Design Synthesis and Biological Evaluation of DYRK1A Inhibitors

A thesis submitted in fulfillment of the requirements for admission to the degree of

Doctor of Philosophy

by

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Declaration

This thesis is a summary of research performed by the author in the School of Chemistry, University of Sydney, under the supervision of Professor Michael Kassiou between September 2014 and September 2017. There is no material that has been accepted for the award of any degree in any University. No other individual's work has been used in this thesis without recognition, and all the efforts have been made to acknowledge previously published material. This thesis contains less than 80,000 words.

One section of original research described in this manuscript has been published in peer-reviewed scientific journal, namely:

“Structural optimization and pharmacological evaluation of inhibitors targeting dual-specificity tyrosine phosphorylation-regulated kinases (DYRK) and CDC-like kinases (CLK) in glioblastoma”


Abstract

This thesis describes the synthesis of a unique library of novel analogues as potent and selective DYRK1A inhibitors based on the lead 7-azaindole scaffold.

DYRK1A is a dual-specificity protein kinase that catalyses not only autophosphorylation on its tyrosine residue but the phosphorylation of serine and threonine residues in its substrates. DYRK1A has been shown to be important for phosphorylation of tau protein and has also been identified as a Down syndrome candidate gene. Overexpression of DYRK1A results in cognitive deficits and impairments in memory, and has been proposed as a significant contributor to the underlying neurodevelopmental abnormalities associated with Down syndrome and Alzheimer’s disease. DYRK1A overexpression has also been found in cancer cells, particularly in glioblastoma, which represents 15% of brain tumours. The increased phosphorylation of sprouty2 mediated by DYRK1A blocks the EGFR degradation as a result of overexpression of EGFR in the cell surface, and the enhanced EGFR signalling eventually leads to tumour survival. On the contrary, DYRK1A inhibition has been found to promote EGFR degradation in glioblastoma cells by triggering the endocytosis and lysosomal degradation, thus reducing the self-renewal ability of tumourigenic cells. Development of small molecule inhibitors of DYRK1A therefore provides attractive treatment strategies.

Despite decades of investigation, the majority of promising DYRK1A inhibitors have not successfully entered clinical trials due to unexpected side effects and pharmacokinetic issues. A recently discovered lead compound, DANDY represents one chemotype of the most potent DYRK1A inhibitors. The work in this thesis explores the importance of the 7-azaindole skeleton to DYRK1A inhibition through the systematic modification to structural features of DANDY. The sequential selectivity of promising inhibitors over other off-targets such as DYRK1B, DYRK2 and CLK1, as well as the functional activity against glioblastoma cells has also been explored.
Preliminary kinase inhibition assays together with cell viability assays have shown promising trends towards the correlation between DYRK1A inhibition activities and cellular efficacies \textit{in vitro}. In this thesis, several promising compounds have shown inhibition for DYRK1A with IC$_{50}$ values being $< 50$ nM. These compounds have also played effective roles for the \textit{in vitro} treatment of glioblastoma cell viability assays.
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**Abbreviations**

Aβ = beta-Amyloid peptide

Ac = acetyl

AD = Alzheimer’s disease

AP4 = Activator protein 4

APCI = atmospheric chemical ionisation

APP = amyloid precursor protein

aq. = aqueous

Ar = aryl

ASF = alternative splicing factor

ASD = Autism spectrum disorder

atm = atmosphere

ATP = adenosine triphosphate

Bn = benzyl

Bu = butyl

Cbz = carboxybenzyl

br. = broad

Calc. = calculated

CDKs = cyclin dependent kinases

CDKLs = CDK-like kinases

CKs = casein kinases
CLKs = CDC-like kinases

$\text{cm}^{-1}$ = wave numbers

CNS = central nervous system

conc. = concentration

CREB = cAMP response element-binding

CRY2 = crystal like protein 2

CTD = $C$-terminal repeated domain

d = doublet

d = deuterated

DANDY = 3,5-diphenol-7-azaindole

dd = doublet of doublets

ddd = doublet of doublet of doublets

DMF = $N,N$-dimethylformamide

DMF-DMA = $N,N$-dimethylformamide dimethyl acetal

DMP = Dess-Martin periodinane

DMSO = dimethylsufoxide

DNA = Deoxyribonucleic acid

dppf = 1,1’-ferrocenediyl-bis(diphenylphosphine)

DS = Down syndrome

DSCR = Down syndrome critical region

dt = doublet of triplets
Abbreviations

DYRKs = dual-specificity tyrosine phosphorylation-related kinases

EC₅₀ = concentration of ligand that produces half maximal efficacy of subjects

EGCG = Epigallocatechin gallate

EGFR = epidermal growth factor receptor

eIF2B = eukaryotic initiation factor 2B

equiv. = equivalents

ESI = electrospray ionisation

Et = ethyl

FGF = fibroblast growth factor

FKHR = forkhead in rhabdomyosarcoma

FOXO = forkhead box O transcriptional factor

FTICR = Fourier Transform Ion Cyclotron Resonance

g = gram (s)

GIST = gastrointestinal stromal tumour

Glu = glutamic acid

Gly = glycine

GSKs = glycogen synthase kinases

h = hour (s)

H19-7 = hippocampal neuroprogenitor cell lines

HD = Huntington’s disease

Hip-1 = huntingtin interacting protein 1
Abbreviations

HIV = human immunodeficiency virus

HOMO = highest occupied molecular orbital

HPLC = high performance liquid chromatography

HPV = human papillomavirus

HRMS = high-resolution mass spectrometry

Hz = Hertz

IC<sub>50</sub> = concentration of ligand that reduces kinase activity by 50%

Ile = isoleucine

IR = Infrared spectrometry

<math>J</math> = coupling constant

LDA = <math>N,N</math>-lithium diisopropylamide

Leu = leucine

LRMS = low-resolution mass spectrometry

LUMO = lowest unoccupied molecular orbital

Lys = lysine

m = metre or milli or multiplet range

M = molar or molecular ion

MAO-A = monoamine oxidase A

MAPks = mitogen-activated protein kinases

<math>m</math>-CPBA = 3-chloroperbenzoic acid

Me = methyl
MeCN = acetonitrile

min = minute (s)

miR = microRNAs

mol = mole (s)

mol% = mole percent

m.p. = melting point

mRNA = messenger ribonucleic acid

Ms = mesylate

MW = microwave irradiation

m/z = mass-to-charge ratio

n = nano or unspecified number of carbons

N/A = not applicable

NBS = N-bromosuccinimide

NFATs = nuclear factor of activated T-cells

NFTs = neurofibrillary tangles

NICD = Notch intracellular domain

NIS = N-iodosuccinimide

NLS = nuclear localisation signal

nm = nanometre

NMR = nuclear magnetic resonance

NR = no reaction
$p$ = para

$P$ = stands for statistical significance

PD = Parkinson’s disease

PDA = photodiode array

Ph = phenyl

PHFs = paired helical filaments

ppm = part(s) per million

Pr = propyl

q = quartet

R = unspecified functional group

$R_f$ = retention factor

RNAPII = Polymerase II

RS = arginine-serine-rich

RT = room temperature

s = singlet

SAR = Structure-activity relationship

sat. = saturated

SEM = standard error of the mean

Ser = serine

$sh$RNAs = short hairpin ribonucleic acids

$s$iRNA = small interfering ribonucleic acid
Abbreviations

SIRT1 = Sirtuin 1

sNPF/NPY = short neuropeptide F/neuropeptide Y

SP = serine-proline repeat

SR = splicing factor containing a C-terminal RS domain

STAT = signal transducer and activator of transcription protein

t = triplet

TBAB = tetra-n-butylammonium bromide

tt = triplet of triplets

$\text{t}_{1/2} = \text{half-life}$

$t$-Bu = tert-butyl

TBAF = tetra-n-butylammonium fluoride

TBS = tert-butyldimethylsilyl

TEA = triethylamine

TFA = trifluoroacetic acid

THF = tetrahydrofuran

Thr = threonine

TLC = thin layer chromatography

TMS = trimethylsilyl

Ts or tosyl = 4-methylbenzenesulfonyl;

Tyr = tyrosine

UV = ultraviolet
Abbreviations

vs = versus

v/v = volume for volume

W = Watt

w/w = weight for weight

X = halide
Chapter 1 - Introduction

1.1 CNS diseases and DYRK1A

1.1.1 Down syndrome and DYRK1A

In 1866, John Langdon Down\textsuperscript{1} first described patients with facial anomalies and mental retardation leading to what is now known as Down syndrome (DS). DS was later recognised by Lejeune \textit{et al}\textsuperscript{2} as a chromosomal disorder resulting from partial or complete trisomy of human chromosome 21, thus DS is the most prevalent intellectual disability arising from a genetic disorder.\textsuperscript{3} Statistical studies\textsuperscript{4-5} showed that DS occurs in approximately 0.1\% of populations in European countries and the United States. Patients with DS generally exhibit language and learning weakness,\textsuperscript{6-7} abnormal motor skills,\textsuperscript{8-10} and short-term and long-term memory deficits.\textsuperscript{11-13} With aging, many patients with DS are prone to developing the symptoms of Alzheimer’s disease (AD), like dementia.\textsuperscript{14-15}

Evidence\textsuperscript{16-17} has shown that cognitive impairments and developmental deficits observed in patients with DS resulted from various pathophysiological changes in the brain. These include changes in brain sizes, reduced neuron numbers,\textsuperscript{18-19} reduced cerebellar volumes,\textsuperscript{20} increased spines on dentrites with augmented spine density\textsuperscript{21} and grey matter density abnormalities.\textsuperscript{22} Since DS has been shown\textsuperscript{23} to be attributed to the overexpression of a large number of trisomic genes, among which, dual-specificity tyrosine phosphorylation-related kinase 1A (DYRK1A) is located within the Down syndrome critical region (DSCR) of chromosome 21.\textsuperscript{24-25} It has also been found to be overexpressed in DS patients.\textsuperscript{26} Previous studies showed that DYRK1A as the dosage sensitive gene on DSCR is responsible for many features of DS including mental retardation and neurodegradation.\textsuperscript{27} Overexpression of DYRK1A leads to the hyperphosphorylation of microtubule-associated tau protein, which further destabilises microtubules, resulting in the release of soluble tau protein. Tau protein eventually forms neurofibrillary tangles (NFTs) by a series of polymerisation and assembly of paired helical filaments (PHFs), which eventually results in neurodegradation \textbf{(Figure 1)}. Alternatively, the increased phosphorylation of ASF (alternative splicing factor) by DYRK1A alters the balance between 3R-tau and 4R-
tau distribution, eventually causing neural death, neurodegradation and mental retardation.\textsuperscript{28-32}

Figure 1. The proposed contribution of DYRK1A to the mechanism of the formation of Neurofibrillary tangles (NFTs) in AD.\textsuperscript{33}

1.1.2 Alzheimer’s disease and DYRK1A

AD is the main cause of dementia.\textsuperscript{34} An estimated 40 million people worldwide have dementia, most of whom are aged over 60.\textsuperscript{35} Generally, AD was thought to result from the aggregates of abnormal beta-Amyloid (Aβ) peptide and NFTs caused by tau protein hyperphosphorylation.\textsuperscript{36-37} Early studies showed that increased phosphorylation of tau by DYRK1A reduced the stabilisation of microtubules, resulting in the release of soluble tau. Subsequent tau dimerisation and polymerisation led to the aggregation of NFTs, which is one of the hallmarks of AD (Figure 1). More recent studies have also indicated that NFTs and Aβ are linked by a key kinase—DYRK1A.\textsuperscript{38} For example, Aβ loading was proven to induce an increased protein
DYRK1A level in neuroblastoma cells, which resulted in tau aggregation.\(^{39}\) In the other pathway, the Aβ precursor protein (APP) gene is also located within human chromosome 21, overexpression of which has caused the increase of Aβ aggregates.\(^{40}\) Mechanistic study indicated that overexpression of APP itself failed to show AD-like pathology.\(^{41}\) Only the phosphorylation of APP by DYRK1A at Thr668 residue \textit{in vitro} and mammalian cells could significantly increase Aβ production in DS and early-onset of AD.\(^{42}\) These results have provided evidence that DYRK1A is a genetic factor for AD.\(^{39,43}\) Additionally, emerging evidence has indicated that DYRK1A exists in much lower plasma concentrations in patients with AD than that in non-AD patients, hinting at its potential as an efficient biomarker for the diagnosis of AD and the development of future therapeutics.\(^{44}\)

### 1.1.3 Other CNS diseases and DYRK1A

Other CNS diseases have been also reported to be associated with the level of DYRK1A expression, such as Parkinson’s Disease (PD).\(^{45}\) PD is a movement disorder or motor syndrome.\(^{46,47}\) The presence of Lewy bodies (intracellular protein aggregates) is one of the hallmarks of PD.\(^{48,49}\) α-Synuclein is the main component of Lewy bodies, and evidence shows that DYRK1A could be potentially correlated with PD by phosphorylating α-synuclein \textit{in vitro} at Ser87 residue.\(^{45}\) The regulation of cytoplasmic α-synuclein inclusion or formation can potentially result in cell apoptosis.\(^{45}\) DYRK1A was also found to directly phosphorylate the causative protein parkin for PD at Ser131, resulting in the loss of its neuroprotective functions.\(^{50}\)

Huntington’s disease (HD) is a fatal genetic brain disorder characterised by uncontrolled motor function, general motor impairments and dementia.\(^{51,52}\) A correlation between DYRK1A binding and direct phosphorylation of huntingtin interacting protein-1 (Hip-1) has been demonstrated in HD. Phosphorylated Hip-1 mediates neuronal differentiation and blockade of cell death in embryonic hippocampal neuroprogenitor (H19-7) cells.\(^{53}\)
DYRK1A has also been implicated with many other diseases, such as Pick disease, diabetes, HIV-1 disease, and Autism spectrum disorder (ASD). Therefore, the normalisation of DYRK1A activity appears to be crucial to improve abnormal phenotypes observed in patients.

1.2 DYRK1A and its family members

DYRKs belong to the CMGC group (named after the initials of some kinase members), which includes other kinases, such as CDKs (cyclin dependent kinases), CDKLs (CDK-like kinases), CK2 (casein kinase 2), CLKs (CDC-like kinases), GSKs (glycogen synthase kinases) and MAPKs (mitogen-activated protein kinases). Among them, CDKs, CKs and MAPK families have been well investigated in terms of their functions in transcription, DNA damage repair, protein degradation and neuronalgenesis. In contrast, the functional roles of DYRKs and CLKs in the signalling pathways of human tissues are currently less clear. DYRKs consist of 5 isoforms including DYRK1A, DYRK1B, DYRK2, DYRK3 and DYRK4, which are subdivided into two classes based on their subcellular localisation. DYRK1A/B belong to class 1, while DYRK2, DYRK3 and DYRK4 are categorised as class 2, and they all possess a kinase domain shown in Figure 2. The CLKs contain CLK1, CLK2, CLK3 and CLK4 in human cells, and all of these members have been found to phosphorylate alternative splicing factors and consequently regulate splicing. Specifically, more details about DYRKs and CLK1 will be reviewed and studied in this thesis.
Figure 2. Domain structure of DYRKs family members.\textsuperscript{54} NLS: nuclear localisation signal; KINASE: kinase domain; PEST: (Pro, Glu, Ser, Thr)-rich domain; His: 13-consecutive-histidine repeat; S/T: (Ser, Thr)-rich region.

As for substrate specificities, DYRKs were originally believed to be proline-directed within a consensus phosphorylation sequence PX(S/T)P, where X is a variable amino acid and S/T (serine/threonine) represents the site of phosphorylation (called P-site) with proline at both P+1 (the first position after P-site in the sequences) and P-1 (the last position before P-site in the sequences) sites.\textsuperscript{76} However, more recent studies have suggested that DYRKs also require an arginine N-terminal to the target site for more efficient phosphorylation.\textsuperscript{77} Therefore, the most preferred consensus sequence for DYRK1A has been reported to be RPX(S/T)P, although proline at P+1 position can be replaced by valine or alanine as well. DYRK1A has preference to recognise serine over threonine,\textsuperscript{78} and phosphorylation on tyrosine was not detectable in exogenous substrates.\textsuperscript{79} In other studies, however, substrates with no proline at P+1 position or arginine at P-2 could be also recognised by DYRK1A. α-Synuclein, mentioned above (Section 1.1.3), does not contain the specificity determinants, but can be phosphorylated by DYRK1A at the Ser87 residue.\textsuperscript{45} Similarly, DYRK1B only phosphorylates at serine/threonine residues but not at tyrosine in exogenous substrates. However, the substrate sites of DYRK1B were not found to correspond very closely with the phosphorylation preferences for DYRK1A as described previously. The sequence for DYRK2 and DYRK3 has been subsequently reported as
Generally, an efficient substrate for phosphorylation is realised through a binding cavity utilisation of protein kinases to specifically recognise complementary charged and/or hydrophobic residues in the substrates. However, CLKs lack such docking grooves in their kinase domains. Rather, the N-terminus attached to CLK1 has been suggested to induce its oligomerisation and thus facilitate its specificity and binding affinity with the RS (arginine-serine-rich) domains of SR proteins (splicing factor containing a C-terminal RS domain). This kinase-substrate combined recognition has been employed to favour highly productive interaction with each other and then facilitate phosphorylation preferences.

1.2.1 DYRK1A

The DYRK1A gene is the only DYRK member located on 21q22.2 of human chromosome 21, which encodes more than 300 annotated genes, one third of which are overexpressed in DS. DYRK1A is a nuclear protein, which contains nuclear localisation signal (NLS) capable of targeting the proteins to the nucleus (Figure 2). However, increased DYRK1A immunoreactivity has also been found in the cytoplasm, which could phosphorylate cytoplasmic protein eIF2B at Ser539 residue in its ε-subunit. DYRK1A also catalyses the phosphorylation of CDKL5 at Ser308 resulting in a site-directed location for subcellular distribution, which facilitates a cytoplasmic location rather than a nuclear location. Furthermore, the kinase domain of DYRK1A incorporates a catalytic domain with its autophosphorylation at the Tyr321 residue, and DYRK1A does not share significant homology sequences with other DYRK2–4 family members outside the kinase domain. DYRK1A also contains a PEST (Pro, Glu, Ser, Thr)-rich domain which is responsible for protein degradation, a repeat of 13 histidine and subsequent serine/threonine residues with functions not yet identified (See Figure 2).

DYRK1A, dual-specificity tyrosine phosphorylation-regulated kinase 1A, as its name implies, has dual specificities. In the kinase domain, DYRK1A can not only catalyse intramolecular tyrosine autophosphorylation (transitional intermediate), but it can also phosphorylate over 20 target substrates at serine/threonine residues (mature active site) in an intermolecular fashion (Figure 3). Therefore, DYRK1A could be targeted
in two approaches by inhibitors: the first approach through inhibiting its autophosphorylation at tyrosine 321 residue during the formation of mature DYRK1A, and the second by targeting the ATP-binding site of mature DYRK1A during the phosphorylation of its substrates at serine/threonine residues. Therefore, dual inhibitor sensitivity is another concept of DYRK1A.\textsuperscript{84}

**Figure 3.** Mechanism of DYRK1A autophosphorylation and mature DYRK1A formation.\textsuperscript{84} As DYRK1A mRNA is translated, the N-terminal polypeptide forms secondary and tertiary structure (I), then polypeptide folding leads to a subsequent intermediate (II) formation with the activation loop as the first substrate, which possesses the tyrosine-kinase activity, the phosphorylated intermediate III at the activation loop is therefore formed and fully translated in its mature formation (IV).

### 1.2.2 DYRK1B

DYRK1B, a highly conserved homolog of DYRK1A, is mapped to human chromosome 19.\textsuperscript{85} DYRK1A and DYRK1B have a high degree of similarity in the N-terminal region and catalytic domain with 84% identical sequences (Figure 2), in which they harbour a conserved nuclear localisation signal. However, DYRK1A and DYRK1B do not show extended sequence similarity in the C-terminal region indicating a different role in functional specialisation. More specifically, DYRK1A is ubiquitously expressed in human and rat tissues, whereas DYRK1B is predominantly
expressed in testes and muscles. Overall, there are fewer than five amino acids variations in the ATP-binding site between DYRK1A and DYRK1B, thus rendering it extremely challenging to identify highly selective inhibitors, especially ATP-competitive inhibitors.

DYRK1B has been found to be overexpressed in several different types of cancer mediating anti-apoptotic functions. For example, DYRK1B is a positive regulator of Hedgehog (HH)/Gli signalling, which plays a causal role in tumour initiation and growth in cancers of the brain, skin and gastrointestinal tract, among others. DYRK1B inhibition has been shown to significantly reduce tumour growth of Gli1-dependent cancer cells such as pancreatic cancer cells with smoothened inhibitor resistance. Furthermore, up-regulation of DYRK1B expression was also found in high-risk human papillomavirus (hr-HPV)-expressing cells, which contributed to HPV E7 cell proliferation by phosphorylating p27 (cyclin-dependent kinase inhibitor 1B) at Ser10 and Thr198. Additional evidence for DYRK1B as a therapeutic target in HPV-associated cancers was also provided.

1.2.3 DYRK2

DYRK2 is another member of DYRK family, predominantly expressed in the cytoplasm. Genetic studies indicated that DYRK2 was overexpressed in all of the tumours showing gene amplification, but the role of DYRK2 in cancer is still controversial. Recent studies demonstrated that DYRK2 induced p53-dependent apoptotic cell death by direct phosphorylation of p53 at Ser46 residue. It also plays a crucial role as a tumour suppressor in human non-small cell lung cancer, and is potentially a predictive marker for chemotherapy.

1.2.4 DYRK3

DYRK3, closely related to DYRK2, is also cytoplasmic containing 54% homology in comparison to DYRK1A. Together with DYRK1A, DYRK3 was demonstrated to promote cell survival through direct phosphorylation and activation
of SIRT1.\textsuperscript{97} On the contrary, knockdown of DYRK3 could in turn activate p53 (also called as tumour protein p53) and thus induce cell death in response to genotoxic stress. Furthermore, DYRK3 was shown to attenuate apoptosis in response to cytokine withdrawal.\textsuperscript{98}

### 1.2.5 DYRK4

In contrast to other family members, data for DYRK4 is barely known. Recently, DYRK4 was identified as a testis-specific kinase with highly restricted testicular expression in spermiogenesis.\textsuperscript{99} Further studies are necessary to obtain further insight into DYRK4 cellular functions.

### 1.2.6 CLK1

CLK1 is a nuclear protein kinase and structurally related to DYRK1A with over 30\% similarity. CLK1 was found to regulate ASF (alternative splicing factor) by phosphorylating SR proteins (splicing factor containing a C-terminal RS domain) more specifically in the RS (arginine-serine-rich) domains except for the serine-proline recognition site.\textsuperscript{80} Increased CLK1 levels significantly enhance the phosphorylation of ASF resulting in the mislocalisation of splicing factors from the nucleus,\textsuperscript{100-101} and the increased expression of splicing factors has been found in numerous carcinomas, such as ovarian cancer.\textsuperscript{102} Analysis of cancer transcriptomes by EST (expressed-sequence-tag) libraries from a wide variety of cancer types has suggested that the cancer cells have “noisier” splicing than that in normal tissue counterparts.\textsuperscript{103-104} These studies indirectly provided the correlation between CLK1 activity and tumour growth. Additionally, CLK1 overexpression in tumours was also detected, and inhibition of CLK1 contributes to cell death in cancer by controlling periodic alternative splicing.\textsuperscript{105-106} A recent report has pointed out that overexpression of CLK1 has strong effect on HIV-1 replication by increasing HIV-1 Gag RNA levels and thus virus production.\textsuperscript{107}

Since CLK1 phosphorylates SR proteins at serine residues, it is involved in the
pathophysiology of AD resulting from the increased release of SR proteins in the nucleoplasm from the nuclear speckles.\textsuperscript{104,108-109} Thus the role of CLK1 appears crucial in the development and discovery of therapeutic potential drug for AD as well.

In summary, DYRK1A has been implicated in the phosphorylation of more than 20 substrates involved in cellular and neuronal process, as well as the regulation of cytoskeletal and synaptic proteins.\textsuperscript{110} Normalisation of DYRK1A expression level appears to be essential. It is for this reason that DYRK1A is the main target of this thesis.

### 1.3 Regulation of DYRK1A expression

DYRK1A is a dosage sensitive gene – dosage imbalance of DYRK1A expression drastically affects brain function and structure. For example, mice with lower levels of DYRK1A expression produced similar phenotypic effects to those overexpressing DYRK1A.\textsuperscript{111} Evidence suggests that haploinsufficiency of DYRK1A might be responsible for the cognitive phenotypes of monosomy 21, thereby leading to autosomal dominant mental retardation.\textsuperscript{112} Therefore, low or high expression of DYRK1A could cause a series of abnormalities. This poses two important questions: 1) what regulates expression level of DYRK1A? 2) How does DYRK1A expression affect diseases?

#### 1.3.1 Factors affecting DYRK1A expression levels

DYRK1A expression is regulated by many factors, including transcriptional factors, tumour suppressor factors, neurogenic factors as well as protein-protein interactions. Emerging studies suggest that the protein-protein interactions could maintain the regulation of DYRK1A. For example, the catalytic activity of DYRK1A is regulated by autophosphorylation at Ser520 \textit{via} an intramolecular mechanism. Autophosphorylation triggers interactions of DYRK1A with 14-3-3β protein that belongs to 14-3-3 protein family. This family is correlated with activation or inactivation of catalytic activities, the formation of macromolecular complexes,
modulation of protein localisation and/or stabilisation. Therefore inducing the conformational changes results in increased catalytic activities of DYRK1A.\textsuperscript{113}

HAN11, the human homologue of AN11, encodes HAN11 protein, which interacts directly with DYRK1A to regulate expression levels. AN11 is predominantly in the cytoplasm, whereas DYRK1A is predominantly in the nuclear, yet binding between the two has been validated in previous studies.\textsuperscript{114} Later reports provided consistent results that HAN11 exists in both the cytoplasm and nucleus, when activated by mDia1, which is a cytoskeletal regulator as a binding partner of HAN11.\textsuperscript{115} Therefore, the interaction between DYRK1A with HAN11 in the cytoplasm is increased by moving from the nucleus to the cytoplasm. As a result, Gli1-dependent gene transcription activity regulated by DYRK1A is decreased by changing the level of nuclear Gli1.

