis*, 2018, **217**, 693-702.

94. A. Llanos-Cuentas, M. Casapia, R. Chuquiyauri, J.-C. Hinojosa, N. Kerr, M. Rosario, S. Toovey, R. H. Arch, M. A. Phillips, F. D. Rozenberg, J. Bath, C. L. Ng, A. N. Cowell, E. A. Winzeler, D. A. Fidock, M. Baker, J. J. Möhrle, R. Hooft van Huijsduijnen, N. Gobeau, N. Araeipour, N. Andenmatten, T. Rückle and S. Duparc, *The Lancet Infectious Diseases*, 2018, **18**, 874-883.
95. S. Kokkonda, X. Deng, K. L. White, J. M. Coteron, M. Marco, L. de las Heras, J. White, F. El Mazouni, D. R. Tomchick, K. Manjalanagara, K. R. Rudra, G. Chen, J. Morizzi, E. Ryan, W. Kaminsky, D. Leroy, M. S. Martínez-Martínez, M. B. Jimenez-Diaz, S. F. Bazaga, I. Angulo-Barturen, D. Waterson, J. N. Burrows, D. Matthews, S. A. Charman, M. A. Phillips and P. K. Rathod, *Journal of Medicinal Chemistry*, 2016, **59**, 5416-5431.
96. P. T. P. Bedingfield, D. Cowen, P. Acklam, F. Cunningham, M. R. Parsons, G. A. McConkey, C. W. G. Fishwick and A. P. Johnson, *Journal of Medicinal Chemistry*, 2012, **55**, 5841-5850.
97. T. C. Leboho, S. F. van Vuuren, J. P. Michael and C. B. de Koning, *Org Biomol Chem*, 2014, **12**, 307-315.
98. W. P. Gallagher and A. Vo, *Organic Process Research & Development*, 2015, **19**, 1369-1373.
99. *USA Pat.,* 2015.
100. M. Fiorenza, A. Mordini, S. Papaleo, S. Pastorelli and A. Ricci, *Tetrahedron Letters*, 1985, **26**, 787-788.
101. M. Yamaguchi, K. Shibato, S. Fujiwara and I. Hirao, *Synthesis*, 1986, **1986**, 421-422.
102. J. Doubský, L. Streinz, L. Lešetický and B. Koutek, *Synlett*, 2003, **2003**, 0937-0942.
103. J. M. Williams, R. B. Jobson, N. Yasuda, G. Marchesini, U.-H. Dolling and E. J. J. Grabowski, *Tetrahedron Letters*, 1995, **36**, 5461-5464.
104. L. De Luca, G. Giacomelli and M. Taddei, *The Journal of Organic Chemistry*, 2001, **66**, 2534-2537.
105. B. Yu, H. Sun, Z. Xie, G. Zhang, L. W. Xu, W. Zhang and Z. Gao, *Org Lett*, 2015, **17**, 3298-3301.
106. A. B. Rode, S.-J. Son and I.-S. Hong, *Bulletin of the Korean Chemical Society*, 2010, **31**, 2061-2064.
107. M. N. S. Rad and S. Behrouz, *Synlett*, 2011, **2011**, 2562-2566.
108. A. S. Karpov and T. J. Müller, *Synthesis*, 2003, **2003**, 2815-2826.
109. M. S. Shvartsberg and E. A. Kolodina, *Mendeleev Communications*, 2008, **18**, 109-111.
110. H. Matter, M. Schudok, W. Schwab, W. Thorwart, D. Barbier, G. Billen, B. Haase, B. Neises, K.-U. Weithmann and T. Wollmann, *Bioorganic & Medicinal Chemistry*, 2002, **10**, 3529-3544.
111. V. Santagada, F. Fiorino, B. Severino, S. Salvadori, L. H. Lazarus, S. D. Bryant and G. Caliendo, *Tetrahedron Letters*, 2001, **42**, 3507-3509.
112. K. Sato, R. Kawata, F. Ama, M. Omote, A. Ando and I. Kumadaki, *CHEMICAL & PHARMACEUTICAL BULLETIN*, 1999, **47**, 1013-1016.
113. Z. Feng, Q.-Q. Min, Y.-L. Xiao, B. Zhang and X. Zhang, *Angewandte Chemie International Edition*, 2014, **53**, 1669-1673.

114. S. Sanoh, Y. Tayama, K. Sugihara, S. Kitamura and S. Ohta, *Drug Metabolism and Pharmacokinetics*, 2015, **30**, 52-63.
115. E. Garattini, R. Mendel, M. J. RomÃO, R. Wright and M. Terao, *Biochemical Journal*, 2003, **372**, 15.
116. Y. Moriwaki, T. Yamamoto, S. Takahashi, Z. Tsutsumi and T. Hada, *Histol. Histopathol.*, 2001, **16**, 745-753.
117. R. Kumar, G. Joshi, H. Kler, S. Kalra, M. Kaur and R. Arya, *Medicinal Research Reviews*, 2018, **38**, 1073-1125.
118. D. C. Pryde, D. Dalvie, Q. Hu, P. Jones, R. S. Obach and T.-D. Tran, *Journal of Medicinal Chemistry*, 2010, **53**, 8441-8460.