Transcriptional activity of nuclear factor of activated T-cells (NFATs) is negatively regulated by DYRK1A.\textsuperscript{116} Phosphorylation of NFAT by the nuclear DYRK1A induces its exit into the cytoplasm, resulting in the loss of transcriptional activity. NFATs stimulate the expression of DYRK1A in both osteoclast precursors and osteoclasts,\textsuperscript{117} which in turn reduces the activity of NFATs by increased DYRK1A expression. This discovery suggested a negative feedback mechanism for DYRK1A-NFATs regulation, and thus predicted a therapeutic strategy for bone homeostasis and bone-destructive diseases. Furthermore, emerging evidence shows that normalisation of DYRK1A expression through MicroRNAs-199b (miR-199b) inhibition by antagonomirs could decrease dysregulated vertebrate development, such as vascular defects and heart failure by promoting calcineurin/NFAT signaling.\textsuperscript{118}

Another transcriptional factor, E2F1, was also indicated to regulate DYRK1A mRNA level. It was suggested that overexpression of E2F1 enhanced the activity of DYRK1A promoter by increasing DYRK1A mRNA level in phoenix cells, which provided additional evidence that DYRK1A plays a crucial role in cell cycle regulation.\textsuperscript{119} Activator protein 4 (AP4) negatively regulated the transcription of DYRK1A in nonneuronal cells as a putative repressive element-binding transcription factor. This occurs by forming a functional complex with corepressor geminin as a repressor complex, indicating that reduced expression of the AP4 repressor complex

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contributes to the premature overexpression of DYRK1A in foetal brain.\textsuperscript{120}

P53, a tumour suppressor factor, has been reported to reduce the level of DYRK1A expression through the induction of miR-1246, overexpression of which reduced DYRK1A levels, resulting in the nuclear retention of NFATc1 and induction of apoptosis.\textsuperscript{121} On the other hand, DYRK1A phosphorylates p53 at Ser15 residue \textit{in vitro}, which results in an impairment of G1/G0 phase transition attenuated H19-7 cells and embryonic neural cells proliferation. This provided evidence that upregulation of DYRK1A makes a contribution to neural proliferation alteration in DS.\textsuperscript{122}

In the cultured neuroblastoma cell line SH-SY5Y and transgenic mice, an increase in DYRK1A mRNA (transcription) was detected as a result of Aβ loading (SH-SY5Y cells were incubated with Aβ peptide). This led to hyperphosphorylation of tau at Thr212 residue, further contributing to AD pathology, which was described previously (Section 1.1.2).\textsuperscript{39} Furthermore, DYRK1A activity was markedly induced by neurogenic factor basic fibroblast growth factor (bFGF), resulting in an increased specific binding between DYRK1A and active site of CREB (cAMP response element-binding) leading to subsequent transcription in neuronal differentiation.\textsuperscript{123}

1.3.2 How does DYRK1A expression affect diseases?

DYRK1A has been found to affect multiple signalling processes by activating or inactivating signals of other factors, such as transcriptional factors, translation factors, splicing factors and miscellaneous proteins including glycogen synthase, caspase-9 and Notch, as well as synaptic proteins phosphorylation.

1.3.2.1 DYRK1A activates transcriptional factors

DYRK1A was suggested to comprise part of RNAPII (Polymerase II)-containing complexes \textit{in vivo}. Studies showed that RNAPII CTD (C-terminal repeated domain), which is responsible for ‘paused’ or ‘elongating’ phase of the transcription cycle, is
also a substrate for phosphorylation at Ser2 and Ser5 residues by DYRK1A. Low levels of DYRK1A expression led to reduced RNAPII association in the targeting regions and increased CTD phosphorylation. In this case, DYRK1A is likely acting as a transcriptional regulator.\textsuperscript{124}

Multiple data sources indicate that the DYRK family is a key protein regulating NFAT1 phosphorylation. DYRKs 2–4 maintain the phosphorylation status of cytoplasmic NFAT in resting cells, whereas DYRK1A re-phosphorylates nuclear NFAT and thus promotes nuclear export in terms of their particular locations (cytoplasm or nucleus). Moreover, DYRK1A-mediated phosphorylation of the conserved serine-proline repeat 3 (SP-3) motif of the NFAT-regulatory domain primes for further phosphorylation of SP-2 and serine-rich region 1 (SRR-1) motifs by CK1 and GSK, thus deactivating NFAT1 by facilitating complete phosphorylation.\textsuperscript{125} Overdosage of DYRK1A associated with DSCR1 was reported to lead to reduced NFATc activity in immune response.\textsuperscript{116}

Sprouty2, as a modulator of tyrosine kinase receptor signalling capable of enhancing EGF-dependent signalling, was suggested to be phosphorylated by DYRK1A, thus blocking epidermal growth factor receptor (EGFR) degradation.\textsuperscript{126} Furthermore, phosphorylation of sprouty2 at Thr75 by DYRK1A was found to positively affect FGF-induced mitogen-activated protein kinase activation.\textsuperscript{127}

DYRK1A was reported as a regulator of the DREAM complex, which is a multi-subunit complex that regulates gene expression during quiescence. Phosphorylation of DREAM subunit LIN52 by DYRK1A at Ser28 was found to regulate the complex formation of the G0 phase. Inhibition of DYRK1A by inhibitors such as INDY led to quiescence inhibition and significantly increased the imatinib-induced gastrointestinal stromal tumour (GIST) cell apoptosis, thereby increasing anti-tumour efficacy.\textsuperscript{128}

DYRK1A also has the capacity to activate CREB, a transcriptional factor implicated in the formation of learning and memory. Phosphorylation and subsequent stimulated CRE-mediated gene transcription in neuronal differentiation resulted in neurite outgrowth.\textsuperscript{123} Additionally, DYRK1A was demonstrated to activate transcriptional
factor FKHR (forkhead in rhabdomyosarcoma) by phosphorylating FKHR at Ser329 specifically in vivo. This has been implicated in the control of gene expression by insulin and regulation of apoptosis. The increased DYRK1A levels also made a contribution to FKHR cellular localisation.\textsuperscript{129}

STAT (signal transducer and activator of transcription protein), a transcriptional factor critical for astrogliogenesis, is another factor activated by DYRK1A. Overexpression of DYRK1A enhanced the phosphorylation and activity of STAT3 at Ser727 residue, a regulatory site that activates STAT3 activity, and in turn, the elevated STAT3 activity increases the differentiation of progenitor cells into astrocytes in vitro and in vivo. However, the knockdown of DYRK1A has no significant effects on STAT3 phosphorylation at Ser727, nor differentiation into astrocytes.\textsuperscript{130} These studies suggested that the DYRK1A-STAT signalling pathway is an implication for the anomalies in DS brain development, since DS is associated with the increased generation of astrocytes resulting from the increased progenitor cells differentiation into astrocytes.\textsuperscript{131}

Tau itself is a poor substrate for GSK3 (glycogen synthase kinase-3), whose phosphorylation by GSK3 could only occur after a first phosphorylation of serine or threonine by other protein kinases like DYRK1A in vitro.\textsuperscript{32} For example, phosphorylation of RCAN1 (Regulator of Calcineurin 1, which is also located in chromosome 21) by DYRK1A at the Ser112 residue primes protein phosphorylation of Ser108 by GSK3\textbeta.\textsuperscript{132} Phosphorylation by DYRK1A at tau Thr212 residue primes tau phosphorylation by GSK3 at the Ser208 residue, and thus resulting in the accumulation increase of neurofibrillary tangles, which, according to in vitro kinase assay, exists in the brain of AD patients.\textsuperscript{133} DYRK1A, as one of the clock-regulated protein kinases, catalyses priming phosphorylation of CRY2 (crystal like protein 2) at Ser557, and this leads to secondary phosphorylation of CRY2 at Ser\textsuperscript{553} by GSK-3\textbeta, thereby leading to CRY2 degradation.\textsuperscript{134} Moreover, DYRK1A priming phosphorylated GSK-3\textbeta at Ser539 in its \epsilon-subunit permits GSK3 to phosphorylate translation factor eIF2B\textepsilon at Ser535 in vitro, and thus suggests that DYRK1A indirectly activated eIF2B\textepsilon.\textsuperscript{32} As a result, increased DYRK1A immunoreactivity was found in the cytoplasm, which provides further evidence that DYRK1A plays an
essential role in the cytoplasm as well, even though its localisation is in the nucleus.

In addition, DYRK1A stimulates the activity of transcriptional factor Gli1 resulting in more efficient association with other transcriptional factors and thus delaying its nucleus export.\textsuperscript{135}

\subsection*{1.3.2.2 DYRK1A activates splicing factors}

DYRK1A has the capacity to activate splicing factors. For example, DYRK1A phosphorylated ASF at Ser227, Ser234 and Ser238 \textit{in vitro}, and its overexpression altered the nuclear distribution of ASF into nuclear speckles, which affected the tau transcript. The resulting imbalance of 3R/4R tau in the DS brain further accelerated neurofibrillary degeneration, this represents a novel mechanism of neurofibrillary degeneration.\textsuperscript{136}

DYRK1A is currently the only DYRK family member that has been found to be involved in the regulation of Cyclin D1 turnover in RG (radial glio) progenitors.\textsuperscript{137} Evidence showed that high-level phosphorylation of Cyclin D1 by DYRK1A at the Thr286 residue might induce proteolytic degeneration of Cyclin D1 in RG progenitors \textit{in vivo}, and this process subsequently caused a steep increase in G1 phase duration of the cell cycle.\textsuperscript{138} This is consistent with previous studies that suggested that the premature neuronal differentiation of neural progenitor cells was caused by Cyclin D1.\textsuperscript{139} In contrast, reduced DYRK1A activity increases Cyclin D1 expression, and the resulting increased expression of the CDK inhibitor p21 causes a competition between Cyclin D1 and p21 expression level, and then controls the cell fate.\textsuperscript{138}

\subsection*{1.3.2.3 DYRK1A activates miscellaneous proteins and synaptic proteins}

Notch proteins are a family of transmembrane proteins. Notch signalling regulates interactions between physically adjacent cells, and it is controlled by several kinases in the nucleus by inducing phosphorylation of the Notch intracellular domain (NICD). DYRK1A has been implied to attenuate Notch signalling in neural cells both in culture and \textit{in vivo}. The interaction between DYRK1A and NICD promotes its
phosphorylation in the ankyrin domain and induces transcription termination.\textsuperscript{140} DYRK1A plays a critical role in maintaining the normal function of the retina – a dosage change of the DYRK1A gene leads to severe alterations in retinal cellularity and the changes in retina cell numbers. Evidence has shown that DYRK1A phosphorylates caspase-9 at the Thr125 residue, and the activation of caspase-9 is associated with apoptosis.\textsuperscript{141} Therefore, correct DYRK1A gene dosage is essential for the survival of retina cells during their development.\textsuperscript{142}

It has been identified that DYRK1A, together with other DYRKs, causes the phosphorylation of GSK3 at the Ser640 residue. This protein-protein interaction subsequently leads to the inactivation of glycogen synthase, a key enzyme in the regulation of glycogen synthesis by insulin. This finding has provided a new mechanism for the regulation of glycogen synthase by DYRK1A.\textsuperscript{114}

Dynamin 1 was suggested\textsuperscript{143-144} as one of the substrates acted upon by DYRK1A as it was directly phosphorylated by DYRK1A mainly at Ser857 in its native state. Phosphorylation of Dynamin 1 resulted in the reduced binding affinity of dynamin to amphiphysin 1, an enzyme involved in endocytosis. In most case, DYRK1A may just function as a regulator controlling the synaptic vesicle recycling, but later studies demonstrated that DYRK1A also directly phosphorylated amphiphysin 1 at Ser293 during the regulation of endocytosis at synapses.\textsuperscript{145}

\textbf{1.3.2.4 Other substrates activated by DYRK1A}

DYRK1A is a critical kinase for the regulation of beta cell growth. DYRK1A-haploinsufficient mice showed severe glucose intolerance and reduction in mass and proliferation of beta cells, eventually causing diabetes.\textsuperscript{146} Upregulation of DYRK1A in beta cells was found to significantly enhance this phenomenon.\textsuperscript{147} However, recent studies\textsuperscript{148} have presented different conclusions concerning the correlation between DYRK1A and beta cell proliferation. For example, DYRK1A inhibition by the adenosine kinase inhibitor steeply stimulated human beta cell proliferation \textit{in vitro} and \textit{in vivo}, as reported in previous parallel studies.\textsuperscript{149-151} Such different results can be explained by the large variance between rodent and human and beta cell replication.
Accumulating studies showed that DYRK1A, together with DYRK3, promoted cell survival and resulted in deacetylation of p53. SIRT1 (Sirtuin 1, also known as NAD-dependent deacetylase sirtuin 1), in this process, acts as the p53 deacetylase, resulting from the phosphorylation and activation directly by DYRK1A at the Thr522 residue. This essential deacetylation is involved in multiple physiological processes including stress response and energy metabolism. On the contrary, knockdown of DYRK1A and DYRK3 activated p53 and induced cell death in response to genotoxic stress. The increased SIRT1 activation by DYRK1A activated FOXO (forkhead box O) transcriptional factor, and turned on the expression of sNPF/NPY (short neuropeptide F/neuropeptide Y) target gene, as a result, increased food intake in fruit flies and mice was observed.

DYRK1A also specifically binds to Hip-1, which is known to be associated with the pathogenesis of HD described previously (Section 1.1.3). High affinity and hyperphosphorylation of Hip-1 by DYRK1A resulted in Hip-1-mediated neuronal differentiation and blockade of cell death in H19-7 cells. DYRK1A directly phosphorylated the GTPase-binding domain of neural Wiskott-Aldrich syndrome protein (NWASP) at the threonine residue, and knockdown of the DYRK1A gene can promote actin filament polymerisation, which is associated with cell migration and endocytosis in cellular process.

Normalising the level of DYRK1A expression rescued the skeletal alterations in DS Ts65Dn mice, including reduction of bone mineral density and changes of facial features. DYRK1A also catalyses the phosphorylation of CDKL5 at the Ser308 residue, subcellular distribution is then altered to facilitate a cytoplasmic location in response to some stimulation in hippocampal neurons.

In summary, hyperphosphorylation mediated by DYRK1A overexpression is associated with multiple pathogenetic differentiations in the development of brain diseases, especially in DS and AD. Therefore, it is necessary to investigate DYRK1A as a novel target for exploration of treatments of abnormal phenotypes in DS or AD patients.
1.4 Roles of DYRK1A in tumours

In DS patients, about 1.5 fold DYRK1A overexpression has been detected resulting from the extra copy of human chromosome 21. However, multiple studies have suggested that patients with DS have a much lower risk of all common tumour types and several malignancies (except testicular cancers and leukaemia) in either females or males at all ages.\textsuperscript{157} Such a low cancer risk can be explained by a slower rate of replication and/or higher possibility of apoptosis resulting from the tumour suppressor genes location on human chromosome 21 as described before (Section 1.3.2.2),\textsuperscript{158} the hyperphosphorylation of cyclin D1 or NFAT mediated by DYRK1A overexpression could be linked to these anti-cancer effects.\textsuperscript{138}

Overexpression of DYRK1A has been found in several tumours. For example, up-regulated DYRK1A in HPV16 infected keratinocyte cells has been identified,\textsuperscript{159} although it was not detected in primary keratinocytes. Increased DYRK1A induced tumour formation and growth by triggering cell anti-apoptosis resulting from the mislocalisation of FKHR mentioned above (Section 1.3.2.1).\textsuperscript{129} In cervical lesions,\textsuperscript{160} higher DYRK1A levels have been observed compared with those in normal tissues.\textsuperscript{161} In gastrointestinal stromal tumour, studies indicated that perturbations in the quiescence regulation by a regulator – DREAM complex contributes to the increased mitotic gene expression levels which are frequently detected in cancers, and DYRK1A inhibition was found to eliminate the DREAM complex and thus enhance imatinib-induced apoptosis.\textsuperscript{162}

Studies have shown that DYRK1A is upregulated in glioblastoma cells, which represent approximately 15\% of brain tumours.\textsuperscript{163} The role of DYRK1A has also been established in the same studies. The increased phosphorylation of sprouty2 mediated by DYRK1A (Section 1.3.2.1) blocks the EGFR degradation as a result of overexpression of EGFR in the cell surface (Figure 4), and the enhanced EGFR signalling eventually leads to tumour survival. On the contrary, DYRK1A inhibition has been found to promote EGFR degradation in glioblastoma cells by triggering the endocytosis and lysosomal degradation, thus reducing the self-renewal ability of
tumourigenic cells.\textsuperscript{163} Therefore, DYRK1A as a novel target in EGFR-dependent glioblastoma represents a promising strategy for cancer therapy.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{dyrk1a_egfr.png}
\caption{DYRK1A-mediated EGFR degradation or recycling.\textsuperscript{65}}
\end{figure}

1.5 DYRK1A inhibitors

Recently, researchers have endeavoured to modulate DYRK1A expression to ameliorate DYRK1A-associated memory deficits and mental retardation. Several approaches have been investigated, such as natural products and derivatives, synthetic inhibitors or \textit{shRNAs} (short hairpin ribonucleic acids).\textsuperscript{164-165} Generally, small-molecules act as a scaffold for protein-protein interactions, whereas if treated by \textit{shRNAs}, proteins may lose activities in other biological functions. Therefore, small, cell-permeable and blood-brain barrier (BBB)-permeable inhibitors of DYRK1A are important tools for investigating effective solutions to counteract alterations in the brain as a therapeutic strategy.
1.5.1 Natural compounds and derivatives

Several compounds with inhibitory activity against DYRK1A have been isolated from natural sources.\textsuperscript{166-167} For example harmine (1a) (Figure 5), a β-carboline alkaloid with a pyrido[3,4-b] indole ring structure, was first isolated from medical herbs — the South American vine \textit{Banisteriopsis caapi}, which was first used to treat Parkinsonism in 1928.\textsuperscript{167} Harmine was later identified as a CDK inhibitor in an ATP-competitive manner and displayed a strong inhibitory activity against cell growth and proliferation.\textsuperscript{151,168} Recently, harmine has been shown to be an ATP-competitive, potent and relatively selective inhibitor of DYRK1A \textit{in vitro},\textsuperscript{169-170} with 5-fold more inhibitory activity against DYRK1A (IC\textsubscript{50} = 33 nM) over DYRK1B (IC\textsubscript{50} = 166 nM), and much more potent than other DYRK family members. Studies have shown that harmine inhibited substrate phosphorylation by DYRK1A more potently than that at tyrosine autophosphorylation of the translational intermediate of DYRK1A (IC\textsubscript{50} = 1.9 μM). Inhibition of substrate phosphorylation by DYRK1A is therefore considered to be the major mode of action.\textsuperscript{170} Moreover, harmine and other β-carboline analogues were demonstrated to inhibit DYRK1A-dependent phosphorylation of tau at multiple sites associated with tau pathology like NFTs in DS and AD.\textsuperscript{171}

Harmine, a β-carboline analogue, has shown additional properties, such as inhibition of monoamine oxidase A (MOA-A) (IC\textsubscript{50} = 5.3 nM), a target for depression.\textsuperscript{172} Recently, by evaluating the characteristic features of the binding pockets of MOA-A and DYRK1A, the reduction of MOA inhibition, whilst maintaining DYRK1A inhibition has been achieved.\textsuperscript{173} In comparison (Figure 6), the methoxy group of harmine does not interact with MAO-A, whereas it does interact with the backbone NH group of hinge residue Leu241 in DYRK1A.\textsuperscript{174} Despite this, the central ring NH group of harmine is essential for MAO-A, but not essential for DYRK1A binding (Figure 5).\textsuperscript{175} Therefore, to improve DYRK1A selectivity, modifications were focused on the central NH group of harmine by significantly changing the binding affinity with MAO-A. As expected, the harmine derivative 1b (Figure 5), with increased size of the substituted ethyl ester dramatically decreased its inhibitory potency against MAO-A (IC\textsubscript{50} > 10000 nM), but retained binding affinity with
DYRK1A (IC$_{50}$ = 52 nM). Surprisingly, by changing the spatial effects of the methyl group on the pyridine ring, the chloro derivative $1c$ (IC$_{50}$ = 56 nM) showed similar potency with $1b$, but it lost all the activity against MAO-A. This improvement gave us confidence in the investigation of potential DYRK1A inhibitors through functional modifications.

![Chemical structures of harmine and its derivatives](image)

**Figure 5.** Chemical structures of harmine and its derivatives.

![Binding pockets of MAO-A (left) and DYRK1A (right) interacting with harmine](image)

**Figure 6.** Binding pockets of MAO-A (left) and DYRK1A (right) interacting with harmine.$^{174}$ Modifications on the central NH group of harmine could significantly decrease the binding affinity with MAO-A, thus it imparts a greater DYRK1A selectivity.

Epigallocatechin gallate (EGCG, 2) is a polyphenol bearing a benzopyrane scaffold (**Figure 7**). It was isolated from green tea leaves as a major catechin component.$^{176}$ EGCG has been reported by Bain and co-workers as a potent inhibitor of DYRK1A with an IC$_{50}$ of 0.33 µM.$^{166}$ However it also showed inhibitory potency against p38 with an IC$_{50}$ of 1 µM by binding to p38 at the threonine residue in the activation loop. As a result, p38 phosphorylation by other kinases was blocked in this case. EGCG was later discovered to be a non-ATP competitive inhibitor *via* a combination of
genetic and biochemical approaches.\textsuperscript{177} Research later has shown that EGCG-activated cell death signals and induced apoptosis in cancer cells selectively, without effecting normal cells.\textsuperscript{178} Overexpression of DYRK1A was found to lead to neuronal plasticity deficits in TgDYRK1A mice model, a transgenic mouse model generated by the overexpression of full-length DYRK1A cDNA with similar phenotypes to DS.\textsuperscript{179} When TgDYRK1A mice were treated with EGCG, its neurogenesis alterations could be significantly rescued.\textsuperscript{180-181} Furthermore, a randomised and double-blind pilot study has demonstrated the safety of EGCG, and its use has shown significant effects on improving cognitive functions in trisomic mice (TG65Dn) and TgDYRK1A mice.\textsuperscript{182} However, some side-effects of EGCG have been detected, such as inhibitory effects on cannabinoid receptor 1 (CB1).\textsuperscript{183} Unfortunately, the poor bioavailability of EGCG has extensively limited its use as long-term therapeutic option.\textsuperscript{184}

\begin{figure}
\centering
\begin{tikzpicture}
\node at (0,0) {\includegraphics[width=\textwidth]{figure7.png}};
\end{tikzpicture}
\caption{Chemical structures of DYRK1A inhibitors 2–3.}
\end{figure}

Indolocarbazoles are a class of natural products isolated mainly from actinomycetes.\textsuperscript{185} Several indolocarbazole analogues have been applied for the treatment of PD in clinical trials.\textsuperscript{186} Staurosporine (3a), as a representative of this family, possesses an indolo[2,3-a]pyrrolo[3,4-c]carbazole ring system attached to a sugar moiety by bonding to both the indole nitrogen atoms. Staurosporine has exhibited potent inhibition against DYRK1A with an \textit{IC\textsubscript{50}} of 19 nM (Figure 7).\textsuperscript{187} Rhamnose (3b), a carbonyl derivative of 3a, showed even stronger inhibition for DYRK1A (\textit{IC\textsubscript{50}} = 4 nM). However, both 3a and 3b were highly non-selective among a large array of kinases including JAK2 (Janus kinase 2), FTL3 (fms-like tyrosine kinase 3), SYK (spleen tyrosine kinase) with subnanomolar inhibition.\textsuperscript{187} These compounds are typically used as screening control molecules in research studies.\textsuperscript{67}
Marine alkaloids, such as meridianins (4), meriolins (5), and variolins (6) were also isolated from natural sources and all exhibited DYRK1A inhibition (Figure 8).\textsuperscript{188-189}

For example, meridianin 4a displayed 30% inhibition towards DYRK1A at 0.5 µM, but it did not show cytotoxicity to neuroblastoma SH-SY5Y cells (IC\textsubscript{50} > 100 µM).\textsuperscript{190}

Meridianin analogue 4b, bearing a 6-bromo substituent, showed potent inhibition against DYRK1A with an IC\textsubscript{50} of 34 nM, whereas it displayed similar activity towards CLK1 (IC\textsubscript{50} = 32 nM).\textsuperscript{191} Meriolins are chemical hybrids between meridianins and variolins, bearing the 7-azaindole scaffolds (Figure 8). Merolin 5a, containing a 2-aminopyrimidine substituted azaindole, was shown to be a potent inhibitor of CDKs, especially CDK9 (IC\textsubscript{50} = 26 nM), and to a lesser extent, other kinases such as DYRK1A (IC\textsubscript{50} of 130 nM).\textsuperscript{192} The 4-hydroxy derivative merolin 5b has shown inhibitory activity against both CDK9 and DYRK1A with IC\textsubscript{50} values of 18 and 35 nM, respectively. Furthermore, due to their potent inhibition of CDKs resulting in cell cycle perturbations, meriolins have been revealed to have significant effects on antiproliferation of multiple cancer cell lines.\textsuperscript{192}

Variolin B (6) was isolated from the difficult-to-access antarctic sponge Kirkpatrickia varialosa found in Antarctica, and represents one of the first examples of an aminopyrimidine substitute on the tricyclic core.\textsuperscript{193} Variolin B was identified as a CDK inhibitor and potent cytotoxic agent by inhibiting colony formation, resulting in cell apoptosis.\textsuperscript{194-196} Later on, variolin B was also demonstrated to inhibit other kinases such as DYRK1A and CK1 with IC\textsubscript{50} values of 80 and 5 nM, respectively.\textsuperscript{192} However, both variolin B and its derivate deoxy-variolin B have limited use in clinical trials due to the solubility issues and complex pharmacokinetic properties.\textsuperscript{196} Therefore, meriolins as synthetic hybrids between meridianins and variolins might be a potent inhibitory scaffold for future cancer therapeutics.
The purine olomoucine (8) was first identified as a potent CDKs (especially CDK2) inhibitor in an ATP-competitive manner (Figure 9). Roscovitine (9) and purvalanol (10), two derivatives from olomoucine, were later described as more potent and selective inhibitors of CDK2. In 2003, Bain and co-workers first tested roscovitine and purvalanol against a large number of kinases including DYRK1A. Purvalanol, as an ATP-competitive inhibitor, showed a more potent activity against DYRK1A (IC$_{50}$ = 300 nM) in comparison to roscovitine (IC$_{50}$ = 3.1 µM). However, both compounds exhibited far more inhibitory potencies against CDK2 with IC$_{50}$ values of 100 nM and 250 nM, respectively.

Leucettamine B (11a), another natural compound bearing an imidazole scaffold, was isolated from the marine sponge leucetta microraphis in 1993 (Figure 10). The biological activity of 11a reported by Debdab and co-workers displayed potent
inhibition against CLK1 (IC$_{50}$ = 400 nM), and to lesser extent, it also exhibited inhibition against DYRK1A with an IC$_{50}$ value of 2.8 µM. Additionally, its derivative leucettine$_{L41}$ (11b) has dramatically increased its inhibitory potency against DYRK1A (IC$_{50}$ = 40 nM), although it inhibited DYRK2 and CLK1 even more potently with IC$_{50}$ values of 35 and 15 nM, respectively.$^{203}$ Specifically, 11b has also displayed neuroprotective effects on glutamate-induced HT22 cell and APP-induced cell death.$^{204}$

![Chemical structure of DYRK1A inhibitors 11.](image)

**Figur 10.** Chemical structure of DYRK1A inhibitors 11.

### 1.5.2 Synthetic inhibitors

As described previously, DYRK1A cannot only catalyse autophosphorylation on its tyrosine 321 residue, but also phosphorylates substrates on serine/threonine residues. Therefore, two targeted approaches to inhibiting DYRK1A may be considered promising, but require different binding modes. The inhibition of DYRK1A autophosphorylation can generally be realised by using small molecules to induce a conformational change, resulting in the loss of DYRK1A function. These inhibitors are classed as type II inhibitors, and might offer new avenues for developing more specific therapeutics.$^{68}$ However, the major problem that exists with this approach is that excessive mutation of DYRK1A itself would cause long term function loss in other biological signalling pathways. Therefore, in most cases, inhibition of specific substrate-phosphorylation may have more benefits than targeting DYRK1A autophosphorylation. Many synthetic inhibitors of DYRK1A that have been developed to date possess higher inhibitory potency against substrate phosphorylation than against tyrosine autophosphorylation.$^{84,205}$ For example, harmine as noted before,
inhibits DYRK1A substrates phosphorylation with an IC$_{50}$ value of 33 nM, but its inhibitory activity against tyrosine autophosphorylation is much weaker with an IC$_{50}$ value of 1.9 µM. Such substantial differences indicate that inhibition of substrate phosphorylation and autophosphorylation of DYRK1A have markedly different SAR (structure-activity relationship) profiles. Inhibitors targeting the ATP binding site of DYRK1A are termed type I inhibitors, or ATP competitive inhibitors in an ATP-competitive manner. 

Ogawa and co-workers have developed a novel DYRK1A inhibitor INDY (12a) – a benzothiazole derivative structurally related to Tg003 (12b) (Figure 11, top). Tg003, was initially identified as a potent CLK inhibitor (especially CLK1 and CLK4), but was later recognised as DYRK1A inhibitor with IC$_{50}$ value of 93 nM. Strikingly, INDY displayed nearly 3-fold greater inhibitory activity against DYRK1A (IC$_{50}$ = 240 nM) compared with Tg003 in an ATP-competitive manner. Furthermore, INDY also inhibited other DYRK family members. DYRK1B, as the most closely related member to DYRK1A, was inhibited by INDY with comparable potency (IC$_{50}$ = 230 nM). Additionally, the co-crystal structure of DYRK1A/INDY complex was also reported in this study (Figure 11, bottom). INDY binds the active site of DYRK1A where the hydroxyl oxygen appears to hydrogen bond to the hinge backbone amide NH of Leu241, while the carbonyl oxygen is anchored through engagement with the side-chain amino group of conserved Lys188. The biological activity of INDY in cells was also confirmed in this study, in which near total inhibition of tau phosphorylation by INDY was observed at 30 µM, and the calcineurin/NFAT signalling could be partially ameliorated in the INDY-treated HEK293 cells. Moreover, a novel in vivo assay was also employed to evaluate the effects of INDY in X.laevis embryo, which is an ideal platform to observe general effects caused by DYRK1A overexpression. However, deformities were observed in morphological aspects including the eyes and head of tadpoles. To rescue these side effects, pro-INDY (12c) was developed with acetyl group attached (Figure 11, top). Encouragingly, pro-INDY has successfully rescued the morphological changes while maintaining cellular activity similar to that of INDY.
Figure 11. Chemical structures of DYRK1A inhibitors 12 (top) and crystal structure of DYRK1A/INDY complex (bottom).\textsuperscript{206}

CX-4945 (13, Figure 12) was originally reported as a potent and selective inhibitor of CK2 through blocking CK2-dependent HIF-1α transcription in cancer cells. It is currently undergoing clinical trials for cancer treatments as an orally bioavailable molecule.\textsuperscript{208} In 2014, CX-4945 was described as a potent CLK inhibitor, especially for CLK2 in an ATP competitive manner to regulate alternative splicing.\textsuperscript{209} As noted previously, many CLK inhibitors also exhibited similar inhibitory potencies against DYRKs because of the high degree conservation between DYRKs and CLKs inside the phylogenetic system.\textsuperscript{58} In 2016, the inhibitory activity of CX-4945 against DYRK1A was investigated by Kim and co-workers.\textsuperscript{210} Intriguingly, CX-4945 was found to be an even more potent inhibitor of DYRK1A than harmine, INDY or proINDY, with an IC\textsubscript{50} value of up to 6.8 nM. It was evaluated to effectively rescue the abnormal phosphorylation of tau and APP in mammalian cells and living
organisms, and significantly ameliorate neurological and phenotypic defects.\textsuperscript{210}

\begin{center}
\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{CX-4945.png}
\caption{Chemical structure of DYRK1A inhibitor 13.}
\end{figure}
\end{center}

More interestingly, it was proposed that the interactions of CX-4945 with DYRK1A may display a similar binding mode with that of harmine with DYRK1A, indicating that the tricyclic appendage was a major reason for the enhanced the inhibitory activity. However, as mentioned before, harmine also showed strong inhibition to MAO-A, so determining the effects of CX-4945 on MAO-A is necessary.\textsuperscript{210}

Labrière and co-workers have recently mimicked the fused tricyclic skeleton of harmine to explore more rigid analogues such as 14a–b (Figure 13).\textsuperscript{211} Interestingly, analogue 14a without substitution at the 2-position showed potent inhibition against DYRK1A, DYRK1B, CLK1 and CLK4 (DYRK1A: $IC_{50} = 25$ nM; DYRK1B: $IC_{50} = 34$ nM; CLK1: $IC_{50} = 23$ nM; CLK4: $IC_{50} = 33$ nM). This can be contrasted with harmine, which showed inhibition against DYRK1A and CLK1 with $IC_{50}$ values of 29 nM and 26 nM, respectively. 14b, possessing a 2-methoxy group (mimicking the 2-methyl group of harmine), showed improved inhibitory potency and selectivity against DYRKs and CLKs (DYRK1A: $IC_{50} = 18$ nM; DYRK1B: $IC_{50} = 42$ nM; CLK1: $IC_{50} = 23$ nM; CLK4: $IC_{50} = 30$ nM).\textsuperscript{211}

\begin{center}
\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{14a-14b-harmine.png}
\caption{Chemical structures of DYRK1A inhibitors 14 versus harmine.}
\end{figure}
\end{center}
Quinazolines represent one class of compounds that possess a chemical structure closely related to the adenine nucleus. NGCG (15a–c) have been evaluated as DYRK1A and CLK inhibitors in an ATP competitive manner (Figure 14).\(^{207,212}\) Within a comprehensive kinome scan, 15a–c all possess activities versus CLK1, CLK4 and DYRK1A below 100 nM through binding at the kinase hinge regions. In particular, 15c was evaluated to have the highest selectivity for DYRK1A over other kinases (DYRK1A: \(IC_{50} = 27\) nM; DYRK1B: \(IC_{50} = 734\) nM; CLK4: \(IC_{50} = 30\) nM).\(^{212}\)

![Figure 14. Chemical structures of DYRK1A inhibitors 15.](image)

In 2013, Gerard Rosse developed a novel class of thiazoloquinazoline compounds with quinazoline-amine scaffolds similar to compounds 15 (Figure 15).\(^{213}\) As exemplified by compounds 16a–e, also designated as EHT (Figure 15, Table 1),\(^{214}\) these have displayed much stronger inhibition against DYRK1A and DYRK1B than harmine with subnanomolar IC\(_{50}\) concentrations.\(^{215}\) Previous studies have indicated that the EHTs series exhibited highly selective inhibition against DYRK1 over other DYRKs and CLKs off-targets.\(^{214-215}\) Additionally, EHT analogues with different di-substitutions (see Table 1) displayed similar or improved inhibition and selectivity between DYRK1A and DYRK1B. For example, para-methoxy substituted 16b showed a better selectivity for DYRK1A over DYRK1B albeit with less potency than 16a with para-chloro substitution. Interestingly, 16e with para-methyl substitution showed the best selectivity between DYRK1A and DYRK1B in comparison to 16a–d with nearly 3-fold more inhibitory activity against DYRK1A over DYRK1B. Although the co-crystal structure of the complex EHT/DYRK1A could not obtained, docking studies of 16a–b and DYRK1A/1B have revealed an unusual binding mode unlike that with harmine (see Figure 16). Compounds 16a or 16b were bound within
the ATP binding site, but the heterocyclic core did not adopt a strong hinge interaction; rather a hydrogen bonding interaction to a salt bridge lysine residue through the quinozaline nitrogen was observed instead. The methyl carbimide did not interact strongly with hinge residue Ile193, with a distance relatively far away from the backbone of the Ile193 by calculation.\textsuperscript{215} Accordingly, the difference in charge and size of substitution on R\textsubscript{1} or R\textsubscript{2} has positively affected the interaction within the cavity as well. Previous studies have described that 16\textit{a} also inhibits DYRK1A-induced tau phosphorylation in cellular assays and normalised Aβ production. Therefore, the class of compounds 16\textit{a}–\textit{e} as selective DYRK1A inhibitors warrant further investigation as effective therapeutics for AD-like diseases.\textsuperscript{214}

![EHTs (16)](image)

**Figure 15.** Chemical structures of DYRK1A inhibitors 16. R\textsubscript{1} and R\textsubscript{2} are clarified in Table 1 below, respectively.

**Table 1.** IC\textsubscript{50} values of 16\textit{a}–\textit{e} for DYRK1A/B.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R\textsubscript{1}</th>
<th>R\textsubscript{2}</th>
<th>DYRK1A</th>
<th>DYRK1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>16\textit{a}</td>
<td>Cl</td>
<td>Cl</td>
<td>0.22</td>
<td>0.28</td>
</tr>
<tr>
<td>16\textit{b}</td>
<td>F</td>
<td>OMe</td>
<td>0.36</td>
<td>0.59</td>
</tr>
<tr>
<td>16\textit{c}</td>
<td>F</td>
<td>F</td>
<td>0.94</td>
<td>1.07</td>
</tr>
<tr>
<td>16\textit{d}</td>
<td>F</td>
<td>Cl</td>
<td>0.99</td>
<td>1.63</td>
</tr>
<tr>
<td>16\textit{e}</td>
<td>H</td>
<td>Me</td>
<td>0.98</td>
<td>2.83</td>
</tr>
<tr>
<td>harmine</td>
<td>-</td>
<td>-</td>
<td>21.83</td>
<td>57.40</td>
</tr>
</tbody>
</table>

\textsuperscript{a}IC\textsubscript{50} values expressed as nM.
A novel bioluminescent-eliciting assay was recently developed to evaluate more specific and selective DYRK1A inhibitors by Sonamoto and co-workers. In particular, CaNDY (17a) was demonstrated to be a potent inhibitor of DYRK1A with an IC\textsubscript{50} value of 7.9 nM in an ATP competitive mechanism (Figure 17). INDY as a comparison in the same assay showed much lower potency in this assay (IC\textsubscript{50} = 122 nM). Two compounds structurally similar to CaNDY, PD0439 (17b) and PD0442 (17c), were also tested (Figure 17). Compound 17b, with a dihydrobenzofuran substituent, inhibited the kinase activity of DYRK1A with an IC\textsubscript{50} value of 16.7 nM, whereas 17c with an indole substituent showed much weaker inhibition against DYRK1A (IC\textsubscript{50} not shown). This implies that the oxygen atom attached to the aromatic of CaNDY is the key pharmacophore for maintaining inhibitory effects on DYRK1A. CaNDY was also evaluated as a selective inhibitor of DYRK1A over other DYRK family members. The \textit{in vitro} study showed that CaNDY possessed 3-fold poorer inhibitory activity against DYRK1B versus DYRK1A. Moreover, CaNDY selectively induced degradation of the DYRK1A molecule in cells and thus suppressed DYRK1A activity.
To date, compounds reported as DYRK-specific or CLK-specific inhibitors are rare, mainly due to the high conservation of ATP binding sites inside CLKs and DYRKs. Bis-indoles of indirubin have attracted the interest of many researchers as a protein kinase inhibitory scaffold. In 2009, Beauchard and co-workers reported the synthesis of indirubin derivatives and their biological activity against CMGC kinases including CDKs, GSK, CK1 and DYRK1A. Although all these derivatives showed weak inhibition against DYRK1A (> 1 µM, data not shown here), they exhibited far better activity than against other kinases such as CDKs, GSK3. Researchers subsequently suggested that indirubin derivatives might be promising inhibitors that selectively inhibit DYRKs rather than other CMGC kinases. Encouragingly, Myrianthopoulos and co-workers had made great improvements by the modification of the bis-indole scaffold (Figure 18). As shown in Table 2, 18a–c all exhibited much higher inhibitory potencies against DYRK1A and DYRK2 versus other kinases, with all IC50 values in the submicromolar range. This increase in inhibitory potencies resulted from the combined presence of bromine and carboxylic acid substituents. According to the co-crystal structure of compound 18a/DYRK2 complex, the bulky bromine instigated a steric clash between compounds within the kinase hinge, while the acidic substitution established a stabilising interaction with lysine in DYRK2.
Achievements in developing DYRK1A inhibitors with selectivity among the structurally and functionally closely related CLKs have been made as recently as 2015.\textsuperscript{219} Kunick and co-workers\textsuperscript{219} reported a class of novel DYRK1A inhibitors 19a–c (Figure 19), which exhibited DYRK1A inhibitory activity with IC\textsubscript{50} values in double digital nanomolar concentrations (Table 3). When the chloro-substituent of compound 19a was substituted for bromine, the resulting analogue (19b) showed an improvement in the inhibition of DYRK1A (IC\textsubscript{50} = 20 nM) but more potent activity against DYRK2 (IC\textsubscript{50} = 16 nM). Interestingly, compound 19c, with an iodo substituent, dramatically inhibited DYRK1A with an IC\textsubscript{50} value in a single digital nanomolar concentration (IC\textsubscript{50} = 6 nM). More importantly, 19c totally lost its inhibitory activity against DYRK2, DYRK3 and CDK9, showing 100-fold less inhibition against DYRK1B, and 30-fold less inhibitory potency against CLK1 or CLK4 (Table 3).\textsuperscript{219} The X-ray co-crystal structures of 19a–c with DYRK1A have

\textbf{Table 2. IC\textsubscript{50} values of the inhibitory activity of indirubin 18a–c against selected CMGC kinases.}\textsuperscript{a}

\begin{tabular}{|c|c|c|c|c|c|}
\hline
Compounds & DYRK1A & DYRK2 & CDK5 & GSK3 & CK1 \\
\hline
18a & 0.21 & 0.13 & > 10 & > 10 & > 10 \\
18b & 0.41 & ND & 1.60 & 1.20 & 4.20 \\
18c & 0.60 & 1.70 & > 10 & > 10 & 7.40 \\
\hline
\end{tabular}

\textsuperscript{a}IC\textsubscript{50} values expressed in µM. [ATP] = 7.7 µM.\textsuperscript{218}
been also determined. The halogen atom of 19a–c may act as halogen bond acceptor forming an analogous bond toward hinge residue Leu241, whereas the acidic substitution acts as a salt bridge between the carboxylate and the conserved lysine residue, keeping the inhibitor in position in the ATP binding site of DYRK1A.\textsuperscript{219} However, potential issues lie in the solubility and cell permeability of potent inhibitor. Further studies are therefore necessary to understand the nature of the interaction between 19c and DYRK1A.

![Figure 19](image)

**Figure 19.** Chemical structures of DYRK1A inhibitors 19a–c.

**Table 3.** IC\textsubscript{50} values of the inhibitory activity of 19a–c against selected CMGC kinases.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Kinases</th>
<th>19a</th>
<th>19b</th>
<th>19c</th>
</tr>
</thead>
<tbody>
<tr>
<td>DYRK1A</td>
<td>31</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>DYRK1B</td>
<td>210</td>
<td>80</td>
<td>600</td>
</tr>
<tr>
<td>DYRK2</td>
<td>40</td>
<td>16</td>
<td>&gt;10</td>
</tr>
<tr>
<td>DYRK3</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CDK9</td>
<td>150</td>
<td>160</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CLK1</td>
<td>130</td>
<td>32</td>
<td>500</td>
</tr>
<tr>
<td>CLK4</td>
<td>45</td>
<td>35</td>
<td>210</td>
</tr>
</tbody>
</table>

\textsuperscript{a}IC\textsubscript{50} values expressed in nM. [ATP] = 7.7 \mu M.

In summary, the inhibition of DYRK1A represents a new avenue to cancer treatments
as well as neurodegenerative pathologies in DS and AD. Natural products with highly potent inhibition for DYRK1A, such as harmine and EGCG, have played crucial roles in investigating more promising DYRK1A inhibitors. These compounds undoubtedly warrant further study by structural modifications aimed at diminishing the side effects while maintaining inhibition. To date, the most promising DYRK1A inhibitors, either from natural sources or synthetic compounds, have still been limited in use, in most cases, due to the undesirable effects on off-targets such as CDKs, CLKs and other members of the DYRK family. Reassuringly, more and more crystal structures of inhibitors within the active site of DYRK1A have been solved, providing more insight into the future rational design of novel, potent, and selective DYRK1A inhibitors.

1.6 Project aims

Undeniably, small molecules devoid of toxicological liabilities represent a new approach to therapeutic use as DYRK1A inhibitors. Therefore, attempts to find more potent and selective DYRK1A inhibitors are taking place. In 2013, a series of compounds based on 7-azaindole scaffolds were reported as DYRK1A inhibitors.220 One such molecule, 20a (Figure 20, right), was reported as one of the most potent DYRK1A inhibitors to date, showing inhibitory activity against DYRK1A with an IC₅₀ value of 23 nM,220 whereas 20a also exhibited inhibition to CLK at a similar concentration. According to the proposed binding mode between the 7-azaindole series of compounds within the active site of DYRK1A (Figure 20, left), the NH and N on the azaindole core displayed hydrogen bonding to the backbone amide of the Glu239 residue and hinge residue Leu241, respectively.220 Furthermore, substituents at C-3 and C-5 positions have offered stabilising interactions with conserved Lys188 residue and Ile165, as well as Ser242.220 This proposed mechanism of binding provided us a further insight into potential modifications on the 7-azaindole scaffolds for more potent and selective DYRK1A inhibitors.
Using *in silico* modelling, we were able to dock a number of potential molecules into the crystal structure ATP-binding site of DYRK1A (Figure 21). From the virtual screening studies, both molecule a (Figure 21, A) and b (Figure 21, B) have shown strong binding affinity within the ATP-binding site of DYRK1A by non-covalent interactions, such as hydrophobic interactions, electrostatic attractions and/or hydrogen bond interactions. The calculated Glidescore values of molecule a and b were found to be the same according to the classical scoring functions. However, when both molecules were tested in DYRK1A inhibition assay, their IC$_{50}$ values were quite different: 6 nM and 31 nM, respectively (Figure 21). These results suggested that the docking approach to designing novel molecules for DYRK1A inhibition is not sufficiently predictive in this case when looking at low nanomolar potency. More complicated environment *in vitro* or *in vivo* can be very different from that *in silico*. We therefore decided to synthesise novel DYRK1A inhibitors in a traditional approach based on the lead compound DANDY. Novel analogues would be made by changing individual segments of DANDY to investigate which segment makes the most important contribution to maintaining DYRK1A inhibition, with the overall aim of accessing more potent and selective DYRK1A inhibitors.

**Figure 20.** *In silico* 7-azaindole series compounds with active site of DYRK1A (left); Representative 7-azaindole (DANDY, 20a) (right).
In order to systematically explore structural features that impart DYRK1A binding affinity and selectivity, I sought to optimise more potent and selective DYRK1A inhibitors based on DANDY. Details about the modifications on the 7-azaindole 20a are shown in Figure 22.
Figure 22. Generalised scaffolds for DANDY 20a to investigate novel derivatives by changing 7-azaindole core (shown in the left box with outline in blue), substituents on the C3 and/or C5 (shown in the bottom box with outline in green) and disconnection of 7-azaindole ring (shown in the right and top box with outline in purple).

In the subsequent chapters, I intend to address the following problems:

1. How important is the 7-azaindole heterocyclic core? Does the pyridine nitrogen play a key role in maintaining DYRK1A inhibition? Are the two nitrogen atoms in a specifically spatial arrangement necessary to contribute to the inhibitory potency against DYRK1A? Is the hydrogen atom of the pyrrole-NH crucial to the binding mode? More diversified scaffolds are needed to answer these questions and will be addressed in Chapter 2.

2. Do compounds that maintain both nitrogen atoms but possess more flexibility through ring-opening exhibit more potent activity against DYRK1A? Do spatial distances between di-phenols contribute to DYRK1A inhibition? Attempts to answer these questions are the subjects of Chapter 3 (Figure 22).

3. What substituents on C-3 or C-5 positions are most tolerated, and improve the DYRK1A inhibition and selectivity over other kinases? Does an amide linker
between the 7-azaindole core and aromatic substituents at C-3 or C-5 increase DYRK1A inhibition activity? Does the DYRK1A inhibition by potent inhibitors correlate with the cellular efficacies? These questions will be addressed in Chapter 4 (Figure 22).
Chapter 2 - Synthesis and biological evaluation of heterocyclic derivatives with diphenols

2.1 Introductory remarks

Structural modification is a popular approach to creating novel compounds from a starting scaffold. For example, some heterocyclic compounds were designed by mimicking the pyrimidine portion of ATP, thus acting as ATP competitive molecules to bind the adenine pocket via hydrogen bonds, hydrophobic effects and/or ionic interactions.\textsuperscript{223-224} Among them, the 7-azaindole core (21, Figure 23) has often been utilised due to the presence of both a hydrogen bond donor and acceptor.\textsuperscript{225} It is also advantageous for accessing diversified analogues through the installation of multiple substituents at the C-3 and C-5 positions.\textsuperscript{226} For example, Hong and co-workers designed [3,5-d]-7-azaindole analogues 22 as potent PI3K (phosphoinositide 3-kinase) inhibitors to show strong effects on cellular proliferation and apoptosis (Figure 23).\textsuperscript{227} This series of analogues was found to exhibit further inhibitory potencies against TrK (tropomyosin receptor kinase) with anticancer and antiangiogenic activities.\textsuperscript{228} 7-azaindole analogue 23 was designed as a potent inhibitor of SGK1 (serine/glucocorticoid regulated kinase 1) and was effective enough to be carried through \textit{in vivo} testing (Figure 23).\textsuperscript{229} Gelbard and co-workers recently reported an orally bioavailable brain-penetrating inhibitor (24) of MLK3 (mixed-lineage protein kinase 3) with excellent \textit{in vivo} activity as a potential strategy for the treatment of Parkinson’s disease.\textsuperscript{230} This 7-azaindole analogue 24 bearing substitution at the C-3 position has also shown neuroprotective and anti-neuroinflammatory properties in both \textit{in vitro} and \textit{in vivo} assays of HIV-1-associated neurocognitive disorders (HAND) (Figure 23).\textsuperscript{231}
Interestingly, several reported DYRK1A inhibitors are based on a 7-azaindole core as well, notably meriolins and DANDYs. DANDY (20a, Figure 24) was reported as one of the most potent DYRK1A inhibitors by Gourdain and co-workers in 2013. Its proposed mechanism of binding is with the ATP-binding site of DYRK1A, which involves hydrogen bonding between the N-H of 20a and the backbone amide of Glu239, and a second hydrogen bond between the nitrogen atom at the 7-position and the hinge residue Leu241 N-H. These interactions contribute to the excellent binding affinity within the active site of DYRK1A. Furthermore, diphenol substitutions at both C-3 and C-5 of the azaindole generate further stabilising interactions with the conserved residues Lys188 and Ile165. However, when testing its inhibitory activity against other related kinases (CLK1, DYRK2), 20a shows equipotent inhibition against CLK1 compared with DYRK1A, and inhibitory activity against DYRK1B was not disclosed in these studies. Very little investigation and exploration of the DANDY series of compounds has been subsequently reported. It is still unclear which individual segment of 20a is crucial to achieve DYRK1A inhibition: is the 7-azaindole core really a key moiety for preserving inhibitory potency against DYRK1A? Do other heterocyclic cores work as well, or better?
In an attempt to answer these questions, I investigated other diverse heterocyclic scaffolds by maintaining the characteristics of diphenols (shown in Figure 24). Initially, I explored derivatives with an indole or indazole core, which differ through the positioning of and/or the second nitrogen atom. Furthermore, different spatial arrangements of two nitrogen atoms were studied by designing heterocycles such as benzimidazole-2-amine or 2-acetamide, pyrazole-pyridine with phenol substitution at the C-4 or C-5 position, bipyridine, naphthyridine, imidazole, and triazolopyrazine series. Additionally, the 7-membered analogue with a ring-shaped diphenol has been also investigated. With these novel analogues in hand, I then explored their inhibitory activity against DYRK1A afterwards.

![Figure 24. Generalised heterocyclic cores based on 7-azaindole core of DANDY.](image)

2.2 Synthesis of DANDY, indole and indazole derivatives 20b–c

The lead compound 20a was synthesised by following previously reported synthetic routes. Commercially available 5-bromo-7-azaindole (25a) was treated with N-iodosuccinimide (NIS) in the presence of KOH to afford the regioselective iodinated intermediate, subsequent 4-methylbenzenesulfonyl (tosyl) protection gave 26a in excellent yield (Scheme 1). Suzuki cross-coupling reaction was employed at both halide positions simultaneously to afford aryl substituted compound 27a. According to the palladium-catalysed cycle of Suzuki cross-coupling (Figure 25), the reaction follows a sequence with an initial oxidative insertion of aryl-halide to a Pd^0-ligand complex to form the Pd(II)-aryl complex intermediate. This can be selectively achieved with an aryl iodide over the bromo equivalent if required. Oxidative addition
is followed by transmetallation with an arylboronic acid, activated by the presence of base, and subsequent reductive elimination from the Pd(II)-diaryl complex intermediate to afford the corresponding biaryl products with regeneration of Pd⁰. The selectivity between the aryl iodides and aryl bromides stems from the greater electrophilicity of the former, therefore promoting faster oxidative addition. Deprotection of the tosyl group was achieved under basic conditions in MeOH to furnish 28a, followed by demethylation by treatment with BBr₃ in CH₂Cl₂ to afford lead compound 20a in moderate yield. The total yield of compound 20a was limited by the demethylation step with BBr₃. After quenching with MeOH, thin layer chromatography showed a spot consistent with the desired product (R_f = 0.45, CH₂Cl₂/Methanol 20:1) and a strong baseline spot that could not be developed by any mobile phase. It was proposed that a boron-complex intermediate was forming and was relatively stable in this case. A process of quenching with MeOH, drying under reduced pressure, diluting with CH₂Cl₂, and quenching again by MeOH and repeating three times, allowed for more efficient isolation of compound 20a. Spectral data clearly matched that which had been previously reported.
Scheme 1. Reagents and conditions: (a) NIS (1.0 equiv.), KOH (0.5 equiv.), CH₂Cl₂, RT, 10 h; (b) NaH (60% in mineral oil, 3.0 equiv.), TsCl (1.2 equiv.), BnEt₃NCl (0.02 equiv.), CH₂Cl₂, 0 °C−RT, 30 min; 80−89% over two steps; (c) arylboronic acid (2.0 equiv.), Pd(PPh₃)₄ (4 mol%), aq. K₂CO₃ (2 M, 4.0 equiv.), toluene/ethanol 3:1, 100 °C, 3.5 h, 84−87%; (d) KOH (5.0 equiv.), MeOH, 80 °C, 2 h, 91−95%; (e) BBr₃ (6.0 equiv.), CH₂Cl₂, 0 °C to RT, 15 h, 58−65%; TsCl = 4-methylbenzenesulfonyl chloride.
By altering the starting material, this procedure could be used to generate several analogues of 20a in moderate yield (Scheme 1). Firstly, the indole core 25b was employed instead of azaindole core to investigate the functionality requirement of the nitrogen atom at the 7-position. The indole-based compound 20b was obtained starting from 5-bromoindole (25b) following the same procedures outlined for 20a. A second functional modification was made, namely the indazole-based compound 25c, to reinstall a nitrogen atom at a different position to investigate different hydrogen bonding requirements. Compound 20c was prepared from 5-bromoindazole (25c) in a similar manner (Scheme 1).

2.3 Synthesis of benzimidazole-based analogues 29 and 30

I initially synthesised benzimidazole-2-amine 29 and benzimidazole-2-acetamide 30 starting from commercially available 2-fluoroaniline (31) (Scheme 2). The selectively brominated intermediate 32 was achieved by treatment with N-bromosuccinimide (NBS) in CHCl₃ in 90% yield. The aniline 32 was then oxidised to nitro compound 33 in the presence of hydrogen peroxide in trifluoroacetic acid (TFA) at 75 °C for 2 h. It was hoped that the subsequent neucleophilic substitution could be achieved by treatment with tert-butyldimethylsilyl ether (OTBS)-aniline 34a under basic
conditions. Unexpectedly, the desired product 35a was not obtained, and the byproduct 35b was produced instead. I proposed that the silicon atom in the TBS protecting group favoured bond formation with the fluorine atom of 33, resulting in the formation of a transition state (Scheme 2), and eventually producing ether 35b.

Scheme 2. Reagents and conditions: (a) NBS (1.0 equiv.), CHCl₃, RT, 2 h, 90%; (b) aq. H₂O₂ (30%, 5.0 equiv.), TFA, 75 °C, 2 h, 75%; (c) 34a (1.1 equiv.), K₂CO₃ (1.5 equiv.), DMF, 90 °C, 3 h, 80%.