119. J. F. Alfaro and J. P. Jones, *The Journal of Organic Chemistry*, 2008, **73**, 9469-9472.
120. D. C. Pryde, T.-D. Tran, P. Jones, J. Duckworth, M. Howard, I. Gardner, R. Hyland, R. Webster, T. Wenham, S. Bagal, K. Omoto, R. P. Schneider and J. Lin, *Bioorganic & Medicinal Chemistry Letters*, 2012, **22**, 2856-2860.
121. R. A. Torres, K. R. Korzekwa, D. R. McMasters, C. M. Fandozzi and J. P. Jones, *Journal of Medicinal Chemistry*, 2007, **50**, 4642-4647.
122. S. Lepri, M. Ceccarelli, N. Milani, S. Tortorella, A. Cucco, A. Valeri, L. Goracci, A. Brink and G. Cruciani, *Proceedings of the National Academy of Sciences*, 2017, **114**, E3178.
123. Y. Xu, L. Li, Y. Wang, J. Xing, L. Zhou, D. Zhong, X. Luo, H. Jiang, K. Chen, M. Zheng, P. Deng and X. Chen, *Journal of Medicinal Chemistry*, 2017, **60**, 2973-2982.
124. F. O'Hara, A. C. Burns, M. R. Collins, D. Dalvie, M. A. Ornelas, A. D. N. Vaz, Y. Fujiwara and P. S. Baran, *Journal of Medicinal Chemistry*, 2014, **57**, 1616-1620.
125. P. L. Ferrarini, C. Mori, G. Primofiore and L. Calzolari, *Journal of Heterocyclic Chemistry*, 1990, **27**, 881-886.
126. P. L. Ferrarini, C. Mori, M. Badawneh, V. Calderone, L. Calzolari, T. Loffredo, E. Martinotti and G. Saccomanni, *European Journal of Medicinal Chemistry*, 1998, **33**, 383-397.
127. T. K. Chen and W. T. Flowers, *Journal of the Chemical Society, Chemical Communications*, 1980, 1139-1140.
128. C. Xu, W.-H. Guo, X. He, Y.-L. Guo, X.-Y. Zhang and X. Zhang, *Nature Communications*, 2018, **9**, 1170.
129. C. Brouwer, K. J. Jenko, S. S. Zoghbi, C. L. Morse, R. B. Innis and V. W. Pike, *European Journal of Medicinal Chemistry*, 2016, **124**, 677-688.
130. C. Lu, H. Lu, J. Wu, H. C. Shen, T. Hu, Y. Gu and Q. Shen, *The Journal of Organic Chemistry*, 2018, **83**, 1077-1083.
131. Z. Feng, Q.-Q. Min and X. Zhang, *Organic Letters*, 2016, **18**, 44-47.
132. P. Liu, L. S. Lin, T. G. Hamill, J. P. Jewell, T. J. Lanza, R. E. Gibson, S. M. Krause, C. Ryan, W. Eng, S. Sanabria, X. Tong, J. Wang, D. A. Levorse, K. A. Owens, T. M. Fong, C.-P. Shen, J. Lao, S. Kumar, W. Yin, J. F. Payack, S. A. Springfield, R. Hargreaves, H. D. Burns, M. T. Goulet and W. K. Hagmann, *Journal of Medicinal Chemistry*, 2007, **50**, 3427-3430.
133. G. S. Lal, G. P. Pez, R. J. Pesaresi, F. M. Prozonic and H. Cheng, *The Journal of Organic Chemistry*, 1999, **64**, 7048-7054.

134. A. L'Heureux, F. Beaulieu, C. Bennett, D. R. Bill, S. Clayton, F. LaFlamme, M. Mirmehrabi, S. Tadayon, D. Tovell and M. Couturier, *The Journal of Organic Chemistry*, 2010, **75**, 3401-3411.
135. S. R. Kandalkar, R. D. Kaduskar, P. A. Ramaiah, D. A. Barawkar, D. Bhuniya and A. M. Deshpande, *Tetrahedron Letters*, 2013, **54**, 414-418.
136. V. Cmiljanovic, P. Hebeisen, F. Beaufils, T. Bohnacker, D. Rageot, A. Sele, M. Wymann and J. Langlois, *Int. Pat.* WO20160751302016, 2016.