Instead, the intermediate 33 was treated with 4-aminophenol and K₂CO₃ in dry DMF at 90 °C (Scheme 3), and the desired amine 35c was successfully obtained in moderate yield. Next nitro reduction by iron powder in AcOH was realised at room temperature in excellent yield. Cyclisation to intermediate 37 was achieved in the presence of cyanogen bromide to proceed in 78% yield at room temperature. The 2-aminobenzimidazole analogue 29 was achieved using a standard Suzuki coupling reaction, followed by acetylation to afford the 2-acetamide benzimidazole analogue 30 in moderate 65% yield (Scheme 3).
Scheme 3. Reagents and conditions: (a) 4-aminophenol (1.1 equiv.), K$_2$CO$_3$ (1.5 equiv.), dry DMF, 90 °C, 3 h, 65%; (b) Fe (5.0 equiv.), acetic acid, RT, 2 h, 95%; (c) BrCN (5 M in CH$_3$CN, 1.2 equiv.), MeCN-H$_2$O, 3:1 (v/v), RT, 16 h, 78%; (d) (4-hydroxyphenyl)boronic acid (1.5 equiv.), Pd(PPh$_3$)$_4$ (2 mol%), aq. K$_2$CO$_3$ (2 M, 3.0 equiv.), 1,4-dioxane, 110 °C, 4 h, 30%; (e) Ac$_2$O (1.1 equiv.), AcOH, 110 °C, 3 h, 65%.

However, a more expedient alternative synthesis of analogue 29 and 30 was devised based on literature, and is shown in Scheme 4. The commercially available 4-bromobenzene-1,2-diamine (38) was treated with cyanogen bromide in a mixture solvent of MeOH and H$_2$O at room temperature to furnish the cyclised intermediate 39 in 80% yield. C-N bond formation was achieved following Chan-Lam type coupling reaction. This reaction was catalysed by copper acetate in the absence of any ligands or base, while the reaction was also open to the air. Regioisomers 41a and 41b were both obtained in this case, and the confirmation of isomer 41b was determined by deprotection of the TBS group to furnish a compound which was consistent with the spectra of intermediate 37 shown in Scheme 3. The desired compound 41b was further treated with (4-hydroxyphenyl)boronic acid under the standard Suzuki coupling conditions to afford the intermediate 42, and subsequent deprotection of the TBS group eventually gave analogue 29. Analogue 30 was achieved under the same conditions described above in moderate yield.
Scheme 4. Reagents and conditions: (a) BrCN (5 M in CH₃CN, 1.2 equiv.), MeOH-H₂O, 3:1 (v/v), RT, 12 h, 80%; (b) 40 (1.1 equiv.), Cu(OAc)₂ (0.2 equiv.), air, MeOH, RT, 12 h, 20% (41a), 45% (41b); (c) (4-hydroxyphenyl)boronic acid (1.5 equiv.), Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 3.0 equiv.), 1,4-dioxane, 110 °C, 4 h, 65%; (d) TBAF (2.0 equiv.), THF, RT, 10 min, 95%; (e) Ac₂O (1.1 equiv.), acetic acid, 110 °C, 3 h, 40%; TBAF = tetra-n-butylammonium fluoride.

2.4 Synthesis of pyrazole-pyridine-based analogues 43 and 44

The synthesis of analogue 43 was initially designed to follow the retrosynthetic approach shown in Scheme 5 from a Suzuki coupling reaction with 5-bromo substituted intermediate 49. Heterocycle formation was to be derived from a 1,3-dione by treatment with hydrazine, and the intermediate 1,3-dione could be afforded from the condensation between ketone and ester. The 2-acetate pyridine could be easily obtained from commercially available 2,5-dibromopyridine 45.
Scheme 5. Retrosynthesis of analogue 43.

Treatment of 2,5-dibromopyridine (45) with n-BuLi in toluene at −78 °C and quenching with N,N-dimethylacetamide afforded acetate 46a in 80% yield (Scheme 6). 46a was treated with methyl 4-(benzyloxy)benzoate 47a under different conditions (Table 4) but none of the desired 1,3-dione 48a was obtained. The byproduct benzoic acid (48b) was isolated when t-BuOK or NaOMe was used as base. Use of LDA led to a complex mixture of products observed by TLC monitoring.

Scheme 6. Reagents and conditions: (a) n-BuLi (1.1 equiv.), N,N-dimethylacetamide (1.5 equiv.), toluene, −78 °C, 1 h, 80%; (b) see Table 4.
Table 4. Conditions screened for step b in Scheme 6.\textsuperscript{238-239}

<table>
<thead>
<tr>
<th>Base</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-BuOK</td>
<td>THF</td>
<td>25 to 70 °C</td>
<td>48b (85%)</td>
</tr>
<tr>
<td>NaOMe</td>
<td>MeOH</td>
<td>25 to 80 °C</td>
<td>48b (80%)</td>
</tr>
<tr>
<td>LDA\textsuperscript{a}</td>
<td>THF</td>
<td>-78 °C to 25 °C</td>
<td>complex mixture</td>
</tr>
</tbody>
</table>

\textsuperscript{a}LDA was freshly made from n-BuLi (1.1 equiv.) and N,N-diisopropylethylamine (1.2 equiv.) at \(-78 \degree\) C.

Considering the low reactivity of benzoate 47a, I elected to use benzaldehyde 47b as the reactant (with subsequent oxidation step) by treatment with 46a in the presence of LDA at \(-78 \degree\) C. Unfortunately, the reaction was sluggish and then yielded a complex mixture of products after stirring for another 12 h at room temperature (Scheme 7).

\begin{align*}
46a + 47b & \rightarrow 48c \\
\text{Scheme 7. Reagents and conditions: (a) 46a (1.05 equiv.), 47b (1.0 equiv.), LDA (1.1 equiv.), THF, \(-78 \degree\) C to RT.}
\end{align*}

We assumed that the HOMO of the nucleophile 46a and the LUMO of the electrophile 47b might not match with each other due to the electronic effects from the aromatic systems. Therefore, I decided to swap the functional groups of ketone 46a and aldehyde 47b to aldehyde 46b and ketone 47c, respectively (Scheme 8). As predicted, the desired aldol product 48d was successfully achieved in 78% yield by treatment with LDA in a similar protocol as described above.
Scheme 8. Functional groups of ketone 46a and aldehyde 47b to aldehyde 46b and ketone 47c were interchanged, respectively. Reagents and conditions: (a) 46b (1.0 equiv.), 47c (1.05 equiv.), LDA (1.1 equiv.), THF, −78 °C to RT, 1 h, 78%.

With the intermediate 48d in hand, subsequent oxidation with Dess-Martin periodinane (DMP) was employed to furnish 1,3-dione 48a in 95% yield (Scheme 9). The formation of pyrazole intermediate 49 was achieved in 75% yield by treatment with hydrazine monohydrate in MeOH at room temperature.240 No reaction occurred when this substrate was treated with (4-hydroxyphenyl)boronic acid under standard Suzuki coupling conditions. A proposed explanation for this lies in the formation of a complex between the Pd catalyst and pyridine-N and pyrazole moieties (a typical bidentate coordination site), thus reducing the catalytic reactivity of Pd catalyst in the reaction. Therefore, the pyrazole-N was protected with a carboxybenzyl (Cbz) group and the resulting substrate 50a was subjected to the Suzuki coupling reaction. Unfortunately, the Cbz protecting group was not tolerated under these conditions and only the Cbz-deprotected compound 49 was isolated (Scheme 9).
Scheme 9. Reagents and conditions: (a) DMP (1.5 equiv.), CH₂Cl₂, RT, 30 min, 95%; (b) N₂H₄•H₂O (2.0 equiv.), MeOH, RT, 48 h, 75%; (c) benzyl carbonobromidate (1.1 equiv.), NaH (60% in mineral oil, 2.0 equiv.), CH₂Cl₂, 0 °C–RT, 30 min, 90%; (d) (4-hydroxyphenyl)boronic acid (1.5 equiv.), Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 3.0 equiv.), 1,4-dioxane, 110 °C, 4 h.

Treating the tosyl-protected analogue 50b with (4-hydroxyphenyl)boronic acid under the Suzuki coupling conditions finally afforded aryl-substituted compound 51b in 85% yield (Scheme 10). Removal of the tosyl and benzyl protecting groups could be easily achieved in two sequential steps, and analogue 43 was eventually furnished in reasonable yield (Scheme 10).

Scheme 10. Reagents and conditions: (a) TsCl (1.1 equiv.), NaH (60% in mineral oil, 2.0 equiv.), CH₂Cl₂, 0 °C–RT, 30 min, 95%; (b) (4-hydroxyphenyl)boronic acid (1.5 equiv.), Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 3.0 equiv.), 1,4-dioxane, 100 °C, 4 h, 85%; (c) KOH (5.0 equiv.), MeOH, 75 °C, 1 h; (d) Pd/C (10 wt. %), H₂, MeOH, RT, 5 h, 45% over two steps.
Next, I explored the synthesis of the pyrazole-pyridine based analogue 44 with the phenol substitution at the C-4 position, which was initially designed to follow the retrosynthetic approach shown in Scheme 11. In a similar manner to that of analogue 43, compound 44 could be derived from the cyclisation of a 1,3-dione, which could be originally achieved from the aldol condensation between aldehyde and ketone with the optimised conditions described above.

Scheme 11. Retrosynthesis of analogue 44.

In order to realise the synthesis of compound 44, I first tried to make the intermediate 52 (Scheme 11) from 2-bromopyridine 58 (Scheme 12) using lithium-halogen exchange. Commercially available pyridin-4-ylboronic acid (54) was coupled with 1-(benzyloxy)-4-bromobenzene (55) to afford 4-aryl intermediate 56 (Scheme 12). The oxidation of 56 was subsequently performed in order to install the bromide selectively at the C-2 position. \(^{241}\) N-oxide 57 was obtained in 80% yield through oxidation with hydrogen peroxide in acetic acid. Unfortunately, bromination of 57 with POBr\(_3\) in toluene at 110 °C for 48 h was unsuccessful.
Scheme 12. Reagents and conditions: (a) 54 (1.50 equiv.), 1-(benzyloxy)-4-bromobenzene (55) (1.0 equiv.), Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 3.0 equiv.), 1,4-dioxane, 100 °C, 4 h, 80%; (b) aq. H₂O₂ (30%, 5.0 equiv.), AcOH, 70 °C, 15 h, 80%; (c) POBr₃ (1.1 equiv.), toluene, 110 °C, 48 h.

As an alternative approach towards synthesis of 44, 2-amino-4-bromopyridine 59 was then chosen as the starting material. It was first coupled with boronic acid 40 to afford 4-aryl intermediate 60 (Scheme 13). The brominated compound 59a was sought from a Sandmeyer reaction by treatment with bromine and isopentyl nitrite in THF (not shown here), but no desired product was obtained. Instead, multiple brominated byproducts were observed and thought to be produced as a result of the electron rich 2-aminopyridine ring. Therefore, multiple conditions that were devoid of electrophiles were screened (Table 5). CuBr was initially used as a bromide source but even heating in refluxing MeCN (80 °C) for 48 h no reaction occurred. No reaction was observed when CuBr₂ or a CuBr/CuBr₂ mixture was used in a similar manner. According to the literature, 242 iodination at the C-2 position could be furnished by using CH₂I₂ as the solvent and halogen source with catalytic HI (Scheme 13). This was achieved to give the desired compound 59b in 25% yield. Increasing the reaction temperature (80 °C) or extending the reaction time failed to give 59b with an improved yield. According to the earlier mechanistic studies, 243 this reaction does not proceed via the diazonium ion as in classical Sandmeyer protocols. Instead, it is proposed to form a diazoaminobenzene intermediate that leads to the formation of aryl radical in all cases, and the iodinated compound 61b was achieved through the radical reaction between the diazoaminobenzene intermediate and the iodo radical from CH₂I₂ (Figure 26). We assumed that the role of HI in this reaction was as a
radical scavenger to capture radicals such as •CH₂I, thus stopping the occurrence of side reactions.

Scheme 13. Reagents and conditions: (a) (4-((tert-butyl dimethylsilyl)oxy)phenyl)boronic acid (40) (1.5 equiv.), Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 3.0 equiv.), 1,4-dioxane, 100 °C, 4 h, 87%; (b) isopentyl nitrite (2.0 equiv.), 48 h, see Table 5 for additional conditions; (c) isopentyl nitrite (20.0 equiv.), aq. HI (57%, 2 mol%), CH₂I₂, RT, 24 h, 25%.

Table 5. The conditions screening for step b in Scheme 13.

<table>
<thead>
<tr>
<th>Halogen source</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuBr</td>
<td>MeCN</td>
<td>25 to 80 °C</td>
<td>N/A</td>
</tr>
<tr>
<td>CuBr₂</td>
<td>MeCN</td>
<td>25 to 80 °C</td>
<td>N/A</td>
</tr>
<tr>
<td>CuBr/CuBr₂ (1:1)</td>
<td>MeCN</td>
<td>25 to 80 °C</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 26. Mechanistic proposal for the transformation of 60 to 61b.²⁴³

With the 2-iodinated intermediate 61b in hand, the formation of analogue 44 could be easily achieved in a similar synthetic approach to that of 43 (Scheme 14). The 2-aldehyde intermediate 52 was furnished by the lithium-halogen exchange and quenching with DMF in a moderate 50% yield. The subsequent aldol condensation with 47c gave the aldol product 62 in 60% yield. Oxidation by DMP and subsequent pyrazole formation yielded intermediate 63 and the two sequential deprotection steps eventually afforded analogue 44.
Scheme 14. Reagents and conditions: (a) n-BuLi (1.1 equiv.), DMF (1.5 equiv.), toluene, −78 °C, 1 h, 50%; (b) 47c (1.05 equiv.), LDA (1.1 equiv.), THF, −78 °C to RT, 1 h, 60%. (c) DMP (1.5 equiv.), CH₂Cl₂, RT, 30 min, 75%; (d) N₂H₄•H₂O (2.0 equiv.), MeOH, RT, 48 h, 70%; (e) Pd/C (10 wt. %), H₂, MeOH, RT, 5 h; (f) TBAF (2.0 equiv.), THF, RT, 30 min, 55% over two steps.

2.5 Synthesis of bipyridine-based analogue 64

Analogue 64 is a known compound reported by Constable and co-workers.⁴⁴ I used 2,2'-bipyridine 65 as the starting material, with initial formation of N-oxide 66. Initial attempts were made by treatment with the oxidant peracetic acid in acetic acid and stirred at 70 °C, but only the starting material 65 was recovered (Entry 1, Table 6). The more reactive oxidant 3-chloroperbenzoic acid (m-CPBA) was attempted and gave the N-oxide 66 in 20% yield, with 20% of bipyridine 65 not consumed (Entry 2, Table 6). The low yield also resulted from the poor recovery during work-up due to the strong polarity of N-oxide (Entry 2, Table 6). The highly improved yield of 66 (90%) was achieved utilising hydrogen peroxide as the oxidant in AcOH (Entry 3, Table 6). This reaction not only went to completion but the work-up was easily
performed through precipitation after cooling to room temperature with the addition of acetone.\textsuperscript{245}

\textbf{Table 6. Attempted optimisation of the formation of N-oxide 66}

<table>
<thead>
<tr>
<th>Entries</th>
<th>Conditions</th>
<th>Product Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH$_2$CO$_2$H (5.0 equiv.), AcOH, RT to 70 °C, 15 h</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>\textit{m}-CPBA (5.0 equiv.), CHCl$_3$, RT to 70 °C, 15 h</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>aq. H$_2$O$_2$ (30%, 5.0 equiv.), AcOH, RT to 70 °C, 15 h</td>
<td>90</td>
</tr>
</tbody>
</table>

With \textit{N}-oxide 66 in hand, the dinitro-substitution at the \textit{para}-positions was achieved by treatment with a mixture of sulfuric acid and nitric acid at 100 °C (Scheme 15). The low yield of 20\% after 20 h of heating presumably resulted from the use of the normal nitric acid instead of fuming nitric acid.\textsuperscript{246} The following chloro-substitution could be achieved by treatment with acetyl chloride in acetic acid at 100 °C to afford 68 in good yield.\textsuperscript{246} Reduction of \textit{N}-oxide 68 was performed in the presence of PCl$_3$ to give 69 in excellent yield, which was further coupled with (4-hydroxyphenyl)boronic acid to furnish the desired compound 64 in moderate overall yield.\textsuperscript{246}
Scheme 15. Reagents and conditions: (a) H₂SO₄ (21.0 equiv.), nitric acid (8.4 equiv.), 100 °C, 20 h, 20%; (b) acetyl chloride (5.0 equiv.), AcOH, 100 °C, 12 h, 85%. (c) PCl₃ (20.0 equiv.), 65 °C, 20 h, 95%; (d) (4-hydroxyphenyl)boronic acid (2.5 equiv.), PdCl₂(dppf) (5 mol%), aq. K₂CO₃ (2 M, 5.0 equiv.), 1,4-dioxane, 110 °C, 4 h, 40%; dppf = 1,1'-ferrocenediyl-bis(diphenylphosphine).

2.6 Synthesis of naphthyridine based analogue 70

Analogue 70 was achieved through known synthetic routes. The regioselective bromination of 2-aminonicotinate 71 gave the 3-bromopyridine 72 in 90% yield, and subsequent esterification under classical conditions furnished 73 in excellent yield (Scheme 16). The cyclisation of 73 was achieved by treatment with N,N-dimethylformamide dimethyl acetal (DMF-DMA) in a sealed tube at high temperature to afford the imine intermediate, followed by the treatment with n-BuLi and MeCN at −78 °C and subsequent addition of acetic acid to give 74 in 35% yield in a one pot reaction sequence. Decyanation of 74 was performed by treatment with concentrated HCl at 150 °C in a sealed tube to give compound 75 in 33% yield after neutralisation with an ammoniacal solution. Given that triflates act as pseudohalides in coupling reactions, I initially attempted the triflation of the hydroxy group on compound 75 (not shown here). However, the triflate intermediate appeared unstable in this heterocyclic system as observed by TLC monitoring. Instead, chloride installation was achieved by treatment with POCl₃ at 105 °C in a sealed tube to give chloro-substituted...
intermediate 76, with subsequent Suzuki coupling achieved using the more reactive PdCl₂(dppf) catalyst and microwave irradiation at 130 °C. Thus, analogue 70 could be obtained in good overall yield.

Scheme 16. Reagents and conditions: (a) Br₂ (1.3 equiv.), AcOH, RT, 20 h, 90%; (b) H₂SO₄ (8.0 equiv.), MeOH, 80 °C, 18 h, 94%. (c) (1) DMF-DMA (10.0 equiv.), 110 °C, 3 h; (2) n-BuLi (2.2 equiv.), MeCN (2.2 equiv.), THF, −78 °C 1 h; (3) AcOH (3.0 equiv.), −40 °C, 2 h, then RT, 20 h, 35% over three steps; (d) aq. HCl (32%), 150 °C, 3 h, 33%; (e) POCl₃, 105 °C, 3 h, 93%; (f) (4-hydroxyphenyl)boronic acid (2.5 equiv.), PdCl₂(dppf) (20 mol%), aq. K₂CO₃ (2 M, 6.0 equiv.), 1,4-dioxane, MW, 130 °C, 3 h, 85%.

2.7 Synthesis of imidazole-based analogue 77

Imidazole-based analogue 77 could be easily obtained from imidazole 78 first by treatment with iodine in the presence of KI and NaOH in H₂O at room temperature. The regioselective diiodinated intermediate 79 was acquired in excellent yield (Scheme 17). The diarylation of 79 was achieved through a double-Suzuki coupling reaction. I initially tried the conditions described previously by using K₂CO₃ as a base, but failed to afford the desired product 77. We assumed that the strong basicity of imidazole combined with the addition of K₂CO₃ increased the pH to a point that was detrimental to the reaction. The addition of the phase transfer
catalyst tetra-\textit{n}-butylammonium bromide (TBAB) increased the interaction between K$_2$HPO$_4$ and organic components to afford analogue 77 in 35% yield. The low yield resulted from poor product recovery in the work-up step because of its strong polarity.

![Scheme 17](image)

**Scheme 17.** Reagents and conditions: (a) NaOH (4 M), KI (5.0 equiv.), I$_2$ (2.2 equiv.), H$_2$O, RT, 10 h, 80%; (b) (4-hydroxyphenyl)boronic acid (3.0 equiv.), Pd(PPh$_3$)$_4$ (6 mol%), K$_2$HPO$_4$ (3.8 equiv.), TBAB (0.1 equiv.), MeOH-H$_2$O, 4:1 (v/v), MW, 100 °C, 60 min, 35%.

### 2.8 Synthesis of triazolopyrazine analogues 80a–b and 81a–b

The next core under investigation was triazolopyrazine. Considering the spatial arrangements of diphenol substitutions on lead compound 20a, we designed triazolopyrazine analogues with linkers between phenols and triazolopyrazine core to give the optimal structural arrangements. Therefore, analogues 80a and 81a were proposed, in addition to meta-hydroxy-substituted 80b and 81b, in order to investigate more diversified analogues (see as Schemes 18 and 19).

The synthesis of triazolopyrazine analogues 80a–b could be achieved from the commercially available 2,6-dichloropyrazine 82 shown in Scheme 18. The formation of hydrazone 83 was achieved in two sequential steps. Firstly, treatment of 82 with hydrazine monohydrate in ethanol gave the hydrazine intermediate, which was reacted with 4-(benzyloxy)benzaldehyde 47b to give hydrazone 83 in 70% yield over two steps. The oxidative heterocyclisation of hydrazone 83 to triazolopyrazine intermediate 84 was easily achieved using PhI(OAc)$_2$ in CH$_2$Cl$_2$ at room temperature in excellent yield. The following nucleophilic substitution reactions with alcohols 85a–b, using 18-crown-6 and KOH, could be achieved to give ethers 86a–b in
moderate yields (69–73%). Hydrogenolysis of \(86a-b\) then gave the desired compounds \(80a-b\) in reasonable yields (Scheme 18).

\[ \text{Scheme 18. Reagents and conditions: (a) } \text{N}_2\text{H}_4 \cdot \text{H}_2\text{O (1.1 equiv.), ethanol, 80 °C, 8 h; (b) } 4\text{-} \text{(benzyloxy)benzaldehyde 47b (1.1 equiv.), ethanol, 80 °C, 10 h, 70% over two steps. (c) } \text{PhI(OAc)}_2 \text{ (1.0 equiv.), CH}_2\text{Cl}_2, \text{RT, 12 h, 85%; (d) } 85a \text{ or } 85b \text{ (1.1 equiv.), KOH (3.0 equiv.), 18-crown-6 (0.07 equiv.), toluene, 40 °C, 4 h, 69–73%; (e) } \text{Pd/C (10 wt. %), H}_2, \text{MeOH, RT, 2 h, 67–70%}. \]

The synthesis of triazolopyrazine analogues \(81a-b\) could be achieved in a similar manner as that described above (Scheme 19). Pyrazine-2-carboxylic acid \(87\) was first oxidised to give N-oxide intermediate as reported previously\(^{254}\). The subsequent chlorination\(^{255}\) was achieved by treatment with phosphorus oxychloride at high temperature to give the chloro-intermediate \(88\) in 70% yield over two steps. Amide formation was performed via the acetyl chloride intermediate, followed by the treatment with para- or meta-OTBS aniline \(34a-b\) and triethylamine in dry \(\text{CH}_2\text{Cl}_2\) to give the amides \(89a-b\) in good yields (80–85%). The following reactions were
performed in a similar manner to those described in Scheme 18, hydrazones 91a–b were obtained in 78–85% yields over two steps and further converted to the cyclised compounds 92a–b in good yields. The deprotection of the TBS groups gave the desired compounds 81a–b in excellent yields.256

Scheme 19. Reagents and conditions: (a) (1) Na₂WO₄•2H₂O (0.025 equiv.), aq. H₂O₂ (30%, 1.1 equiv.), H₂SO₄ (1 M), pH = 2, RT to 80 °C, 2 h; (2) POCl₃, 120 °C, 2 h, 70% over two steps; (b) (1) oxalyl chloride (1.5 equiv.), DMF (0.1 equiv.), CH₂Cl₂, 0 °C to RT, 2 h; (2) 34a or 34b (1.0 equiv.), triethylamine (1.1 equiv.), CH₂Cl₂, RT, 2 h, 80–85% over two steps; (c) N₂H₄•H₂O (1.1 equiv.), ethanol, 80 °C, 8 h; (d) 4-((tert-butyldimethylsilyl)oxy)benzaldehyde 90 (1.1 equiv.), ethanol, 80 °C, 10 h, 78–85% over two steps. (e) Phl(OAc)₂ (1.0 equiv.), CH₂Cl₂, RT, 12 h, 80–87%; (f) TBAF (2.0 equiv.), THF, RT, 30 min, 95–97%.
2.9 Synthesis of 7-membered analogue 93

The synthesis of 7-membered analogue 93 could be achieved from commercially available starting material 94. Nucleophilic substitution with 4-methoxy-2-nitroaniline was unsuccessful under basic conditions at high temperatures (Scheme 20). However, a Buchwald-Hartwig cross coupling reaction could be utilised by first protecting the aldehyde 94 as the dioxolane 95, then coupling with 4-methoxy-2-nitroaniline to obtain the desired secondary amine 96. The following acetal deprotection was easily carried out by treatment with TFA in chloroform to give the desired intermediate 97 in 90% yield. Compound 98 could be obtained from 97 in one pot via a cascade process. Initial reduction of the nitro moiety to the aniline was followed by intramolecular condensation with the aldehyde. Subsequent reduction in the presence of hydrogen gave 98. Finally, the 7-membered analogue 93 was successfully obtained after demethylation under classical conditions in reasonable yield (Scheme 20).
Scheme 20. Reagents and conditions: (a) NaH (60% in mineral oil, 1.5 equiv.), 4-methoxy-2-nitroaniline (1.0 equiv.), THF, 0 to 70 °C, 24 h; (b) TsOH (0.01 equiv.), ethane-1,2-diol (5.0 equiv.), toluene, 125 °C, 24 h, 95%; (c) 4-methoxy-2-nitroaniline (1.0 equiv.), Pd(OAc)$_2$ (10 mol%), Cs$_2$CO$_3$ (1.5 equiv.), toluene, 120 °C, 3 h, 89%; (d) TFA (6.0 equiv.), CHCl$_3$, RT, 30 min, 90%; (e) Pd/C (10 wt. %), H$_2$, MeOH, RT, 4 h, 75%; (f) BBr$_3$ (6.0 equiv.), CH$_2$Cl$_2$, RT, 10 h, 50%; TsOH = p-toluenesulfonic acid.

2.10 Structure-activity relationship study

With these derivatives in hand, their potency for DYRK1A inhibition was determined by performing a kinase assay in triplicate with Woodtide as a substrate and an ATP concentration of 100 µM. The lead compound 20a was confirmed to have inhibitory potency against DYRK1A with an IC$_{50}$ of 14 nM (See Table 7). I then tested indole- and indazole-based analogues 20b and 20c, however, both were inactive with IC$_{50}$ values of up to 1000 nM (Entries 2–3, Table 7). This result confirmed the importance of the two nitrogen atoms contained within the 7-azaindole core, and their specific arrangements. Next, I tested the other analogues, which maintain the two nitrogen atoms in similar positioning to that of lead compound 20a. Again, all of these
derivatives did not exhibit inhibitory potencies against DYRK1A at $>1000$ nM (Table 7).

Table 7. DYRK1A inhibition assay.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structures</th>
<th>DYRK1A (IC$_{50}$, nM)</th>
<th>Compounds</th>
<th>Structures</th>
<th>DYRK1A (IC$_{50}$, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20a</td>
<td><img src="image1.png" alt="Image" /></td>
<td>14</td>
<td>64</td>
<td><img src="image2.png" alt="Image" /></td>
<td>$&gt;1000$</td>
</tr>
<tr>
<td>20b</td>
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<tr>
<td>43</td>
<td><img src="image11.png" alt="Image" /></td>
<td>$&gt;1000$</td>
<td>81a</td>
<td><img src="image12.png" alt="Image" /></td>
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<tr>
<td>44</td>
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<td>$&gt;1000$</td>
<td>81b</td>
<td><img src="image14.png" alt="Image" /></td>
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<tr>
<td>93</td>
<td><img src="image15.png" alt="Image" /></td>
<td>$&gt;1000$</td>
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</tbody>
</table>

Even though all these results showed poor inhibitory activities against DYRK1A, different potencies of all these analogues were further investigated by looking into the remaining activity of DYRK1A at different concentrations (1 µM vs 10 µM) compare with 100% activity of DYRK1A. Very useful information was provided as shown in
Both indole 20b and indazole 20c displayed some inhibition against DYRK1A at higher concentrations (10 µM). In comparison, benzimidazole analogues 29 or 30 showed stronger potency against DYRK1A than 20b–c, which suggested that N-C-NH moiety is more important than that of N-NH or NH alone. Interestingly, pyrazole-pyridine-based analogue 43 failed to show any inhibitory potency against DYRK1A even at higher concentrations. Analogue 44, with a closer distance between the two phenol substituents exhibited much more potent activity. DYRK1A activity could be reduced to 10% when the concentration of compound 44 was ramped up to 10 µM, which suggests that the two phenols in a specific distance between each other may play an important role in maintaining DYRK1A inhibition. Furthermore, naphthyridine 70, the most structurally related compound to the lead compound 20a with the NH moiety replaced with N, still completely lost inhibitory potency against DYRK1A, highlighting the importance of the NH moiety in maintaining DYRK1A inhibition. Para-hydroxy analogue 80a exhibited relatively more potent activity than that of meta-hydroxy 80b, which is in line with results obtained with structurally related compounds 81a and 81b. Compound 93 with its fused ring structure showed very poor activity against DYRK1A even at 10 µM concentration.
Table 8. DYRK1A inhibition assay at different concentrations (1 µM vs 10 µM).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structures</th>
<th>DYRK1A activity at 1 µM</th>
<th>DYRK1A activity at 10 µM</th>
<th>Compounds</th>
<th>Structures</th>
<th>DYRK1A activity at 1 µM</th>
<th>DYRK1A activity at 10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>20a</td>
<td><img src="image" alt="Structure" /></td>
<td>2%</td>
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<td>64</td>
<td><img src="image" alt="Structure" /></td>
<td>78%</td>
<td>85%</td>
</tr>
<tr>
<td>20b</td>
<td><img src="image" alt="Structure" /></td>
<td>100%</td>
<td>46%</td>
<td>70</td>
<td><img src="image" alt="Structure" /></td>
<td>44%</td>
<td>20%</td>
</tr>
<tr>
<td>20c</td>
<td><img src="image" alt="Structure" /></td>
<td>92%</td>
<td>36%</td>
<td>77</td>
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<td>68%</td>
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<tr>
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<td><img src="image" alt="Structure" /></td>
<td>77%</td>
<td>42%</td>
<td>80a</td>
<td><img src="image" alt="Structure" /></td>
<td>77%</td>
<td>89%</td>
</tr>
<tr>
<td>30</td>
<td><img src="image" alt="Structure" /></td>
<td>92%</td>
<td>78%</td>
<td>80b</td>
<td><img src="image" alt="Structure" /></td>
<td>84%</td>
<td>77%</td>
</tr>
<tr>
<td>43</td>
<td><img src="image" alt="Structure" /></td>
<td>100%</td>
<td>100%</td>
<td>81a</td>
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<td>75%</td>
<td>87%</td>
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<tr>
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<td>62%</td>
<td>10%</td>
<td>81b</td>
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<td>95%</td>
<td>88%</td>
</tr>
<tr>
<td>93</td>
<td><img src="image" alt="Structure" /></td>
<td>100%</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.11 Concluding remarks

I have generated 14 novel compounds with different heterocyclic cores and then subjected them to DYRK1A inhibition assays. However, none of these derivatives showed any DYRK1A inhibition with all the IC$_{50}$ values of over 1000 nM. Regardless, these results have provided us with the valuable knowledge that the 7-azaindole heterocyclic core shows more potent activity against DYRK1A than other heterocycles, even when maintaining the diphenolic characteristics. By comparing analogues 43 with 44 we have shown that the distance between the two phenols has strong effects on the DYRK1A inhibition. Comparing analogue 70 to lead compound 20a has hinted that the NH moiety is important, probably to act as a hydrogen bond donor in the binding modes. These data will be of use for designing further structure-activity relationship studies to further improve the selective inhibition of DYRK1A.

Based on the results of this chapter, further structural modification for the production of novel compounds will be attempted in the next chapter. As is shown in Figure 27, the aims could be realised by disconnecting the pyrrole ring of 20a to generate pyridine based diphenols 99, that have different spacing by virtue of different carbon linkers. Further disconnection of the pyridine ring could afford the linear analogues 100 or 101, also with different carbon linkers between the two phenols. The methylated analogues would also be explored to again probe the importance of the NH functionality. The synthesis and screening of these analogues is described in Chapter 3.

![Figure 27](image.png)

**Figure 27.** Further exploration for 7-azaindole core-opening derivatives 99–101.
Chapter 3 - Synthesis and biological evaluation of ring-opened analogues

3.1 Introductory remarks

As described in Chapter 2, the 7-azaindole core has provided the best potency for DYRK1A inhibition in comparison to a small panel of other heterocycles. In this chapter, again starting from 7-azaindole DANDY (20a), I chose to investigate diverse skeletons with disconnection of lead compound 20a, while retaining the hydrogen bonding characteristics to explore its inhibition potentials against DYRK1A (Figure 28). Regardless of DYKR1A potency, we would also test the cytotoxicity of all these novel compounds against cancer cells, in particular, glioblastoma cell lines, as a global test of non-DYRK1A potency. Glioblastoma represents the most common type of primary tumour in the central nervous system. Established A172 glioblastoma cell lines express higher quantities of DYRK1A than that in the patient-derived glioblastoma cell lines. As DYRK1A activity correlates with DYRK1A protein quantity, higher DYRK1A quantity (thus higher DYRK1A activity) in the established glioblastoma cells could render these cells more sensitive to DYRK1A inhibition. For this reason, we wanted to screen all analogues in A172 glioblastoma cells to investigate their functional activity in cancer cells. To that end, we first examined lead compound 20a, making disconnections to the 5-membered ring giving rise to the 1-amino pyridine compounds 99 with various linker lengths (n = 0 to 3) (Figure 28). This was then further deconstructed, opening the pyridine ring to produce the secondary or tertiary chain compounds 100 or 101, respectively, again examining various linker lengths (n = 0 to 2).

![Figure 28](image)

**Figure 28.** Design of 7-azaindole core-opening derivatives from disconnection of lead compound 20a.
3.2 Synthesis of compounds with disconnection of pyrrole ring

The formation of compound 99a was initially designed to follow the retrosynthetic approach shown in Figure 29, starting from commercially available 102a with the fluoride as a good leaving group. The strategy involved nucleophilic substitution with an aniline to afford the corresponding secondary amine, followed by a standard Suzuki coupling reaction and sequential deprotection to afford 99a. However, this was not successful when I treated 102a with 34a under several different conditions (See Table 9). I first tried NaH as a base in THF, and heated to 70 °C (Entry 1, Table 9), but no desired compound was obtained after 24 h. Then the higher boiling point and polarity solvent DMF was trialed, but again no product was obtained, and both starting materials were recovered. Finally, pyridine was used as both the base and solvent, and the mixture was heated to 100 °C with stirring for 24 h. These conditions also proved unsuccessful.

![Figure 29. Retrosynthesis of analogue 99a from 102a.](image-url)
Table 9. Conditions screening for nucleophilic substitution of 102a

<table>
<thead>
<tr>
<th>Entries</th>
<th>Base</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaH</td>
<td>THF</td>
<td>25 to 70 °C</td>
<td>NR</td>
</tr>
<tr>
<td>2</td>
<td>NaH</td>
<td>DMF</td>
<td>25 to 100 °C</td>
<td>NR</td>
</tr>
<tr>
<td>3</td>
<td>pyridine</td>
<td>pyridine</td>
<td>25 to 100 °C</td>
<td>NR</td>
</tr>
</tbody>
</table>

In light of these results, I chose 102b as the starting material (Scheme 21). Compound 103 was successfully obtained by a copper-catalysed boronic acid cross-coupling reaction at room temperature in air in moderate yield. This Chan-Lam type reaction was realised in the absence of ligand and base, and a mechanism has been proposed in previous studies by Rao and co-workers (Figure 30). The obtained compound 103 subsequently underwent a standard Suzuki coupling reaction with boronic acid 40 to furnish compound 104 in good yield (80%), followed by the deprotection of both TBS groups in the presence of TBAF to afford analogue 99a.

Scheme 21. Reagents and conditions: (a) 40 (1.2 equiv.), Cu(OAc)₂ (0.1 equiv.), MeOH, RT, 12 h, 60%; (b) 40 (1.2 equiv.), Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 2.0 equiv.), 1,4-dioxane, 90 °C, 3 h, 80%; (c) TBAF (1.5 equiv.), THF, RT, 10 min, 95%.
Figure 30. Tentative mechanistic proposal for the transformation of 102b to 103 catalysed by Cu(OAc)$_2$ in the air.$^{259}$

Analogues 99b and 99d were both achieved from the same starting material 102b as used previously (Scheme 22). As for the reductive amination reaction in the step a, I initially tried to treat 102b with 4-hydroxybenzaldehyde in EtOH at 70 °C for 1 h, followed by addition of NaBH(OAc)$_3$, but no product was obtained, which we assumed was due to the failure of the required imine formation. The addition of a catalytic amount of AcOH to this reaction was still unsuccessful. Alternatively, using AcOH as solvent and heating to 80 °C for 1 h before adding NaBH(OAc)$_3$, afforded trace amounts of the desired product. Interestingly, when I treated 102b and 4-hydroxybenzaldehyde with CF$_3$CH$_2$OH as solvent, the reaction was realised at room temperature to get compound 105 in moderate yield (68%).$^{260}$ Similarly, I then treated 102b with compound 106 under the same conditions to afford compound 107 in good yield (89%). A Suzuki coupling reaction between compounds 105 or 107 with boronic acid was subsequently performed to afford 99b and 108 respectively. Hydrogenolysis of 108 furnished the desired compound 99d in excellent yield.
Scheme 22. Reagents and conditions: (a) 106 or 4-hydroxybenzaldehyde (1.0 equiv.), NaBH(OAc)$_3$ (1.5 equiv.), CF$_3$CH$_2$OH, RT, 12 h, 68–89%; (b) (4-hydroxyphenyl)boronic acid (1.2 equiv.), Pd(PPh$_3$)$_4$ (2 mol%), aq. K$_2$CO$_3$ (2 M, 2.0 equiv.), 1,4-dioxane, 90 °C, 3 h, 70–80%; (c) H$_2$, Pd/C (10 wt. %), MeOH, RT, 2 h, 90%.

The synthesis of analogue 99c was accomplished by starting from commercially available 2,5-dibromopyridine (109) (Scheme 23), which was treated with nucleophilic $p$-methoxyphenyletheneamine to give intermediate 110, followed by a Suzuki cross-coupling reaction resulting in aryl-substituted pyridine 111. Subsequent demethylation of 111 using BBr$_3$ afforded the final compound 99c in moderate yield (59%) (Scheme 23).

Scheme 23. Reagents and conditions: (a) 2-(4-methoxyphenyl)ethan-1-amine (1.2 equiv.), K$_2$CO$_3$ (3.0 equiv.), DMF, 120 °C, 8 h, 63%; (b) (4-methoxyphenyl)boronic acid (1.5 equiv.), Pd(PPh$_3$)$_4$ (2 mol%), aq. K$_2$CO$_3$ (2 M, 2.0 equiv.), 1,4-dioxane, 90 °C, 5 h, 90%; (c) BBr$_3$ (6.0 equiv.), CH$_2$Cl$_2$, 0 °C to RT, 15 h, 59%.
3.3 Synthesis of compounds with disconnection of pyridine ring

Compound 100a was synthesised\(^\text{262}\) from 4-aminophenol 112a and glyoxal to give the corresponding Schiff base without further purification (Scheme 24), followed by the reduction by NaBH\(_4\) to give the secondary amine 100a in good yield. Tertiary amine 101a could be easily obtained by reductive amination by using 37% formaldehyde and NaBH\(_4\) in EtOH. Similarly, condensation of \(p\)-hydroxybenzaldehyde (113) with ethylenediamine afforded the diimine intermediate that, following reduction, gave 100b. An additional reductive amination of 100b produced tertiary amine 101b in comparable yield.

Scheme 24. Reagents and conditions: (a) glyoxal (40% in ethanol, 0.9 equiv.), ethanol, RT, 4 h; (b) NaBH\(_4\) (1.0 equiv.), MeOH/CH\(_2\)Cl\(_2\), 1:1 (v/v), 0 °C to RT, 30 min, 86–93% over two steps; (c) (1) formaldehyde (37%, 10.0 equiv.), ethanol, RT, 30 min; (2) NaBH\(_4\) (1.0 equiv.), RT, 30 min, 88–90% over two steps; (d) ethylenediamine (0.5 equiv.), toluene, 110 °C, 20 min.

The synthesis of 100c and 101c initially started from commercially available 4-methoxyphenylethylamine 114 (Scheme 25), whose treatment with diethyl oxalate afforded oxalamide 115 in high yield (91%).\(^\text{263}\) Subsequent reduction in the presence of LiAlH\(_4\) furnished secondary amine 116 in 92% yield. Demethylation of 116 was initially attempted by treatment with BBr\(_3\) in dry CH\(_2\)Cl\(_2\), but failed to give the demethylated product returning unreacted starting material. Alternatively, methylated amine 116 was treated with concentrated HBr (48%) and stirred at 120 °C for 5 h and the desired compound 100c was obtained in good yield.\(^\text{264}\) Secondary amine 100c was
then subjected to reductive amination under standard conditions described previously to give tertiary amine \(101c\) in excellent yield (90% over two steps). However, considering the multiple steps in this synthetic approach, the synthesis of \(100c\) and \(101c\) was then devised by using a more efficient approach shown in \textbf{Scheme 24}. Compound \(100c\) was efficiently prepared from 4-(2-aminoethyl)phenol amine \(112b\), followed by the standard reductive amination in two consecutive steps to afford \(100c\) in excellent yield. An additional reductive amination of \(100c\) successfully produced tertiary amine \(101c\) in a similar manner (\textbf{Scheme 24}).

\begin{center}
\begin{tikzpicture}
    \node (114) at (0,0) {\(114\)};
    \node (115) at (1,1) {\(115\)};
    \node (116) at (2,2) {\(116\)};
    \node (100c) at (3,1) {\(100c\)};
    \node (101c) at (1,3) {\(101c\)};
    \draw[->] (114) -- node[above]{a} (115);
    \draw[->] (115) -- node[above]{b} (116);
    \draw[->] (116) -- node[above]{c} (101c);
    \draw[->] (101c) -- node[above]{d} (100c);
\end{tikzpicture}
\end{center}

\textbf{Scheme 25.} Reagents and conditions: (a) diethyl oxalate (0.55 equiv.), toluene, 110 °C, 12 h, 91%; (b) LiAlH\(_4\) (10.0 equiv.), dry THF, 0 to 70 °C, 12 h, 92%; (c) aq. HBr (48%), 120 °C 5 h, 80%; (d) formaldehyde (37%, 10.0 equiv.), ethanol, RT, 30 min; (2) NaBH\(_4\) (1.0 equiv.), RT, 30 min, 90% over two steps.

\textbf{3.4 Structure-activity relationship study}

With these derivatives in hand, potency for DYRK1A inhibition was determined by performing a kinase assay in triplicate with Woodtide as a substrate and an ATP concentration of 100 µM. The lead compound \(20a\) was confirmed to have inhibitory potency against DYRK1A with an IC\(_{50}\) of 14 nM (See \textbf{Table 10}). I first tested the pyrrole ring opened 2-aminopyridine compounds \(99a–d\). The diarylamine \(99a\) showed no inhibition at >1000 nM (\textbf{Table 10}) with a greater than 100-fold loss of potency in comparison to lead compound \(20a\). We assumed that the unreasonable distance and angles between the two phenols prohibited inhibitory activity. Compounds \(99b–d\), which avoided this problem by increasing the distance between the two phenols, were
also tested, but showed similar results, IC$_{50}$ values were increased to over 1000 nM for all aminopyridine compounds (Table 10).

Despite the undesirable results obtained, we expected that compounds 100a–c and 101a–c, where the pyridine ring was also disconnected, would show better results, which was rationalised in two ways. Firstly, that these compounds were now symmetrical, giving greater malleability within the binding pocket. Secondly, the two nitrogen atoms are essential to inhibition\textsuperscript{258} and by increasing the flexibility between these two atoms, and not just the phenol groups, we thought ideal interactions could be achieved. The hydrogen bond donor/acceptor and not just acceptor was also explored with compounds 100 and 101, respectively. Unfortunately these rationalisations proved to be unfounded, as compounds 100a–c did not improve any inhibitory potency against DYRK1A. All IC$_{50}$ values were shown to be greater than 1000 nM (Table 10). These results were replicated with the methylated tertiary amines 101a–c with values also greater than 1000 nM (Table 10).
Table 10. Kinase inhibition assay of 20a, 99a–d, 100a–c and 101a–c.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structures</th>
<th>DYRK1A (IC₅₀, nM)</th>
<th>Compounds</th>
<th>Structures</th>
<th>DYRK1A (IC₅₀, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20a</td>
<td><img src="image1" alt="Structure" /></td>
<td>14</td>
<td>101a</td>
<td><img src="image2" alt="Structure" /></td>
<td>&gt; 1000</td>
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<td>99a</td>
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<td>&gt; 1000</td>
<td>100b</td>
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<td>99b</td>
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<tr>
<td>99d</td>
<td><img src="image9" alt="Structure" /></td>
<td>&gt; 1000</td>
<td>101c</td>
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<td>100a</td>
<td><img src="image11" alt="Structure" /></td>
<td>&gt; 1000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

With the undesired results for DYRK1A inhibition, we next wanted to test the cytotoxicity of all the compounds against cancer cells, in particular, the established A172 glioblastoma cell lines. In our investigation, lead compound 20a was also tested, which showed cytotoxicity against A172 cell lines with EC₅₀ value of 3.4 μM. In comparison, analogue 99d, which bears three-carbon chain linker between phenol amine and pyridine ring, has showed moderate cytotoxic activity despite the total loss of DYRK1A inhibition, although analogues 99a–c exhibited EC₅₀ values of more than 50 μM (Table 11). More interestingly, secondary amine 100a with disconnection of both the pyrrole and pyridine rings showed the best cytotoxicity potential against A172 cells with EC₅₀ value of 2.4 μM. This means that while it did not show any inhibition against DYRK1A, it exhibited more cytotoxicity than that of lead compound 20a. When testing methylated tertiary amine 101a, much weaker activity was observed, though some moderate cytotoxicity was still observed (EC₅₀ = 22.9 μM). Secondary amines 100b–c or tertiary amines 101b–c with longer distance
totally lost their cytotoxic behaviour in this study. More interestingly, when both 100a and 101a were tested of their cytotoxicity in astrocyte cells (non-tumor cells), neither of them kills astrocytes even at extremely high concentration (up to 50 µM) (Figure 31).

Table 11. Cell viability assay of compounds 20a, 99a–d, 100a–c and 101a–c in glioblastoma cell lines (A172).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structures</th>
<th>A172 (EC₅₀, µM)</th>
<th>Compounds</th>
<th>Structures</th>
<th>A172 (EC₅₀, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20a</td>
<td><img src="image1" alt="Structure" /></td>
<td>3.4 ± 0.0</td>
<td>101a</td>
<td><img src="image2" alt="Structure" /></td>
<td>22.9 ± 2.8</td>
</tr>
<tr>
<td>99a</td>
<td><img src="image3" alt="Structure" /></td>
<td>49.9 ± 0.5</td>
<td>100b</td>
<td><img src="image4" alt="Structure" /></td>
<td>&gt;50</td>
</tr>
<tr>
<td>99b</td>
<td><img src="image5" alt="Structure" /></td>
<td>&gt;50</td>
<td>101b</td>
<td><img src="image6" alt="Structure" /></td>
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<tr>
<td>99c</td>
<td><img src="image7" alt="Structure" /></td>
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<td>100c</td>
<td><img src="image8" alt="Structure" /></td>
<td>&gt;50</td>
</tr>
<tr>
<td>99d</td>
<td><img src="image9" alt="Structure" /></td>
<td>20.2 ± 0.8</td>
<td>101c</td>
<td><img src="image10" alt="Structure" /></td>
<td>&gt;50</td>
</tr>
<tr>
<td>100a</td>
<td><img src="image11" alt="Structure" /></td>
<td>2.4 ± 0.2</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
I have generated a library of 10 novel compounds and subjected them to inhibition assays against DYRK1A. Unfortunately, for all the new compounds reported, 99a–d, 100a–c and 101a–c, nanomolar potency of DYRK1A inhibition was not obtained. These results may implicate the importance of the 7-azaindole motif for DYRK1A inhibition. It is hypothesised that it is not just the positioning of two nitrogen atoms in a similar spatial arrangement that affords strong potency, but the importance of aromatic skeleton with specific spatial arrangements of the two nitrogen atoms can be confirmed. These data will be of use for designing further structure-activity relationship studies to further improve the selective inhibition of DYRK1A.

Additionally, an interesting finding of this study is that although ring-opened compounds have completely lost inhibitory activity against our initial target DYRK1A, some of them exhibit markedly potent cytotoxicity against glioblastoma cells. In particular, analogue 100a has killed cancer cells even more potently than lead compound 20a with an EC₅₀ value of 2.4 μM. More impressively, when both 100a
and 101a were tested of their cytotoxicity in astrocyte cells (non-tumor cells), neither of them kills astrocytes even at extremely high concentration (up to 50 µM) (Figure 31), this finding has provided us with extremely important information to step further for the mechanism studies of their potency against tumour cells rather than non-tumour cells.

In summary, this study has provided us more information and references for future studies on modifications for more novel compounds. For example, the flexible spatial position of two nitrogen atoms has dramatically affected the inhibitory activity against DYRK1A, which means that the aromaticity of the di-nitrogen arrangement is necessary to keep DYRK1A inhibition. Therefore, the subsequent chapter will detail the design and synthesis of novel analogues with diverse functional groups at the C-3 and C-5 position of the 7-azaindole core, and their inhibition of DYRK1A and selectivity over other off-targets such as DYRK1B, DYRK2 and CLK1.
Chapter 4 - Synthesis and biological evaluation of 7-azaindole-based analogues

4.1 Introductory remarks

The work described within this chapter has formed the basis of a publication in The Journal of Medicinal Chemistry.258

Glioblastomas, as very aggressive brain tumours, have been studied for decades. One of the therapeutic strategies involves targeting the EGFR kinase, overexpression of which has been found in half of glioblastoma.265 Accordingly, EGFR expression in glioblastoma is associated with diverse pathological and functional features, such as invasive behavior.266 Recent studies indicated that DYRK1A is highly expressed in glioblastoma, expression of which is indirectly correlated to that of EGFR. Furthermore, inhibition of DYRK1A expression was found to promote EGFR degradation in glioblastoma, resulting in the reduced number of tumourigenic cells.267 To address the therapeutic potential of simultaneously targeting DYRKs, we developed a series of novel DYRK inhibitors and evaluated their efficacy, including DYRK1A inhibition, selectivity, cell viability, clonogenic survival and cell invasion in stem cell-like glioblastoma cell lines.

Interestingly, DYRK1B and CLK1 are also overexpressed in glioblastoma, indicative of cell cycle deregulation through various signalling pathways.128,268 CLK1 is a prime regulator of alternative splicing. It not only increases cell proliferation,105 but also increases expression of anti-apoptotic proteins Bcl-XL (B-cell lymphoma-extra large) and Mcl-1 (induced myeloid leukemia cell differentiation protein),269 that results in resistance to apoptotic stimuli. DYRK1B, however, has the capacity to induce reversible cell arrest in a quiescent state, and its inhibition has been suggested to renew the cell cycle and sensitize cancer cells to chemotherapy and molecularly targeted therapy.88-89,270-272 Encouraged by these studies, it occurred to us that inhibitors targeting multiple kinases (such as DYRK1A, DYRK1B and CLK1) might be beneficial for treatment of a complex disease—cancer. Therefore, compounds as multiple kinases inhibitors against DYRK1A, DYRK1B as well as CLK1 are specifically explored in glioblastoma cells for their cellular efficacies in this chapter.
According to previous studies in Chapters 2 and 3, the 7-azaindole moiety is important, providing not only hydrogen bond donor/acceptor groups, but also possessing a flat ring shape with aromatic positioning of two nitrogen atoms. Herein, we sought to investigate the functionality of DANDY (20a) to improve its inhibitory activity for DYRK1A, and also explore inhibition selectivity over other structurally related kinases such as DYRK1B, DYRK2 and CLK1. Two separate areas of functionality would be investigated: 1) different functionality at the C-3 and C-5 positions, while maintaining the hydrogen bond donor characteristics, and 2) different linkages between the azaindole core and the phenol substituents (namely amide linkages) (Figure 32). The compounds obtained were evaluated in a glioblastoma-relevant cell model.

4.2 Synthetic route of aryl-azaindole derivatives

We explored compounds with different substituents at the C-3 and C-5 positions. Firstly, derivatives 119a–d with varying substituents at the C-3 position were designed and synthesised while maintaining the phenol group at the C-5 position. These derivatives could be synthesised from the same intermediate 26a as employed in the synthesis of 20a in Chapter 2 by utilising sequential Suzuki cross-coupling reactions. To maximise yield, the temperature in the first Suzuki coupling reaction must be below 100 °C (90 °C was found to be optimal) with a slight excess of arylboronic acid. Under these conditions, the synthesis of compounds 117a–c proceeded in 78–85% yields. Electron-rich boron derivatives are more favoured in
transmetallation\textsuperscript{236} thus the lowest yield was obtained with the electron-poor (4-nitrophenyl)boronic acid (Scheme 26).

The second cross coupling required the installation of 4-hydroxyphenyl substituent. Considering the low yield resulting from demethylation with BBr\textsubscript{3}, (4-hydroxyphenyl)boronic acid was used instead of (4-methoxyphenyl)boronic acid in the second Suzuki coupling reaction (condition b, Scheme 26). To our delight, good to excellent yields were obtained with the only modification for the second step being a higher temperature (reflux vs 90 °C). Compound 118c was treated with TMSN\textsubscript{3} under microwave conditions\textsuperscript{274} to obtain the cyclised tetrazole compound 118d in good yield. Tosyl deprotection to afford compounds 119a–c was achieved using the standard procedure described previously, which typically proceeded in 90–95% yield. The resulting compound 119a was subsequently reduced in the presence of iron powder under acidic conditions to afford aniline 119d in moderate yield (Scheme 26).