137. M. McPhillie, Y. Zhou, K. El Bissati, J. Dubey, H. Lorenzi, M. Capper, A. K. Lukens, M. Hickman, S. Muench, S. K. Verma, C. R. Weber, K. Wheeler, J. Gordon, J. Sanders, H. Moulton, K. Wang, T.-K. Kim, Y. He, T. Santos, S. Woods, P. Lee, D. Donkin, E. Kim, L. Fraczek, J. Lykins, F. Esaa, F. Alibana-Clouser, S. Dovgin, L. Weiss, G. Brasseur, D. Wirth, M. Kent, L. Hood, B. Meunieur, C. W. Roberts, S. S. Hasnain, S. V. Antonyuk, C. Fishwick and R. McLeod, *Scientific Reports*, 2016, **6**, 29179.
138. K. Arnold, L. Bordoli, J. Kopp and T. Schwede, *Bioinformatics*, 2005, **22**, 195-201.
139. P. Benkert, M. Biasini and T. Schwede, *Bioinformatics*, 2010, **27**, 343-350.
140. M. Biasini, S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. G. Cassarino, M. Bertoni, L. Bordoli and T. Schwede, *Nucleic Acids Research*, 2014, **42**, W252-W258.
141. V. Gillet, A. P. Johnson, P. Mata, S. Sike and P. Williams, *Journal of Computer-Aided Molecular Design*, 1993, **7**, 127-153.
142. J. Baldwin, A. M. Farajallah, N. A. Malmquist, P. K. Rathod and M. A. Phillips, *Journal of Biological Chemistry*, 2002, **277**, 41827-41834.
143. A. M. Baca and W. G. J. Hol, *International Journal for Parasitology*, 2000, **30**, 113-118.
144. U. K. Laemmli, *Nature*, 1970, **227**, 680-685.
145. M. M. Bradford, *Analytical Biochemistry*, 1976, **72**, 248-254.
146. N. V. Grishin, A. L. Osterman, E. J. Goldsmith and M. A. Phillips, *Proteins: Structure, Function, and Bioinformatics*, 1996, **24**, 272-273.
147. R. W. Miller, in *Methods in Enzymology*, Academic Press, 1978, vol. 51, pp. 63-69.
148. N. Guex and M. C. Peitsch, *ELECTROPHORESIS*, 1997, **18**, 2714-2723.
149. V. Mariani, F. Kiefer, T. Schmidt, J. Haas and T. Schwede, *Proteins: Structure, Function, and Bioinformatics*, 2011, **79**, 37-58.
150. J. Bell, Y. Cao, J. Gunn, T. Day, E. Gallicchio, Z. Zhou, R. Levy and R. Farid, *International Tables for Crystallography*, 2012, 534-538.
151. E. Harder, W. Damm, J. Maple, C. Wu, M. Reboul, J. Y. Xiang, L. Wang, D. Lupyan, M. K. Dahlgren, J. L. Knight, J. W. Kaus, D. S. Cerutti, G. Krilov, W. L. Jorgensen, R. Abel and R. A. Friesner, *Journal of Chemical Theory and Computation*, 2016, **12**, 281-296.
152. C. Rossy, E. Fouquet and F.-X. Felpin, *Beilstein journal of organic chemistry*, 2013, **9**, 1426-1431.
153. S. Wentzler, Y. El-Ahmad, B. Filoche-Romme, C. Nemecek, C. Venot and A. Hittinger, *USA Pat.* 2007/0093480, 2007
154. D. Hoover and K. Witter, *Int. Pat.* WO/2008/004117, 2008.

155. S. B. Lang, R. J. Wiles, C. B. Kelly and G. A. Molander, *Angewandte Chemie International Edition*, 2017, **56**, 15073-15077.
156. D. Billen, N. Chubb, D. Gethin, K. Hall, L. Roberts and N. Walshe, *USA Pat.* 2005/148649*,* 2005.
157. W. R. Dolbier, S. K. Lee and O. Phanstiel, *Tetrahedron*, 1991, **47**, 2065-2072.
158. W. Metz, S. Wentzler, D. Lesuisse and Y. El-Ahmad, *USA Pat*. 2008045561, 2008.
159. H. Dyke, L. Gazzard and K. Williams, *Int. Pat.* WO2011073263, 2011.
160. A. Abengózar, B. Abarca, A. M. Cuadro, D. Sucunza, J. Álvarez-Builla and J. J. Vaquero, *European Journal of Organic Chemistry*, 2015, **2015**, 4214-4223.
161. C. Yang, X. Zhang, Y. Wang, Y. Yang, X. Liu, M. Deng, Y. Jia, Y. Ling, L.-H. Meng and Y. Zhou, *ACS medicinal chemistry letters*, 2017, **8**, 875-880.
162. T. Yu, N. Li, C. Wu, A. Guan, Y. Li, Z. Peng, M. He, J. Li, Z. Gong, L. Huang, B. Gao, D. Hao, J. Sun, Y. Pan, L. Shen, C. Chan, X. Lu, H. Yuan, Y. Li, J. Li and S. Chen, *ACS medicinal chemistry letters*, 2018, **9**, 256-261.
163. A. Hideki, F. Kenichi and M. Makoto, *Japan Pat.* JP2012167047, 2012.