Scheme 26. Reagents and conditions: (a) arylboronic acid, Pd(PPh\textsubscript{3})\textsubscript{4} (2 mol%), aq. K\textsubscript{2}CO\textsubscript{3} (2 M, 2.0 equiv.), toluene/ethanol 3:1, 90 °C, 3–5 h, 78–85%; (b) arylboronic acid, Pd(PPh\textsubscript{3})\textsubscript{4} (2 mol%), aq. K\textsubscript{2}CO\textsubscript{3} (2 M, 2.0 equiv.), toluene/ethanol 3:1, 110 °C, 3–5 h, 80–93%; (c) TBAF (1.0 equiv.), TMSN\textsubscript{3} (10.0 equiv.), sodium ascorbate (0.2 equiv.), CuSO\textsubscript{4}•5H\textsubscript{2}O (0.1 equiv.), d-BuOH/H\textsubscript{2}O, 1:1 (v/v), MW (100 W, 80 °C), 2 h, 81–83%; (d) KOH (5.0 equiv.), MeOH, 80 °C, 2 h, 90–95%; (e) Fe powder (10.0 equiv.), aq. HCl (3 M, 0.03 M), 110 °C, 3 h, 70–84%.
Modifications at the C-5 position were preceded by a first installation of the phenol group at C-3 position to afford compound 117d, again using (4-hydroxyphenyl)boronic acid with no protection necessary (condition a, Scheme 26). The desired compounds 119e–h were successfully obtained over two or three steps from intermediate 117d with no significant decrease in yield compared to compounds 119a–d.

With compound 119h in hand, further modification of the aniline moiety was performed (Table 12). Selectivity for the NH₂ over the indole NH or phenolic OH was achieved due to a greater nucleophilicity of NH₂ towards the chosen electrophiles. Acetamide 120a (entry 1, Table 12) was obtained by acetylation of 119h by treatment with acetic anhydride in acetic acid at 110 °C. Sulfonamide derivative 120b was furnished in mild conditions and moderate yield by treating 119h with methanesulfonyl chloride (MsCl) in H₂O at room temperature (entry 2, Table 12). The relatively low isolated yield resulted from work-up, which was performed by extracting the reaction mixture with organic solvent (ethyl acetate or CH₂Cl₂). The resulting organic layers were monitored by TLC again which indicated that the formed sulfonamide 120b was slowly converted into the starting material 119h in nonacidic conditions. Compound 120b was stable to be characterised and persisted when kept as a solid under an inert atmosphere. However, these observations do pose problems for biological stability and need to be taken into account when considering results. Urea derivative 120c was obtained in 70% yield by using a low melting mixture of citric acid, urea and mannitol at 80 °C in the absence of additional solvent. Addition of I₂ in this reaction has been reported to act as a Lewis acid to activate the carbonyl of urea to afford the desired compound 120c in a shorter time. In the absence of I₂, reported procedures are quite sluggish and result in a much lower yield compared with that in the presence of I₂.
Table 12. Design and synthesis of 119h derivatives 120a–c.

<table>
<thead>
<tr>
<th>Entries</th>
<th>Conditions</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ac₂O (1.2 equiv.), AcOH, 110 °C, 5 h</td>
<td>120a</td>
<td>77%</td>
</tr>
<tr>
<td>2</td>
<td>MsCl (1.5 equiv.), H₂O, RT, 10 h</td>
<td>120b</td>
<td>64%</td>
</tr>
<tr>
<td>3</td>
<td>Urea (15.0 equiv.), I₂ (0.1 equiv.), citric acid, mannitol, 80 °C, 6 h</td>
<td>120c</td>
<td>70%</td>
</tr>
</tbody>
</table>

4.2.1 Structure-activity relationship study of synthesised compounds 119b–h and 120a–c

All the novel compounds prepared above were then subjected to inhibition assays at DYRK1A, DYRK1B, DYRK2 and the structurally related CLK1. Kinase assays were performed with Woodtide as a substrate for DYRK kinases and RS peptide as a substrate for CLK1. ATP concentration (100 µM) was kept constant to allow for comparison of potencies across the kinases. In comparison, harmine (not shown in Table 13) and lead compound 20a inhibited DYRK1A with IC₅₀ values of 360 and 14 nM, respectively. Inhibitor 20a showed nearly equal potency against DYRK1B (15
nM, Table 13), which had not been previously reported.\textsuperscript{220} In addition, 20a showed a 10-fold weaker inhibitory potency against DYRK2 (166 nM, Table 13), but also displayed inhibitory activity at CLK1 with an IC\textsubscript{50} value of 48 nM.

Table 13. Kinase inhibition and cellular efficacy of DANDY (20a) and C3-modified analogues 119b–d.

<table>
<thead>
<tr>
<th>R</th>
<th>DYRK1A (nM)</th>
<th>DYRK1B (nM)</th>
<th>DYRK2 (nM)</th>
<th>CLK1 (nM)</th>
<th>RN1 (µM)</th>
<th>WK1 (µM)</th>
<th>JK2 (µM)</th>
<th>SJH1 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20a</td>
<td>14 ± 2</td>
<td>15 ± 5</td>
<td>166 ± 22</td>
<td>48 ± 7</td>
<td>2.0 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>5.1 ± 0.6</td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td>119b</td>
<td>169 ± 52</td>
<td>83 ± 25</td>
<td>571 ± 149</td>
<td>117 ± 35</td>
<td>5.7 ± 1.3</td>
<td>6.2 ± 2.3</td>
<td>6.3 ± 1.7</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>119c</td>
<td>280 ± 53</td>
<td>172 ± 53</td>
<td>416 ± 46</td>
<td>85 ± 5</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>119d</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

Next, we tested how modification of the phenol at the C-3 position affects kinase inhibition. Bioisosteric replacement of the phenol moiety with indole (119b) resulted in 10-fold weaker DYRK1A inhibition (IC\textsubscript{50} = 169 nM) and comparable loss of activity was observed against DYRK1B (IC\textsubscript{50} = 83 nM), DYRK2 (IC\textsubscript{50} = 571 nM) and CLK1 (IC\textsubscript{50} = 117 nM). Substitution of phenol for a phenyltetrazole moiety at C-3 as seen in 119c further reduced kinase inhibition activity (85–280 nM) and the aniline analogue (119d) resulted in complete loss of kinase inhibition (IC\textsubscript{50} > 1 µM) (Table 13).
Compounds with differing substitution on C-5 showed interesting results (Table 14). Both 4-nitrophenyl (119e) and indole (119f) analogues showed weaker DYRK1A inhibition (212 and 204 nM, respectively), but indole-analogue 119f potently inhibited CLK1 (IC\textsubscript{50} = 26 nM) and DYRK1B (IC\textsubscript{50} = 51 nM). Analogues with phenyltetrazole (119g) or aniline (119h) both showed inhibitory potencies against DYRK1A, DYRK1B and CLK1 with IC\textsubscript{50} range from 32 nM to 86 nM. Interestingly, reversing the substitutions on C3 and C5, aniline 119h (IC\textsubscript{50} up to 32 nM) had greater than 20-fold higher activity than 119d (IC\textsubscript{50} > 1000 nM) (Table 13). These results provide evidence that phenol substitution at the C-3 position plays a crucial role in maintaining potent inhibition, while greater variability was tolerated at the C5 position. Compounds with further modifications of the aniline 119h showed excellent results. The sulfonamide (120b) or urea (120c) inhibited DYRK1A with IC\textsubscript{50} values in double-digit nanomolar potency (31–86 nM), although comparable nanomolar potency was determined for these inhibitors against DYRK1B and CLK1. Selectivity over DYRK2 was detected with 10-fold difference between IC\textsubscript{50} values. Whether or not the sulfonamide moiety in compound 120a survived the testing conditions is uncertain, but due to the different results compared to the aniline 119h it would suggest that this was indeed the case. The introduction of phenylacetamide at C-5 (120a) led to the strongest DYRK1A inhibition (6.6 nM) with 5-fold selectivity over DYRK1B (38 nM) and CLK1 (35 nM), and 80-fold selectivity over DYRK2 (536 nM).
Table 14. Kinase inhibition and cellular efficacy of C-5 analogues.

<table>
<thead>
<tr>
<th></th>
<th>Kinase inhibition assay (IC&lt;sub&gt;50&lt;/sub&gt;, nM)</th>
<th>Cell viability assay (EC&lt;sub&gt;50&lt;/sub&gt;, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;i&gt;DYRK1A&lt;/i&gt;</td>
<td>&lt;i&gt;DYRK1B&lt;/i&gt;</td>
</tr>
<tr>
<td>119e</td>
<td>212 ± 36</td>
<td>198 ± 45</td>
</tr>
<tr>
<td>119f</td>
<td>204 ± 46</td>
<td>51 ± 8</td>
</tr>
<tr>
<td>119g</td>
<td>86 ± 17</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>119h</td>
<td>43 ± 9</td>
<td>32 ± 16</td>
</tr>
<tr>
<td>120a</td>
<td>6.6 ± 1.2</td>
<td>38 ± 15</td>
</tr>
<tr>
<td>120b</td>
<td>56 ± 17</td>
<td>130 ± 37</td>
</tr>
<tr>
<td>120c</td>
<td>31 ± 10</td>
<td>17 ± 8</td>
</tr>
</tbody>
</table>
4.3 Synthesis of indole-based derivatives with selected functional groups

According to the biological activities described above, 7-azaindole based analogues with different substituents at the C-3 or C-5 position showed variable potencies against DYRKs and CLK1. With these results, it is of scientific interest to incorporate an alternatively substituted indole in place of the azaindole in a similar manner, despite the loss of activity observed by the indole-based diphenol analogue 26b in chapter 2. Therefore, I investigated three more derivatives with selected functional groups (indole, tetrazole-phenyl and tetrazole) based on an indole scaffold (Scheme 27). The synthesis of analogues 120d–e could be achieved in a similar fashion to the methods described in Scheme 26, starting from the key intermediate 26b mentioned in Chapter 2. The obtained methoxy intermediates 119i and 119j could be further treated with BBr3 to give demethylated compounds 120d and 120e, respectively.

Scheme 27. Reagents and conditions: (a) arylboronic acid, Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 2.0 equiv.), toluene/ethanol 3:1, 90 °C, 3 h, 93–94%; (b) arylboronic acid (1.2 equiv.), Pd(PPh₃)₄ (2 mol%), aq. K₂CO₃ (2 M, 2.0 equiv.), toluene/ethanol 3:1, 110 °C, 5 h, 87–90%; (c) TBAF (1.0 equiv.), TMSN₃ (10.0 equiv.), sodium ascorbate (0.2 equiv.), CuSO₄•5H₂O (0.1 equiv.), t-BuOH/H₂O, 1:1 (v/v), MW (100 W, 80 °C), 2 h, 74%; (d) KOH, MeOH, 80 °C, 2 h, 89–94%; (e) BBr₃ (6.0 equiv.), CH₂Cl₂, 0 °C to RT, 10 h, 67–70%.

Analogue 120f with tetrazole substitution at the C-3 position was prepared from starting material 25b (Scheme 28). Cyanated compound 117g was generated by
cyanation at the C-3 position by treatment with chlorosulfonylisocyanate and TEA in acetonitrile\textsuperscript{279} and subsequent tosyl protection of indole nitrogen in moderate yield over two steps. The following arylation at the C-5 position was realised \textit{via} a Suzuki coupling reaction to give compound 118l in excellent yield. Tetrazole-containing 119k was furnished in a similar manner described above in Scheme 26, and the final compound 120f was eventually obtained from the demethylation of 119k by treatment with BBr\textsubscript{3} in reasonable yield.

\textbf{Scheme 28:} Reagents and conditions: (a) (1) chlorosulfonylisocyanate (1.1 equiv.), TEA (1.05 equiv.), CH\textsubscript{3}CN, 0 °C to RT; (2) NaH (60\% in mineral oil, 3.0 equiv.), TsCl (1.2 equiv.), BnEt\textsubscript{3}NCl (0.02 equiv.), CH\textsubscript{2}Cl\textsubscript{2}, 0 °C to RT, 30 min; 72\% over two steps; (b) (4-methoxyphenyl)boronic acid (1.5 equiv.), Pd(PPh\textsubscript{3})\textsubscript{4} (2 mol\%), aq. K\textsubscript{2}CO\textsubscript{3} (2 M, 2.0 equiv.), toluene/ethanol 3:1, 110 °C, 3 h, 92\%; (c) TBAF (1.0 equiv.), TMSN\textsubscript{3} (10.0 equiv.), sodium ascorbate (0.2 equiv.), CuSO\textsubscript{4}•5H\textsubscript{2}O (0.1 equiv.), \textit{t}-BuOH/H\textsubscript{2}O, 1:1 (v/v), MW (100 W, 80 °C), 2 h, 77\%; (d) KOH (5.0 equiv.), MeOH, 80 °C, 2 h, 96\%; (e) BBr\textsubscript{3} (6.0 equiv.), CH\textsubscript{2}Cl\textsubscript{2}, 0 °C to RT, 10 h, 70\%.

\textbf{4.3.1 Structure-activity relationship study of synthesised compounds 120d–f}

With indole-based derivatives 120d–f in hand, kinases inhibition assays were evaluated shown in Table 15. Unfortunately, none of them showed active potency against either DYKRs or CLK with all IC\textsubscript{50} values of over 1000 nM. Compound 120d, in comparison to 119f (DYRK1A, IC\textsubscript{50} = 204 nM, Table 14), has lost its
activity against DYRK1A by replacement of the 7-azaindole core with an indole core. This suggests that the 7-nitrogen is crucial in maintaining the kinase inhibition. Despite compounds 120e-f exhibiting inactivity against all the tested kinases, compound 119c, in comparison with the only difference in 7-position displayed moderate potency against DYRK1A with its IC$_{50}$ value of 280 nM. This further confirms the importance of 7-azaindole scaffold compared with other heterocyclic rings.

Table 15. Kinase inhibition and cellular efficacy of 120d–f.
4.4 Synthesis of novel derivatives with amide linker

Since 7-azaindole core has been confirmed as crucial, I next investigated diverse skeletons with different geometries and increased spatial flexibility. The next set of targets therefore contained a linker between the 7-azaindole core and phenol group. The hydroxy position of the phenol group connected with an amide linker was investigated at both the \textit{para}- and \textit{meta}-positions of phenyl ring (\textbf{Scheme 29}). 5-carboxamide derivatives 126a–b were made from starting material 25a. After protection with a tosyl group, the given compound 121 was transformed to carboxylic acid derivative 122. This was achieved by treating with freshly prepared acetic-formic anhydride (synthesised according to the reported routes\textsuperscript{280–281} and palladium acetate with dpff as the ligand. The related catalytic mechanism was proposed previously (\textbf{Figure 33}).\textsuperscript{282–283} CO was generated \textit{in situ} in the presence of base, followed by the formation of the acylpalladium complex (RCOPdX), which subsequently reacted with acetate to generate the anhydride intermediate (RCOOAc) to eventually achieve the carboxylic acid after hydrolysis. This method allows for the safe \textit{in situ} genesis of CO without handling toxic gases.
Scheme 29. Reagents and conditions: (a) TsCl (1.2 equiv.), NaH (60% in mineral oil, 1.5 equiv.), THF, 0 °C, 1 h, 90%; (b) Pd(OAc)$_2$ (5 mol%), dppf (10 mol%), acetic-formic anhydride (5.0 equiv.), $i$-Pr$_2$NEt (5.0 equiv.), DMF, 110 °C, 5 h, 83%; (c) HBTU (1.2 equiv.), $i$-Pr$_2$NEt (2.5 equiv.), aniline 34a or 34b (1.1 equiv.), DMSO, 0 °C–RT, 2 h, 61–79%; (d) KOH (5.0 equiv.), MeOH, 80 °C, 1 h, 94–96%; (e) I$_2$ (1.2 equiv.), KOH (3.0 equiv.), DMF, RT, 8 h, 80–86%; (f) arylboronic acid, Pd(PPh$_3$)$_4$ (2 mol%), aq. K$_2$CO$_3$ (2 M, 2.0 equiv.), toluene/ethanol 3:1, 100 °C, 3–5 h, 60–65%; (g) trichloroacetyl chloride (1.2 equiv.), AlCl$_3$ (2.5 equiv.), 0 °C, 3 h; (h) NaOH (3 M), RT, 5 h, 70% over two steps; (i) TBAF (1.5 equiv.), THF, RT, 30 min, 93–95%; HBTU = (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate).
Figure 33. Proposed mechanism of carboxylic acid formation via in situ genesis of CO.\textsuperscript{282}

With compound 122 in hand, the amide coupling was attempted by treating 122 with oxalyl chloride and catalytic DMF in CH\textsubscript{2}Cl\textsubscript{2} to form the acyl chloride intermediate, followed by addition of aniline derivative 34a or 34b. Unfortunately, the desired amide compound was not successfully obtained. Neither aniline nor acid was consumed, so we assumed that the formation of acyl chloride intermediate was not successful in this case. Alternatively, the treatment of 122 with aniline derivative 34a or 34b in the presence of coupling reagent HBTU afforded desired compounds 123a–b in comparable yields.\textsuperscript{284} The amides obtained were initially treated with I\textsubscript{2} and KOH to iodinate at the C-3 position but no reaction was observed and starting material was recovered. It was hypothesised that this was a result of the electron withdrawing tosyl group which reduced the nucleophilicity of C-3. Therefore, deprotection of the tosyl group was performed by KOH in MeOH under reflux. However, the TBS group was not tolerated under these conditions and as a result, the concomitant deprotection of both TBS group and tosyl group gave compounds 124a–b.\textsuperscript{285} The following iodination\textsuperscript{286} at the C-3 position with I\textsubscript{2} and KOH was successful and gave compounds 125a–b in good yields. The final compounds 126a–b could then be easily obtained by a standard Suzuki cross-coupling reaction described previously.
Similarly, compounds 130a–b could be obtained by again starting with 25a (Scheme 29). The tosyl protected compound 121 was first employed in a Friedel-Crafts reaction by treatment with trifluoroacetic anhydride in the presence of Lewis acid AlCl₃, but no product was obtained with starting material still present. The more reactive electrophile trichloroacetyl chloride was tested yet compound 121 again failed to react. Instead, the unprotected compound 25a was reacted directly. The trifluoroacetic anhydride method was unsuccessful but by treating 25a with trichloroacetyl chloride, the trichloroacetyl intermediate was obtained with all starting material consumed. Subsequent hydrolysis afforded the carboxylic acid 127 in 70% yield over two steps. Subsequent amide formation with 34a–b proceeded by using the optimised HBTU conditions described above to afford 128a–b. Suzuki-coupling gave 129a–b which could be TBS deprotected with TBAF to afford final compounds 130a–b (Scheme 29).

4.4.1 Structure-activity relationship study of amides

We next explored the effect of an amide linker at position C-3 or C-5 on kinase inhibition (Table 16). Compounds 130a or 130b with an amide linker at the C-3 position resulted in complete loss of kinase inhibition (IC₅₀ > 1 µM) which was in line with results obtained with other C-3 modified analogues 119b–d. The same modification at the C-5 position was better tolerated – analogues 126a and 126b inhibited DYRK1A with higher IC₅₀ values (344 and 127 nM, respectively). Interestingly, both 126a and 126b showed selective inhibition against CLK1 over DYRKs members. With these results in hand, we wanted to further investigate the effect of these compounds on cell viability and cell-based functional evaluation, such as cellular thermal shift assay, EGFR degradation, clonogenic survival, migration and invasion.
Table 16. Kinase inhibition and cellular efficacy of analogues with an amide linker.

| R¹ and R²          | DYRK1A (IC₅₀, nM) | DYRK1B (IC₅₀, nM) | DYRK2 (IC₅₀, nM) | CLK1 (IC₅₀, nM) | RN1 (IC₅₀, nM) | WK1 (EC₅₀, µM) | JK2 (EC₅₀, µM) | SJH1 (EC₅₀, µM) |
|--------------------|--------------------|--------------------|------------------|-----------------|----------------|----------------|----------------|----------------|----------------|
| 126a               | 344 ± 84           | 128 ± 31           | >1,000           | 12              | 12.5 ± 2.9     | 4.7 ± 3.4      | ± 2.9          | ± 2.2          |
| 126b               | 127 ± 54           | 138 ± 51           | 847 ± 50         | 34              | 13.8 ± 1.2     | 1.2 ± 0.1      | ± 0.1          | ± 0.1          |
| 130a               | >1,000             | >1,000             | >1,000           | >1,000          | >50            | >50            | >50            | >50            |
| 130b               | >1,000             | >1,000             | >1,000           | >1,000          | >50            | >50            | >50            | >50            |
4.5 Cell-based screening

Glioblastoma is a heterogeneous cancer with great variability between patients and even within the same patient. Glioblastoma tumours have been sub-classified into four molecular subtypes – classical, proneural, neural and mesenchymal. Therefore, we have chosen to screen all potent inhibitors in patient-derived glioblastoma cells (RN1, JK2, WK1 and SJH1, respectively). These cells were grown as stem cells under defined conditions in order to maintain the phenotype and genotype of the primary resected tumours (Figure 34B). Viability of cells treated with test compounds was determined in the Alamar blue assay (Table 13–16).

Figure 34. Expression of DYRK/CLK1 kinases and genotype of patient-derived glioblastoma cell lines. (A) Cell lysate of untreated cells were analysed with Western blotting using indicated antibodies (representative image of 2 independent blots). (B) Subtype and genotype of patient-derived glioblastoma cell line RN1, JK2, WK1 and SJH1 as previously described.

The lead compound 20a diminishes the viability of RN1 cells with an EC50 of 2.0 µM with a comparable reduction in other glioblastoma cell (Table 13). Compounds 119h and 120a, as the two most potent kinase inhibitors developed in this study, have reduced classical RN1 viability with 1.1 µM and 2.1 µM potency, respectively (Table 14). More excitingly, mesenchymal WK1 cells are the most responsive cells to these inhibitors (EC50 = 0.9–2.1 µM), furthermore the viability of the neural SJH1 and proneural JK2 cell lines were also reduced by 119h and 120a with the EC50 values
Chapter 4

ranging 3.2–8.6 µM. Compounds 120b and 120c, however, were 13 times less potent in the cell viability assays (EC50 > 13 µM) versus 119h (EC50 = 1.1 µM). This reduction in efficacy could arise from the increased polarity and reduced cell membrane permeability, though off-target effects could also be a contributing factor. For the remaining compounds, cellular efficacy correlated with the potency in the kinase inhibition assay (see as Table 15 and 16).

4.5.1 Cell-based functional evaluation

To evaluate the most potent inhibitors, we further tested analogues 119h and 120a in cell-based assays. Analogue 119d was employed as a negative control, which lacks the kinase inhibition activity and was not cytotoxic to primary cells (Table 13).

4.5.1.1 Cellular thermal shift assay (CETSA)

To demonstrate target engagement in cells, compound 119h was assessed for its ability to penetrate cells and bind to DYRK1A. We used a cellular thermal shift assay (CETSA)293-294 to assess DYRK1A engagement in U251 cells as this cell line expresses high quantities of DYRK1A (See Figure 34A). The method was implemented as follows: U251 cells were treated with analogue 119h (10 µM) and cell suspensions were heated to different temperatures to induce protein denaturation. Soluble proteins were extracted with an aqueous buffer and analysed with Western blotting. Inhibitors that bind to their cognate target in cells would yield a stabilised protein, which will be detected at higher temperatures compared to the resultant denaturation profile in cells without drug treatment.

As a result, 119h efficiently stabilised DYRK1A, but not the structurally unrelated p38α MAPK (Figure 35A–B). To investigate 119h concentration effects, we derived the isothermal dose-response fingerprint (ITDRF_{CETSA}), which is a characteristic ligand-induced protein stabilisation at a constant temperature. Indeed, the DYRK1A biochemical potency of 119h (IC50 = 43 nM) translated well into its cellular target engagement (ITDFR_{CETSA} = 711 nM; Figure 35C–D).
Figure 35. Treatment of U251 cells with inhibitor 119h stabilises DYRK1A and decreases cell viability. CETSA melt curves (40–67 °C) for DYRK1A and p38α (A) and quantification (mean ± SEM) of 3 independent experiments (B) were determined in intact U251 cells treated with compound 119h (1 h, 10 µM). Representative image (C) and quantification (mean ± SEM) of 2 independent experiments (D) of ITDRF_{CETSA} in intact U251 cell treated with compound 119h (0–20 µM, 1 h) at 54 °C.

4.5.1.2 EGFR degradation

To confirm DYRK1A inhibition in cells treated with 119h, we performed an EGFR degradation assay. Overexpression of DYRK1A has been shown to block EGFR degradation, resulting in reduction of cell proliferation.\textsuperscript{295} We therefore tested 119h for its ability to increase EGFR degradation in U251 cells. EGFR protein translation was blocked with cyclohexamide and EGFR degradation was induced with EGF (100 ng/mL). Cell lysates were collected at indicated time points and the amount of non-
degraded EGFR quantified by Western blotting (Figure 36A). Pre-treatment of cells with 119h (2.5 µM) reduced EGFR half-life to 11.3 ± 2.5 min in comparison to that in untreated U251 cells (25.6 ± 7.6 min) (Figure 36B). Importantly, a similar effect was observed with genetic knockdown of DYRK1A. Using siRNA approach, we achieved on average 80% reduction in DYRK1A expression (Figure 36C) and this DYRK1A down-regulation reduced EGFR half-life to 3.3 ± 2.5 min (Figure 36D).

Figure 36. DYRK1A inhibition induces EGFR degradation. (A) U251 cells were treated with DMSO (Ctr) or compound 119h (2.5 µM) for 4 h. Cells were incubated with cycloheximide (30 µg/mL; 1 h) and EGF (100 ng/mL) was added for indicated time points. (B) U251 cells were treated with scramble (Ctr) or DYRK1A-targeting siRNA for 24 h. Cells were incubated with cycloheximide (30 µg/mL; 1 h) and EGF (100 ng/mL) was added for indicated time points. Cell lysates were analysed by Western Blotting using indicated antibodies. (C–D) EGFR levels were normalised to loading control (β-actin) and are expressed as percentage of untreated cells (0 min). Half-life (t_{1/2}) values were calculated by non-linear regression analysis and represent mean ± SEM from 3 independent experiments.
4.5.2 Clonogenic survival

Based on kinase inhibition \textit{in vitro} and in cells, we evaluated compounds \textbf{119h} and \textbf{120a} for their ability to reduce long-term survival of glioblastoma cells. Compounds \textbf{119h} and \textbf{120a} dose-dependently inhibited clonogenic survival of RN1 with EC\textsubscript{50} values of 0.8 and 2.1 µM (Figure 37A–B), respectively; mirroring the EC\textsubscript{50} values obtained in the short-term viability assay (Table 14). Compound \textbf{119h} was more efficacious at reducing the survival of the established U251 cell line (EC\textsubscript{50} = 0.18 µM, Figure 37C).

![Figure 37. Clonogenic survival following drug treatment.](image)

Patient-derived RN1 glioblastoma cells were treated with \textbf{119h} (A) or \textbf{120a} (B) for 10 days. Established U251 glioblastoma cells were treated with \textbf{119h} (C) for 10 days. Colonies were fixed, stained with Toluidine Blue and counted using the ImageJ software. Data were normalised to vehicle-treated controls (set as 100% survival) and EC\textsubscript{50} values calculated by non-linear regression analysis. EC\textsubscript{50} values represent mean ± SEM from 3 independent experiments performed in duplicate. One-way ANOVA, followed by Dunnett’s multiple comparison test was used to determine statistical significance. (*P < 0.05, *** P < 0.001, ****P < 0.0001 in relation to vehicle-treated cells).

4.5.3 Migration and invasion

We finally assessed \textbf{119h}, \textbf{120a}, and the inactive analogue \textbf{119d} using \textit{in vitro} assays of cell migration and invasion (Table 17). We employed U251, A172 and RN1 cell lines, all of which without treatment by any compounds (Conc. = 0 µM) show high
basal velocity of migration and invasion (Table 17). When treated with 119h, migration of A172, U251 and RN1 cells were all reduced up to 75% when a concentration of 5 µM was reached (Table 17), and invasiveness of A172 and U251 cells in a dose-dependent manner were also blocked (Table 17). Similar effects were observed across all assays with the analogue 120a, whereas the inactive analogue 119d was not effective at all in the migration and invasion assays (Table 17).

Table 17. Inhibition of migration and invasion of glioblastoma cells by CMGC inhibitors 119h and 120a, and inactive analogue 119d. Values represent mean ± SEM from 3 independent experiments.

<table>
<thead>
<tr>
<th>Conc. (µM)</th>
<th>U251 migration (µm/h)</th>
<th>U251 invasion (µm/h)</th>
<th>A172 migration (µm/h)</th>
<th>A172 invasion (µm/h)</th>
<th>RN1 migration (µm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2,163 ± 133</td>
<td>1,337 ± 93</td>
<td>3,123 ± 103</td>
<td>2,659 ± 674</td>
<td>1,174 ± 158</td>
</tr>
<tr>
<td>119h</td>
<td>1.0</td>
<td>1,247 ± 176</td>
<td>1,139 ± 112</td>
<td>2,828 ± 271</td>
<td>2,675 ± 475</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>127 ± 56</td>
<td>234 ± 22</td>
<td>728 ± 308</td>
<td>1,538 ± 696</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>129 ± 52</td>
<td>-149 ± 12</td>
<td>131 ± 129</td>
<td>714 ± 585</td>
</tr>
<tr>
<td>120a</td>
<td>0.0</td>
<td>2,159 ± 48</td>
<td>1,710 ± 97</td>
<td>2,739 ± 428</td>
<td>3,047 ± 506</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1,125 ± 93</td>
<td>736 ± 117</td>
<td>2,891 ± 147</td>
<td>2,731 ± 431</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>173 ± 38</td>
<td>34 ± 153</td>
<td>2,296 ± 564</td>
<td>2,027 ± 419</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>111 ± 1</td>
<td>-118 ± 37</td>
<td>651 ± 300</td>
<td>1,718 ± 517</td>
</tr>
<tr>
<td>119d</td>
<td>0.0</td>
<td>2,653 ± 395</td>
<td>1,151 ± 133</td>
<td>2,650 ± 675</td>
<td>2,990 ± 997</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2,331 ± 143</td>
<td>1,451 ± 69</td>
<td>3,123 ± 745</td>
<td>2,411 ± 241</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>2,370 ± 39</td>
<td>1,481 ± 181</td>
<td>3,065 ± 718</td>
<td>2,232 ± 498</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>2,702 ± 239</td>
<td>1,380 ± 73</td>
<td>2,827 ± 709</td>
<td>2,707 ± 491</td>
</tr>
</tbody>
</table>
4.6 Concluding remarks

While others have concentrated on the development of selective DYRK1A inhibitors, our goal was to understand the SAR across the DYRK family and CLK1. In our studies, potent inhibitor 120a showed about 6-fold more potent activity against DYRK1A over DYRK1B, which demonstrated that selective inhibition of DYRK1A is possible within the CMGC family of protein kinases. The potency of tested analogues to inhibit DYRK1A and DYRK1B activity was comparable to the potency of inhibitors against CLK1; with exception of compound 126a and 126b which inhibited CLK1 with >10-fold higher potency than DYRK1A/B, these interesting results are quite significant for future design of CLK1 inhibitors. Finally, all compounds displayed selectivity as their inhibitory action at DYRK2 with >10-fold higher IC₅₀ values. After screening anti-cancer efficacy in established and stem cell-like glioblastoma cell lines, we found that the potent inhibitor 119b for both DYRK1A/DYRK1B and CLK1 (IC₅₀ ≤ 50 nM) significantly decreased viability, clonogenic survival, migration and invasion of glioblastoma cells than that of the most potent inhibitor 120a for DYRK1A only, which demonstrated that inhibitors targeting multiple kinases are beneficial in the treatment of tumours. We also demonstrate that DYRK1A’s thermal stability in cells was increased upon compound treatment, confirming binding in cells by using cellular thermal shift assay.

Accordingly, 7-azaindole based compounds, as is shown in Figure 38, have been further analysed in terms of their SAR. The N-C-NH as a key moiety plays an
important role in keeping potent inhibitory activities against DYRKs as both a hydrogen bond donor and acceptor. Moreover, we found that C3-modified analogues failed to inhibit all kinases, underlining the importance of the C-3 phenolic group attached to the 7-azaindole core. In particular, aniline 119d completely lost its inhibitory potency against DYRK1A with an IC<sub>50</sub> value of up to 1000 nM, presumably its NH<sub>2</sub> can be protonated which decreases its electrostatic interaction with the ammonium ion of conserved Lys188 residue. However, the reason why potency of other analogues such as 119b and 119c with different hydrogen bond donors at the C-3 position varied in kinase inhibition remains elusive. Interestingly, the C-5 position offered scope for improvement of activity and yielded analogues with low nanomolar potency. In particular, acetamide 120a has shown selectivity between DYRK1A and DYRK1B with a ratio of 5:1. The potency and selectivity probably results from several factors including, 1) the acetamide moiety acts as a hydrogen bond donor/acceptor; 2) it has a decreased spatial distance between 120a and Ile165 residue compared with the hydroxy group of DANDY; 3) the methyl group of acetamide improves its hydrophobicity and thus increases its hydrophobic interaction with the hydrocarbon side chain of hinge residue Ile165.

In summary, the protein kinases of the DYRK family are emerging as potential targets for small molecule cancer therapy. We have presented a library of novel potent and cell-active DYRK kinase inhibitors based on the 7-azaindole scaffold. Future work will be focused on the biological evaluation of these potent analogues in an in vivo animal model of glioblastoma, including the permeability in cells and BBB, as well as the tolerance abilities in the actual environment in vivo.
Chapter 5 - Summary and future work

5.1 Summary and conclusion

In the work described in this Thesis, I have systematically generated a small library of compounds based on structural modifications to the lead compound DANDY (20a), and all of these compounds have been subjected to DYRK1A inhibition assays. In some instances, selectivity against other off-targets including DYRK1B, DYRK2 and the structurally related CLK1 has been also evaluated. Additionally, for the most promising compounds functional activity was investigated in glioblastoma cells, which have provided us relevant information for further studies in the future.

The nature of the 7-azaindole core of DANDY (20a) was found to be essential for DYRK1A inhibition and selectivity over other off-targets. Replacement by other heterocyclic rings such as indole, indazole, benzimidazole, pyrazole-pyridine, bipyridine, naphthyridine, imidazole or triazolopyrazine has resulted in dramatic reduction in DYRK1A inhibition.

In terms of the ring-opened analogues, investigations have revealed that relatively more flexible compounds failed to exhibit nanomolar potency against DYRK1A, even though they possess similar spatial arrangements of two nitrogen atoms mimicking the 7-azaindole scaffold. Interestingly, some of ring-opened compounds have showed markedly potent cytotoxicity against glioblastoma cells by cell viability assays. In particular, linear compound 100a killed cells more effectively than DANDY. Since these ring-opened analogues’ cell cytotoxicity was not correlated with the capacity for DYRK1A inhibition, the internal mechanism is still unknown, which drives us to further explore their mode of action.

Exploration of the nature of the biaryl substitutions on C-3 or C-5 position of the 7-azaindole core has revealed that various modifications and substituents at the C-5 position were much more tolerated than that of the C-3 position. Analogue 120a with acetamide rather than hydroxy exhibited the most potent activity against DYRK1A with its IC₅₀ up to 6 nM in comparison to DANDY (IC₅₀ = 14 nM). More importantly,
derivative 120a has also showed improved selectivity for DYRK1A over DYRK1B with a ratio of 5:1 compared with DANDY with that of about 1:1. This is a big improvement over previous studies since DYRK1B only differs by a small number of amino acids in the ATP-binding site compared with DYRK1A, which makes it extremely difficult to design inhibitors only targeting DYRK1A. Additionally, aniline 119h has shown a broad inhibition for DYRK1A, DYRK1B and CLK1 with all the IC₅₀ values of being < 50 nM, as a result, 119h has played effective roles in targeting multiple kinases in the therapeutic treatment of glioblastoma in kinases inhibition assays and cell viability assays in vitro.

Considering that 7-azaindole scaffold is essential in keeping DYRK1A inhibition, the introduction of an amide linkage between the phenol group at the C-3 or C-5 position of the 7-azaindole core has been further investigated. However, the analogues 130a–b have totally lost their inhibition for DYRKs and CLK1 by the addition of this amide linker on C-3 position. This further confirms our observation that phenol substitution at the C-3 position is quite essential in maintaining the activity. Interestingly, analogues 126a–b with the amide linker at the C-5 position have shown moderate potency against DYRK1A with their IC₅₀ values in three digit nanomolar range, even though this does make them less effective than DANDY. More surprisingly, amide 130a–b exhibited selective potency against CLK1 with two digit nanomolar concentration (12 nM and 34 nM, respectively), and in the same case, they showed relatively cytotoxic activity in all the subtypes of glioblastoma cells. These results have provided us very useful directions for our future investigations on CLKs inhibitors.

According to the preliminary studies, potent DYRK1A inhibitors such as 119h and 120a have displayed a very similar trend to the corresponding cellular efficacies in vitro assay. This indicates that these promising results warrant further exploration of the in vivo activity of the novel inhibitors in animal models, in the hope that these promising inhibitors can be utilised in clinical studies for therapeutic treatment of cancers. Such efforts are currently underway.
5.2 Future work

As mentioned above, the future work of this project will first involve further biological evaluation of these promising inhibitors through *in vivo* assays, with the aim of a potential use of inhibitors for targeting cancer cells.

According to recent studies on DYRK1A inhibitors, Guillou and co-workers have reported a novel compound 131a as a potent DYRK1A inhibitor (Figure 39), and in their studies, an interesting result has been obtained when a methoxy group was switched from the 6-position of the 7-azaindole skeleton to the 7-position by a replacement of the 7-nitrogen, the resulting analogue 131b has exhibited much more potency against DYRK1A than 131a with its IC₅₀ value of 18 nM. This finding has given us a hint in the future designs based upon DANDY shown in Figure 39 below. The introduction of the methoxy group at the C-6 to give compound 132a might provide improvement of its binding affinity within th DYRK1A active site, since there is still some space left between the 6-position and the hinge residue according to the binding mode described in Chapter 1. Additionally, a switching of the methoxy group from the 6-postion to the 7-position to give compound 132b might be worthwhile by a replacement of the 7-nitrogen. The oxygen atom of the methoxy group has similar properties as a hydrogen bond acceptor in binding modes with that of nitrogen atom.
In 2013, Luk and co-workers have reported a new class of DYRK1A inhibitors (Figure 40), the exemplified \textit{133a} showed very poor inhibitory activity against DYRK1A with its IC$_{50}$ value over 1000 nM. However, with the addition of a phenyl group on the long carbon chain, the resulting racemic compound \textit{133b} has been dramatically increased for its DYRK1A inhibition with its IC$_{50}$ value in single digital nanomolar concentration (9 nM). This interesting finding has driven us to refocus on our inactive compounds \textit{99a–d} described in Chapter 3 (Figure 40), which have totally lost DYRK1A inhibition (IC$_{50}$ > 1000 nM). Similarly, it might be promising to install an additional phenyl group on the carbon chain, increasing π–π interactions in the binding modes, and eventually improving its binding affinity within the DYRK1A active site. Importantly, it was unknown which confirmation of \textit{133b} has made a bigger contribution to DYRK1A inhibition in this study. Therefore, more endeavours on this aspect are necessary as well in our future directions.

\textbf{Figure 39.} Design for novel DYRK1A inhibitors \textit{132a–b}. 

\begin{center}
\includegraphics[width=\textwidth]{figure39.png}
\end{center}
Figure 40. Design for novel DYRK1A inhibitors 134.
Chapter 6 - Experimental

6.1 General details

Unless noted otherwise, commercially obtained reagents were used as purchased without further purification. Solvents for flash chromatography were distilled prior to use, or used as purchased for HPLC grade, with the eluent mixture reported as the volume/volume ratio (v/v). Flash chromatography was performed using Merck Kieselgel 60 (230–400 mesh) silica gel. Analytical thin-layer chromatography (TLC) was performed using Merck aluminum-backed silica gel 60 F254 (0.2 mm) plates (Merck, Darmstadt, Germany), which were visualised using shortwave (254 nm) ultraviolet fluorescence. Melting points were measured with a rate of 5 °C/min and are uncorrected. Infrared absorption spectra were reported as vibrational frequency (cm⁻¹). Nuclear magnetic resonance spectra were recorded at 300 K on Bruker Advance DRX200, DRX300, DRX400 or DRX500 spectrophotometers. The data are reported as chemical shift (δ ppm) relative to the residual protonated solvent resonance (CDCl₃: δ 7.26, d-DMSO: δ 2.50, d-MeOD: δ 3.31, D₂O: δ 4.79 and d-acetone: δ 2.05), the carbon of the solvent resonance (CDCl₃: δ 77.16, d-DMSO: δ 39.52, d-MeOD: δ 49.00 and d-acetone: δ 29.84/206.26), relative integral, multiplicity (s = singlet, br.s = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, etc.) and coupling constants (J Hz). Low-resolution mass spectra (LRMS) was obtained from a ThermoQuest Finnigan LCQ Deca ion trap mass spectrometer with electro-spray ionisation in either positive (+ESI) or negative (-ESI) mode. Data is expressed as observed mass (m/z), assignment (M = molecular ion), and relative intensity (%). High-resolution mass spectra (HRMS) was performed on a Bruker Apex Qe 7T Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer equipped with an Apollo II ESI dual source. Samples were run with syringe infusion at 150 µL/hr on a Cole Palmer syringe pump into electrospray ionization (ESI). Atmospheric pressure chemical ionization (APCI) was performed by utilising MeOH. High performance liquid chromatography (HPLC) analysis of organic purity was conducted on a Waters Alliance 2695 instrument using a SunFire™ C18 column (5 µm, 2.1 x 150 mm) and detected using a Waters 2996 photodiode array (PDA) detector set at 254 nm. Separation was achieved using water (solvent A) and acetonitrile (solvent B) at flow rate of 0.2 mL/min and a gradient of
Chapter 6

0% B to 100% (HPLC Method A) or 0% B to 80% (HPLC Method B) or 0% B to 40% (HPLC Method C) over 30 min. HPLC data is reported as percentage purity and retention time (RT) in minutes.

6.2 Synthesis of compounds from Chapter 2

**General procedure A:** synthesis of iodinated derivatives

![Chemical structure](image)

The compounds were prepared following a literature procedure.\(^{232}\) **Step 1:** To a solution of indole-based compound (1.0 equiv.) in CH\(_2\)Cl\(_2\) (0.05 M) was added KOH (0.5 equiv.) at RT. After 30 min, NIS (1.0 equiv.) was added, and the mixture was stirred for 10 h, quenched with sat. Na\(_2\)S\(_2\)O\(_3\) (aq.) and extracted with CH\(_2\)Cl\(_2\). The combined organic layers were dried over MgSO\(_4\) and concentrated \textit{in vacuo}. The crude product was used in the next step without further purification.

**Step 2:** To a solution of the iodinated intermediate or 5-bromo-7-azaindole or cyanated intermediate (1.0 equiv.) in CH\(_2\)Cl\(_2\) (0.05 M) was added NaH (60% in mineral oil, 3.0 equiv.) and benzyltriethylammonium chloride (0.02 equiv.) under nitrogen at 0 °C. After 30 min, TsCl (1.2 equiv.) was added at 0 °C, and the mixture was allowed to warm to RT. After 2 h, H\(_2\)O was added and extracted with CH\(_2\)Cl\(_2\). The combined organic layers were dried over MgSO\(_4\) and concentrated \textit{in vacuo}.

**5-bromo-3-iodo-1-tosyl-1H-pyrrolo[2,3-b]pyridine (26a)**

![Chemical structure](image)

This compound was prepared according to **general procedure A** by treating 25a (1.97 g, 10 mmol) with NIS (2.25 g, 10 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 15:1 → 10:1) to give the product as a white solid (3.86 g, 81% over two steps). **m.p.** 186–187 °C; \(R_f\) (hexane/ethyl acetate...
10:1): 0.65; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.44 (1H, d, $J$ = 2.1 Hz), 8.06–8.04 (2H, m), 7.86 (1H, s), 7.78 (1H, d, $J$ = 2.1 Hz), 7.29 (2H, d, $J$ = 8.1 Hz), 2.37 (3H, s); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 146.7, 146.0, 144.7, 134.7, 132.5, 131.4, 130.0, 128.4, 126.7, 116.0, 60.4, 21.8; LRMS (+ESI): $m/z$ 499/501 (100/97, [M + Na]$^+$). IR (neat, cm$^{-1}$): 3138, 1619, 1371, 1167, 1140, 1015, 527. The spectroscopic data matched that reported in the literature.$^{273}$

**5-bromo-3-iodo-1-tosyl-1H-indole (26b)**

![Structure of 5-bromo-3-iodo-1-tosyl-1H-indole (26b)](image)

This compound was prepared according to general procedure A by treating 25b (1.96 g, 10 mmol) with NIS (2.25 g, 10 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 20:1 → 10:1) to give the product as a white solid (4.24 g, 89% over two steps). $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 7.83 (1H, d, $J$ = 8.7 Hz), 7.75 (2H, d, $J$ = 8.4 Hz), 7.68 (1H, s), 7.51 (1H, d, $J$ = 1.7 Hz), 7.45 (1H, dd, $J$ = 1.9, 8.7 Hz), 7.25 (2H, d, $J$ = 8.0 Hz), 2.36 (3H, s); $^{13}$C NMR (50 MHz, CDCl$_3$): $\delta$ 145.8, 134.8, 134.4, 133.3, 131.1, 130.3, 128.8, 127.1, 125.0, 117.7, 115.0, 65.5, 21.8. The spectroscopic data matched that reported in the literature.$^{300}$

**5-bromo-3-iodo-1-tosyl-1H-indazole (26c)**

![Structure of 5-bromo-3-iodo-1-tosyl-1H-indazole (26c)](image)

This compound was prepared according to general procedure A by treating 25c (1.97 g, 10 mmol) with NIS (2.25 g, 10 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 20:1 → 10:1) to give the product as an off-white solid (3.82 g, 80% over two steps). m.p. 160–161 °C; R$_f$ (hexane/ethyl acetate 12:1): 0.50; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.05 (1H, dd, $J$ = 0.5, 8.9 Hz), 7.86 (2H, dd, $J$ = 1.7, 6.7 Hz), 7.69 (1H, dd, $J$ = 1.8, 8.9 Hz), 7.60 (1H, d, $J$ = 1.6 Hz), 7.27 (2H, d, $J$ = 8.1 Hz), 2.38 (3H, s); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 146.2, 139.2, 134.1, 133.5, 131.9, 130.2, 127.9, 125.0, 118.2, 114.8, 102.4, 21.8; HRMS (ESI+)

IR (neat, cm⁻¹): v 3102, 3071, 2923, 1593, 1373, 1238, 663, 531.

**General procedure B for di-Suzuki Coupling Reaction**

To a solution of 26a–c (1.0 equiv.) in toluene/ethanol 3:1 (0.02 M) was added (4-methoxyphenyl)boronic acid (2.0 equiv.), aq. K₂CO₃ (2 M, 4.0 equiv.) and Pd(PPh₃)₄ (4 mol%), and the reaction was heated to 110 °C for 5 h under argon. The reaction mixture was cooled to RT, concentrated *in vacuo*, and then partitioned between H₂O and ethyl acetate. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were dried over MgSO₄ and concentrated *in vacuo*. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 3:1) to give the product.

**3,5-bis(4-methoxyphenyl)-1-tosyl-1H-pyrrolo[2,3-b]pyridine (27a)**

This compound was prepared according to general procedure B by treating 26a (2.38 g, 5 mmol) with (4-methoxyphenyl)boronic acid (1.51 g, 10 mmol) to give the product as a white solid (2.10 g, 87%). m.p. 158–160 °C; R_f (hexane/ethyl acetate 2:1): 0.45; ¹H NMR (400 MHz, CDCl₃): δ 8.64 (1H, d, J = 2.1 Hz), 8.14 (2H, d, J = 2.0 Hz), 8.12 (1H, d, J = 1.8 Hz), 7.81 (1H, s), 7.54 (2H, dd, J = 2.2, 6.7 Hz), 7.49 (2H, dd, J = 2.2, 6.7 Hz), 7.29 (2H, dd, J = 0.6, 8.6 Hz), 7.01 (4H, dt, J = 2.2, 8.8 Hz), 3.87 (3H, s), 3.85 (3H, s), 2.38 (3H, s); ¹³C NMR (100 MHz, CDCl₃): δ 159.6, 159.5, 146.8, 145.3, 144.2, 135.7, 132.7, 131.1, 129.8, 128.9, 128.7, 128.2, 126.8, 125.3,
122.7, 122.0, 120.4, 114.8, 114.7, 55.5 (two overlapping signals), 21.8; **HRMS** (ESI+) Calc. for C_{28}H_{24}N_{2}O_{4}S [M + H]^+ 485.1529, found 485.1525. **IR** (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 2920, 2836, 1596, 1381, 1173, 1157, 577.

3,5-bis(4-methoxyphenyl)-1-tosyl-1H-indole (27b)

![Diagram of 3,5-bis(4-methoxyphenyl)-1-tosyl-1H-indole (27b)]

This compound was prepared according to **general procedure B** by treating 26b (2.38 g, 5 mmol) with (4-methoxyphenyl)boronic acid (1.51 g, 10 mmol) to give the product as a white solid (2.08 g, 86%). **m.p.** 168–170 °C; **Rf** (hexane/ethyl acetate 2:1): 0.45; **\(^1\)H NMR** (500 MHz, CDCl\(_3\)): \(\delta\) 8.08 (1H, d, \(J = 10.4\) Hz), 7.86 (1H, s), 7.83 (2H, d, \(J = 8.0\) Hz), 7.64 (1H, s), 7.56–7.54 (3H, m), 7.51 (2H, d, \(J = 8.2\) Hz), 7.23 (2H, d, \(J = 8.0\) Hz), 7.02 (2H, d, \(J = 8.2\) Hz), 6.97 (2H, d, \(J = 8.2\) Hz), 3.86 (3H, s), 3.84 (3H, s), 2.34 (3H, s); **\(^{13}\)C NMR** (125 MHz, CDCl\(_3\)): \(\delta\) 159.3, 159.1, 145.1, 136.9, 135.3, 134.6, 134.0, 130.2, 130.0, 129.2, 128.5, 127.0, 125.6, 124.3, 124.1, 123.0, 118.5, 114.5, 114.3, 114.1, 55.5, 55.4, 21.7; **HRMS** (ESI+) Calc. for C_{29}H_{25}N_{2}O_{4}S [M + Na]^+ 506.1396, found 506.1394. **IR** (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 2954, 2932, 2834, 1609, 1367, 1170, 835, 576.

3,5-bis(4-methoxyphenyl)-1-tosyl-1H-indazole (27c)

![Diagram of 3,5-bis(4-methoxyphenyl)-1-tosyl-1H-indazole (27c)]

This compound was prepared according to **general procedure B** by treating 26c (2.38 g, 5 mmol) with (4-methoxyphenyl)boronic acid (1.51 g, 10 mmol) to give the product as a white solid (2.03 g, 84%). **m.p.** 115–117 °C; **Rf** (hexane/ethyl acetate 2:1): 0.45; **\(^1\)H NMR** (400 MHz, CDCl\(_3\)): \(\delta\) 8.27 (1H, dd, \(J = 0.6, 8.8\) Hz), 7.98 (1H,
dd, $J = 0.7, 1.6$ Hz), 7.92 (2H, dd, $J = 1.7, 6.7$ Hz), 7.89 (2H, dd, $J = 2.1, 6.8$ Hz), 7.75 (1H, dd, $J = 1.7, 8.8$ Hz), 7.53 (2H, dd, $J = 2.1, 6.7$ Hz), 7.23 (2H, d, $J = 8.0$ Hz), 7.01 (4H, ddd, $J = 2.1, 6.8, 12.4$ Hz), 3.87 (3H, s), 3.85 (3H, s), 2.34 (3H, s);

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 160.9, 159.5, 151.9, 145.3, 141.1, 137.9, 134.8, 133.2, 129.9, 129.7, 128.7, 128.6, 127.7, 125.3, 124.1, 119.3, 114.5, 114.4, 113.9, 55.5 (two overlapping signals), 21.7; HRMS (ESI+) Calc. for C$_{38}$H$_{24}$N$_2$O$_4$S [M + H]$^+$ 485.1529, found 485.1527. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 2934, 2836, 1609, 1303, 1274, 837, 589, 578.

**General procedure C** for the deprotection of tosyl group

![General procedure C](image)

To a solution of tosyl protected substrate (1.0 equiv.) in MeOH (0.02 M) was added KOH (5.0 equiv.), and the reaction was heated to 70 °C for 2 h. After completion monitored by TLC, the solvent was removed under reduced pressure, and then partitioned between H$_2$O and ethyl acetate. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were dried over MgSO$_4$ and concentrated in vacuo.

3,5-bis(4-methoxyphenyl)-1H-pyrrolo[2,3-b]pyridine (28a)

![3,5-bis(4-methoxyphenyl)-1H-pyrrolo[2,3-b]pyridine](image)

This compound was prepared according to **general procedure C** by treating 27a (2.00 g, 4 mmol) with KOH (1.12 g, 20 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as a yellow solid (1.25 g, 95%). m.p. 193–194 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.35; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 11.50 (1H, s), 8.53 (1H, s), 8.37 (1H, d, $J = 1.6$ Hz), 7.59–7.51 (5H, m), 7.03 (4H, d, $J = 8.6$ Hz), 3.87 (6H, s); $^{13}$C NMR (75 MHz,
CDCl₃): δ 159.4, 158.6, 147.3, 140.2, 131.6, 129.9, 128.6, 128.5, 127.6, 127.2, 123.0, 119.8, 116.7, 114.6 (two overlapping signals), 55.5 (two overlapping signals). The spectroscopic data matched that reported in the literature. 232

3,5-bis(4-methoxyphenyl)-1H-indole (28b)

This compound was prepared according to general procedure C by treating 27b (1.45 g, 3 mmol) with KOH (0.84 g, 15 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as a yellow solid (938 mg, 95%). m.p. 153–154 °C; Rf (hexane/ethyl acetate 1:1): 0.35; ¹H NMR (400 MHz, d-acetone): δ 10.39 (1H, bs), 8.03 (1H, t, J = 1.0 Hz), 7.67 (2H, dd, J = 2.2, 6.6 Hz), 7.61 (2H, dd, J = 2.2, 6.6 Hz), 7.53–7.51 (2H, m), 7.42 (1H, dd, J = 1.8, 8.4 Hz), 7.04 (1H, d, J = 2.2 Hz), 7.02 (2H, t, J = 2.5 Hz), 7.00 (1H, d, J = 2.2 Hz), 3.84 (3H, s), 3.83 (3H, s); ¹³C NMR (100 MHz, d-acetone): δ 159.6, 159.0, 137.4, 136.2, 133.7, 129.5, 129.1, 128.9, 127.3, 123.6, 122.0, 118.1, 117.9, 115.1, 115.0, 112.9, 55.6, 55.5; HRMS (ESI+) Calc. for C₂₂H₁₉NO₂ [M + Na]⁺ 352.1308, found 352.1307. IR (neat, cm⁻¹): ν 3401, 2953, 2834, 1503, 1273, 837.

3,5-bis(4-methoxyphenyl)-1H-indazole (28c)

This compound was prepared according to general procedure C by treating 27e (2.0 g, 4 mmol) with KOH (1.12 g, 20 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as a pale yellow solid (1.20 g, 91%). m.p. 180–181 °C; Rf (hexane/ethyl acetate 2:1): 0.50; ¹H NMR (500 MHz, d-DMSO): δ 13.13 (1H, s), 8.12 (1H, s), 7.98 (2H, d, J = 8.7 Hz), 7.66
(2H, d, J = 8.6 Hz), 7.63 (2H, d, J = 5.9 Hz), 7.10 (2H, d, J = 8.7 Hz), 7.03 (2H, d, J = 8.6 Hz), 3.83 (3H, s), 3.80 (3H, s); $^{13}$C NMR (125 MHz, d-DMSO): δ 158.9, 158.5, 143.5, 140.7, 133.4, 133.2, 128.2, 128.1, 126.3, 125.6, 120.6, 117.6, 114.3, 114.3, 110.9, 55.1 (two overlapping signals); HRMS (ESI+) Calc. for C$_{21}$H$_{18}$N$_2$O$_2$ [M + H]$^+$ 331.1441, found 331.1440. IR (neat, cm$^{-1}$): ν 3128, 3024, 2906, 1611, 1243, 804.

**General procedure D for demethylation**

![Chemical structure of 4,4'-((1H-pyrrolo[2,3-b]pyridine-3,5-diyldiphenol (20a))](image)

To a solution of methylated substrates (1.0 equiv.) in dry CH$_2$Cl$_2$ (0.05 M) was added BBr$_3$ (1 M, 6.0 equiv.) under nitrogen. The reaction mixture was allowed to warm to room temperature for 10 h, then quenched at 0 °C with MeOH and concentrated in vacuo.

4,4'-((1H-pyrrolo[2,3-b]pyridine-3,5-diyldiphenol (20a)

This compound was prepared according to general procedure D by treating 28a (330 mg, 1 mmol) with BBr$_3$ (6 mL, 6 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 100:1 → 50:1) to give the product as a pale yellow solid (196 mg, 65%). m.p. 156–158 °C; R$_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.45; $^1$H NMR (500 MHz, d-DMSO): δ 11.83 (1H, s), 9.69–9.23 (1H, br s), 8.47 (1H, d, J = 2.0 Hz), 8.30 (1H, d, J = 2.0 Hz), 7.71 (1H, d, J = 2.5 Hz), 7.56 (4H, dd, J = 0.9, 8.6 Hz), 6.87 (4H, dq, J = 2.0, 8.6 Hz), one OH signal not observed; $^{13}$C NMR (125 MHz, d-DMSO): δ 156.8, 155.7, 147.2, 140.5, 129.5, 128.7, 128.2, 127.7, 125.6, 125.3, 123.2, 117.9, 115.8, 115.7, 115.0; HRMS (ESI+) Calc. for C$_{19}$H$_{14}$N$_2$O$_2$ [M + H]$^+$ 303.1128, found 303.1130. IR (neat, cm$^{-1}$): ν 3107, 2943, 1611, 1249. HPLC: 98.9% (HPLC Method A), RT: 16.4 min.
4,4’-(1H-indole-3,5-diyldiphenol (20b)

This compound was prepared according to general procedure D by treating 28b (329 mg, 1 mmol) with BBr₃ (6 mL, 6 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 50:1) to give the product as a white solid (181 mg, 60%). m.p. 246–248 °C; Rᵣ (CH₂Cl₂/MeOH 20:1): 0.50; ¹H NMR (500 MHz, d-MeOD): δ 7.90 (1H, d, J = 1.0 Hz), 7.48 (4H, dd, J = 8.6, 23.2 Hz), 7.42 (1H, d, J = 8.5 Hz), 7.35–7.33 (2H, m), 6.86 (4H, dd, J = 8.5, 14.8 Hz), NH and OH signals not observed; ¹³C NMR (125 MHz, d-MeOD): δ 157.2, 156.5, 137.6, 135.8, 134.2, 129.4, 129.1, 129.0, 127.6, 123.1, 122.0, 118.6, 117.9, 116.5, 116.4, 112.6; HRMS (ESI+) Calc. for C₂₀H₁₅NO₂ [M + Na]⁺ 324.0995, found 324.0998. IR (neat, cm⁻¹): ν 3402, 3022, 1514, 1219, 1170, 798. HPLC: >99.9% (HPLC Method A), RT: 21.7 min.

4,4’-(1H-indazole-3,5-diyldiphenol (20c)

This compound was prepared according to general procedure D by treating 28c (330 mg, 1 mmol) with BBr₃ (6 mL, 6 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 50:1) to give the product as a white solid (175 mg, 58%). m.p. 251–252 °C; Rᵣ (CH₂Cl₂/MeOH 20:1): 0.50; ¹H NMR (500 MHz, d-MeOD): δ 8.11 (1H, s), 7.97 (1H, d, J = 8.7 Hz), 7.82 (2H, d, J = 7.4 Hz), 7.73 (1H, d, J = 8.7 Hz), 7.51 (2H, d, J = 7.4 Hz), 7.06 (2H, d, J = 7.4 Hz), 6.89 (2H, d, J = 7.2 Hz), NH and OH signals not observed; ¹³C NMR (125 MHz, d-MeOD): δ 161.6, 158.7, 144.6, 141.4, 139.2, 133.2, 132.6, 131.1, 129.5, 120.3, 119.6, 119.5,
117.7, 116.9, 112.9; **HRMS** (ESI+) Calc. for C_{19}H_{14}N_{2}O_{2} [M + H]^+ 303.1128, found 303.1131. **IR** (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3343, 2980, 1609, 1497, 1263, 1182, 809, 507. **HPLC**: 98.8% (HPLC Method A), RT: 19.9 min.

4-bromo-2-fluoroaniline (32)

This compound was prepared following a literature procedure.\(^{301}\) To a suspension of 2-fluoroaniline 31 (2.22 g, 20 mmol) in CHCl\(_3\) (50 mL) was added NBS (3.56 g, 20 mmol), and the resulting mixture was stirred at RT for 2 h. After completion monitored by TLC, the mixture was quenched with sat. Na\(_2\)S\(_2\)O\(_3\) (aq.) and extracted with CH\(_2\)Cl\(_2\) (3 x 20 mL). The combined organic layers were dried over MgSO\(_4\) and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as orange oil (3.42 g, 90%); \(R_f\) (hexane/ethyl acetate 6:1): 0.35; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.14 (1H, dd, \(J = 2.1, 10.5\) Hz), 7.05 (1H, d, \(J = 8.4\) Hz), 6.65 (1H, t, \(J = 9.0\) Hz), 3.72 (2H, br.s); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 151.5 (d, \(J_{CF} = 241.5\) Hz), 133.9 (d, \(J_{CF} = 12.8\) Hz), 127.5 (d, \(J_{CF} = 3.0\) Hz), 118.8 (d, \(J_{CF} = 21.8\) Hz), 117.9 (d, \(J_{CF} = 3.8\) Hz), 109.0 (d, \(J_{CF} = 9.0\) Hz). The spectroscopic data matched that reported in the literature.\(^{301}\)

4-bromo-2-fluoro-1-nitrobenzene (33)

This compound was prepared following a literature procedure.\(^{301}\) To a solution of 32 (1.26 g, 6.6 mmol) in TFA (13 mL) was added aq. H\(_2\)O\(_2\) (30%, 3.4 mL, 33 mmol) dropwise over 30 min, and the resulting mixture was stirred at 75 °C for 1 h. After completion monitored by TLC, the mixture was poured into ice (20 mL), the pale yellow precipitate was obtained and filtered, washed by cold water and dried under reduced pressure to get the desired compound without further purification (1.09g, 75%). \(R_f\) (hexane/ethyl acetate 10:1): 0.45; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.97 (1H, t, \(J = 8.0\)Hz), 7.50 (1H, dd, \(J = 2.0, 10.0\) Hz), 7.47–7.44 (1H, m); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 155.4 (d, \(J_{CF} = 268\) Hz), 136.4, 129.4 (d, \(J_{CF} = 9.0\) Hz), 128.1
(d, $J_{CF} = 4.0$ Hz), 127.1 (d, $J_{CF} = 2.0$ Hz), 122.1 (d, $J_{CF} = 24.0$ Hz). The spectroscopic data matched that reported in the literature.\textsuperscript{301}

**General procedure E for the TBS protection**

The compounds could be prepared according to the literature.\textsuperscript{302} To a solution of hydroxy substrate (1.0 equiv.) in dry DMF (0.05 M) was added imidazole (2.5 or 5.0 equiv.) at 0 °C, followed by the addition of TBSCl (1.5 or 3.5 equiv.). The reaction mixture was allowed to warm to RT for 12 h. After completion monitored by TLC, the mixture was extracted with ethyl acetate (3 x 30 mL) and H$_2$O (50 mL), the organic layers were dried over MgSO$_4$ and concentrated \textit{in vacuo}.

4-((\textit{tert}-butyldimethylsilyl)oxy)aniline (34a)

This compound was prepared according to general procedure E by treating 4-aminophenol (2.18 g, 0.02 mol), imidazole (3.2 g, 0.05 mol) and TBSCl (4.53 g, 0.03 mol). The residue was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as pale yellow oil (4.0 g, 87%). $R_f$ (hexane/ethyl acetate 2:1): 0.45; $^1$H NMR (300 MHz, d-DMSO): $\delta$ 6.53 (2H, d, $J = 8.7$ Hz), 8.46 (2H, d, $J = 8.7$ Hz), 4.59 (2H, s), 0.93 (9H, s), 0.11 (6H, s); $^{13}$C NMR (75 MHz, d-DMSO): $\delta$ 145.5, 142.8, 119.9, 114.9, 25.6, 17.8, −4.6. The spectroscopic data matched that reported in the literature.\textsuperscript{303}

4-(5-bromo-2-nitrophenox)aniline (35b)

To a solution of 33 (220 mg, 1 mmol) in DMF (10 mL) was added K$_2$CO$_3$ (207 mg, 1.5 mmol), followed by the addition of 4-((\textit{tert}-butyldimethylsilyl)oxy)aniline 34a (245 mg, 1.1 mmol). The resulting mixture was heated to 90 °C and stirred for 3 h. After completion monitored by TLC, the mixture was extracted with ethyl acetate (3 x
10 mL) and H$_2$O. The combined organic layers were dried over MgSO$_4$ and concentrated *in vacuo*. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 2:1) to give the product as a pale yellow semisolid (247 mg, 80%); $R_f$ (hexane/ethyl acetate 4:1): 0.25; $^1$H NMR (400 MHz, $d$-DMSO): $\delta$ 7.96 (1H, $d$, $J = 8.8$ Hz), 7.42 (1H, $dd$, $J = 2.0, 8.8$ Hz), 6.96 (1H, $d$, $J = 2.0$ Hz), 6.88 (2H, $dd$, $J = 2.0, 10.8$ Hz), 6.65 (2H, $dd$, $J = 2.0, 10.8$ Hz), 5.26 (2H, br.$s$); $^{13}$C NMR (100 MHz, $d$-DMSO): $\delta$ 152.6, 146.7, 143.6, 138.8, 127.4, 127.2, 125.1, 121.1, 120.3, 115.0; HRMS (ESI+) Calc. for C$_{12}$H$_9$N$_2$O$_3$Br $[M + Na]^+$ 330.9689/332.9668, found 330.9691/332.9670. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3475, 3385, 3098, 3085, 1615, 1505, 1466, 1231, 824, 528.

4-((5-bromo-2-nitrophenyl)amino)phenol (35c)

To a solution of 33 (220 mg, 1 mmol) in dry DMF (10 mL) was added K$_2$CO$_3$ (207 mg, 1.5 mmol), followed by the addition of 4-aminophenol (120 mg, 1.1 mmol). The resulting mixture was heated to 90 °C and stirred for 3 h. After completion monitored by TLC, the mixture was extracted with ethyl acetate (3 x 10 mL) and H$_2$O. The combined organic layer was dried over MgSO$_4$ and concentrated *in vacuo*. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 4:1) to give the product as a pale orange solid (201 mg, 65%); m.p. 151–152 °C; $R_f$ (hexane/ethyl acetate 4:1): 0.30; $^1$H NMR (300 MHz, $d$-DMSO): $\delta$ 9.65 (1H, br.$s$), 9.40 (1H, br.$s$), 8.02 (1H, $d$, $J = 9.0$ Hz), 7.14 (2H, $d$, $J = 8.1$ Hz), 6.92–6.84 (4H, m); $^{13}$C NMR (75 MHz, $d$-DMSO): $\delta$ 156.1, 145.0, 131.0, 130.2, 129.0, 128.2, 127.7, 119.4, 117.7, 116.3; HRMS (ESI+) Calc. for C$_{12}$H$_9$N$_2$O$_3$Br $[M + Na]^+$ 330.9689/332.9668, found 330.9692/332.9672. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3349, 3089, 1602, 1556, 1475, 1241, 1198, 747, 531.
4-((2-amino-5-bromophenyl)amino)phenol (36)

To a solution of 35c (308 mg, 1 mmol) in acetic acid (15 mL) was added iron powder (280 mg, 5 mmol), and the resulting mixture was stirred at RT for 12 h. After completion monitored by TLC, the volatile was removed under reduced pressure and the residue was washed with sat. NaHCO$_3$ (aq.) and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 100:1) to give the product as a brown solid (265 mg, 95%); m.p. 145–147 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.25; $^1$H NMR (400 MHz, d-DMSO): $\delta$ 6.91 (1H, dd, $J$ = 2.4, 8.4 Hz), 6.75 (2H, dd, $J$ = 2.4, 6.8 Hz), 6.68 (1H, d, $J$ = 8.4 Hz), 6.59 (2H, dd, $J$ = 2.4, 6.8 Hz), 6.55 (1H, d, $J$ = 2.4 Hz), 5.08 (2H, br.s), 4.97 (1H, br.s); $^{13}$C NMR (100 MHz, d-DMSO): $\delta$ 146.1, 145.7, 145.3, 138.4, 125.1, 120.2, 118.4, 116.2, 114.8, 105.5; HRMS (ESI+) Calc. for C$_{12}$H$_{11}$BrN$_2$O [M + H]$^+$ 279.0128/281.0107, found 279.0125/281.0104. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3500, 3400, 2896, 1610, 1598, 1510, 1220, 756, 520.

4-(2-amino-6-bromo-1H-benzo[d]imidazol-1-yl)phenol (37)

This compound was prepared following a literature procedure.$^{237}$ To a solution of 36 (200 mg, 0.72 mmol) in the mixture solvent of MeCN-H$_2$O (4:1 (v/v), 10 mL) was added cyanogen bromide (158 µL, 0.79 mmol, 5 M in MeCN) at 0 °C. and the resulting mixture was allowed to warm to RT for 16 h. After completion monitored by TLC, the mixture was treated with sat. NaHCO$_3$ (aq.) (10 mL) and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over MgSO$_4$ and concentrated in vacuo. The residue was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 100:1 → 20:1) to give the product as a pale pink solid (171 mg, 78%); m.p. 273–274 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 15:1): 0.20; $^1$H NMR (400 MHz, d-
DMSO): δ 9.98 (1H, s), 7.24 (2H, dd, J = 2.0, 6.4 Hz), 7.14 (1H, dd, J = 0.4, 8.4 Hz), 7.11 (1H, dd, J = 1.6, 8.4 Hz), 6.96 (2H, dd, J = 2.0, 6.4 Hz), 6.81 (1H, dd, J = 0.4, 1.6 Hz), 6.35 (2H, s); $^{13}$C NMR (100 MHz, $d$-DMSO): δ 157.8, 155.3, 141.6, 136.8, 128.5, 125.0, 123.8, 116.8, 116.4, 110.4, 110.3; HRMS (ESI+) Calc. for C$_{13}$H$_{10}$BrN$_3$O [M + H]$^+$: 304.0080/304.0059, found 304.0083/306.0062. IR (neat, cm$^{-1}$): v 3330, 3205, 1647, 1514, 1449, 1272, 1247, 1122, 800, 727, 586, 432.

**General procedure F for mono-Suzuki Coupling Reaction**

![Diagram of the Suzuki coupling reaction](image)

To a solution of aryl-halide (1.0 equiv.) in toluene/ethanol 3:1 or 1,4-dioxane (0.02 M) was added arylboronic acid (1.2 equiv.), aq. K$_2$CO$_3$ (2 M, 2.0 equiv.), and Pd(PPh$_3$)$_4$ (2 mol%), and the reaction was heated to 90–110 °C for 4 h under argon. After completion monitored by TLC, the reaction mixture was cooled to RT, solvent was removed under reduce pressure, and then partitioned between H$_2$O and ethyl acetate. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were dried over MgSO$_4$ and concentrated *in vacuo*.

**4,4’-(2-amino-1H-benzo[d]imidazole-1,6-diyl)diphenol (29)**

![Structure of 4,4’-(2-amino-1H-benzo[d]imidazole-1,6-diyl)diphenol (29)](image)

This compound was prepared according to general procedure F by treating 37 (61 mg, 0.2 mmol) with (4-hydroxyphenyl)boronic acid (33 mg, 0.24 mmol) in 1,4-dioxane at 110 °C. The residue was purified by flash column chromatography (CH$_2$Cl$_2$: MeOH = 50:1 → 10:1) to afford the product as a pale yellow solid (10 mg, 30%). m.p. 249–250 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 10:1): 0.35; $^1$H NMR (400 MHz, $d$-DMSO): δ 9.85 (1H, br.s), 9.36 (1H, s), 7.42 (2H, dd, J = 2.0, 6.4 Hz), 7.35 (1H, d, J = 1.6 Hz), 7.26 (2H, dd, J = 2.0, 6.8 Hz), 7.06 (1H, dd, J = 1.6, 8.0 Hz), 6.97 (2H, dd, J = 2.0, 6.8 Hz), 6.82 (2H, dd, J = 2.0, 6.4 Hz), 6.79 (1H, d, J = 8.0 Hz), 6.14 (2H,
N-(1,6-bis(4-hydroxyphenyl)-1H-benzo[d]imidazol-2-yl)acetamide (30)

To a solution of 29 (30 mg, 0.10 mmol) in acetic acid (1 mL) was added acetic anhydride (10 µL, 0.11 mmol) at RT. And the resulting mixture was stirred at 110 °C for 3 h. After completion monitored by TLC, the solvent was removed under reduced pressure and the residue was neutralised with sat. NaHCO₃ (aq.) and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (CH₂Cl₂: MeOH = 50:1 → 10:1) to afford the product as a pale yellow solid (25 mg, 38%). m.p. 207–209 °C; Rf (CH₂Cl₂/MeOH 10:1): 0.25; ¹H NMR (400 MHz, d-DMSO): δ 10.28 (1H, br.s), 9.86 (1H, s), 9.45 (1H, s), 7.77 (1H, s), 7.50 (2H, d, J = 8.4 Hz), 7.43 (1H, d, J = 8.0 Hz), 7.24 (2H, d, J = 8.0 Hz), 7.16 (1H, d, J = 8.4 Hz), 6.93 (2H, d, J = 8.4 Hz), 6.85 (2H, d, J = 8.4 Hz), 1.95 (3H, s); ¹³C NMR (100 MHz, d-DMSO): δ 170.3, 157.9, 157.1, 141.7, 141.6, 135.6, 134.6, 132.2, 128.3, 128.0, 126.5, 122.0, 116.5, 116.4, 116.2, 110.7, 23.0; HRMS (ESI+) Calc. for C₂₁H₁₇N₅O₃ [M + H]⁺ 360.1343, found 360.1346. IR (neat, cm⁻¹): ν 3274, 2953, 2921, 2852, 1587, 1550, 1513, 1366, 1203, 834, 808, 560. HPLC: 96.6% (HPLC Method A), RT: 16.6 min.
6-bromo-1H-benzo[d]imidazol-2-amine (39)

This compound was prepared following a literature procedure.\textsuperscript{237} To a solution of 4-bromobenzene-1,2-diamine 38 (206 mg, 1.1 mmol) in the mixture solvent of MeCN-H\textsubscript{2}O (4:1 (v/v), 10 mL) was added cyanogen bromide (240 \(\mu\)L, 1.2 mmol, 5M in MeCN) at 0 °C. and the resulting mixture was allowed to warm to RT for 16 h. After completion monitored by TLC, the mixture was treated with sat. NaHCO\textsubscript{3} (aq.) (10 mL) and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over MgSO\textsubscript{4} and concentrated \textit{in vacuo}. The crude product was purified by flash chromatography (CH\textsubscript{2}Cl\textsubscript{2}/MeOH 100:1 \(\rightarrow\) 10:1) to give the product as a tannish solid (186 mg, 80%); \(R_f\) (CH\textsubscript{2}Cl\textsubscript{2}/MeOH 10:1): 0.20; \textsuperscript{1}H NMR (400 MHz, \textit{d}-DMSO): \(\delta\) 7.24 (1H, d, \(J = 2.0\) Hz), 7.04 (1H, d, \(J = 8.4\) Hz), 6.97 (1H, dd, \(J = 2.0, 8.4\) Hz), 6.39 (2H, br.s); \textsuperscript{13}C NMR (100 MHz, \textit{d}-DMSO): \(\delta\) 156.2, 141.2, 137.1, 121.3, 114.4, 112.5, 111.1. The spectroscopic data matched that reported in the literature.\textsuperscript{304}

(4-((\textit{tert}-butyldimethylsilyl)oxy)phenyl)boronic acid (40)

This compound was prepared according to general procedure E by treating (4-hydroxyphenyl)boronic acid (1.38 g, 0.01 mol) with imidazole (3.2 g, 0.05 mol) and TBSCI (5.29 g, 0.035 mol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 \(\rightarrow\) 3:1) to give the product as a white solid (2.27 g, 90%). \(R_f\) (hexane/ethyl acetate 1:1): 0.30; \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 8.11 (2H, d, \(J = 8.4\) Hz), 6.96 (2H, d, \(J = 8.4\) Hz), 1.02 (9H, s), 0.26 (6H, s); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}): \(\delta\) 159.9, 137.6, 119.9, 111.0, 25.8, 18.4, –4.2. The spectroscopic data matched that reported in the literature.\textsuperscript{305}
5-bromo-1-(4-((tert-butyldimethylsilyl)oxy)phenyl)-1H-benzo[d]imidazol-2-amine (41a)

This compound was prepared following a literature procedure. To a solution of 39 (106 mg, 0.50 mmol) in MeOH (5 mL) was added (4-((tert-butyldimethylsilyl)oxy)phenyl)boronic acid 40 (139 mg, 0.55 mmol), followed by the addition of Cu(OAc)$_2$ at RT. and the resulting mixture was stirred at RT for 12 h. After completion monitored by TLC, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 200:1 → 50:1) to give the product as an off-white solid (71 mg, 34%); m.p. 198–199 °C; R$_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.45; $^1$H NMR (300 MHz, d-DMSO): δ 7.34–7.32 (3H, m), 7.04 (2H, d, $J = 7.8$ Hz), 6.99 (1H, d, $J = 8.1$ Hz), 6.72 (1H, d, $J = 8.1$ Hz), 6.43 (2H, br.s), 0.99 (9H, s), 0.26 (6H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 156.5, 154.2, 142.6, 134.1, 128.1, 126.9, 122.9, 121.7, 118.8, 114.8, 109.5, 25.6, 18.2, −4.4; HRMS (ESI+) Calc. for C$_{19}$H$_{24}$N$_3$O$_2$SiBr [M + H]$^+$ 418.0945/420.0924, found 418.0941/420.0922. IR (neat, cm$^{-1}$): ν 3359, 3060, 2955, 2856, 1654, 1510, 1268, 915, 840, 802, 519.

6-bromo-1-(4-((tert-butyldimethylsilyl)oxy)phenyl)-1H-benzo[d]imidazol-2-amine (41b)

Pale pink solid (106 mg, 51%); m.p. 194–196 °C; R$_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.40; $^1$H NMR (300 MHz, d-DMSO): δ 7.34 (2H, d, $J = 8.1$ Hz), 7.15–7.10 (2H, m), 7.05 (2H, d, $J = 8.1$ Hz), 6.84 (1H, s), 6.36 (2H, s), 1.00 (9H, s), 0.27 (6H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 156.6, 128.1, 128.0, 126.8, 125.1, 122.9, 121.8, 117.1, 112.7, 111.5,
109.6, 25.6, 18.2, –4.3. **HRMS (ESI+)** Calc. for C_{19}H_{24}N_{3}OSiBr [M + H]^+ 418.0945/420.0924, found 418.0942/420.0922. **IR** (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3467, 2954, 2928, 2856, 1644, 1607, 1508, 1255, 908, 837, 804, 513.

**General procedure G** for the deprotection of TBS ethers

The compounds were prepared following a literature procedure.\(^{256}\) To a solution of TBS-protected substrate (1.0 equiv.) in THF (0.05 M) was added TBAF (1 M, 2.0 equiv.). The resulting mixture was stirred at RT for 30 min. After completion monitored by TLC, the solvent was removed in vacuo.

4-(2-amino-6-bromo-1H-benzo[d]imidazol-1-yl)phenol (37)

This compound was prepared according to **general procedure G** by treating 41b (41.8 mg, 0.1 mmol) with TBAF (0.2 mL, 0.2 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 100:1 \(\rightarrow\) 20:1) to give the product as a pale pink solid (29.3 mg, 93%). The spectroscopic data matched that shown as above.

4-(2-amino-1-(4-((tert-butyldimethylsilyl)oxy)phenyl)-1H-benzo[d]imidazol-6-yl)phenol (42)

This compound was prepared according to **general procedure F** by treating 41b (41.8 mg, 0.1 mmol) and (4-hydroxyphenyl)boronic acid (16.6 mg, 0.12 mmol) in 1,4-dioxane at 110 °C. The crude product was purified by flash chromatography.
(CH$_2$Cl$_2$: MeOH = 50:1 → 20:1) to afford the product as an off-white solid (24 mg, 55%). m.p. 217–219 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 15:1): 0.35; $^1$H NMR (400 MHz, d-MeOD): $\delta$ 7.51–7.49 (3H, m), 7.32 (2H, d, $J = 8.8$ Hz), 7.21 (1H, d, $J = 8.4$ Hz), 7.04 (2H, d, $J = 8.4$ Hz), 6.92–6.90 (3H, m), 1.04 (9H, s), 0.25 (6H, s); $^{13}$C NMR (100 MHz, d-MeOD): $\delta$ 159.6, 156.3, 156.0, 142.6, 136.9, 136.7, 135.7, 129.6, 129.1, 127.0, 121.3, 120.3, 117.9, 113.9, 109.5, 26.2, 19.1, −4.3; HRMS (ESI+) Calc. for C$_{25}$H$_{29}$N$_3$O$_2$Si [M + H]$^+$ 432.2102, found 432.2099. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3483, 3327, 2953, 2925, 2854, 1631, 1606, 1513, 1470, 1253, 911, 837, 801, 779.

4,4′-(2-amino-1H-benzo[d]imidazole-1,6-diyl)diphenol (29)

This compound was prepared according to general procedure G by treating 42 (21.6 mg, 0.05 mmol) and TBAF (0.1 mL, 0.1 mmol). The residue was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 → 10:1) to give the product as a pale yellow solid (15 mg, 95%). The spectroscopic data matched that shown as above.

**General procedure H for condensation reactions**

The compounds were prepared following a literature procedure. To a solution of halogenated pyridine substrate (1.0 equiv.) in dry toluene (0.05 M) was added $n$-BuLi (1.1 equiv., 2.5 M in hexane) dropwise at −78 °C, and the resulting mixture was stirred for 30 min. $N$, $N$-dimethylacetamide or DMF (1.5 equiv.) was added dropwise and the mixture was stirred for another 30 min. After completion, the reaction was quenched with sat. NH$_4$Cl (aq.) and extracted with ethyl acetate (3 x 15 mL). The organic layer was dried over MgSO$_4$ and concentrated in vacuo.
1-(5-bromopyridin-2-yl)ethan-1-one (46a)

This compound was prepared according to **general procedure H** by treating 2,5-dibromopyridine 45 (237 mg, 1 mmol) with N, N-dimethylacetamide (44 µL, 1.5 mmol) in dry toluene. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as a white solid (160 mg, 80%); \( R_f \) (hexane/ethyl acetate 8:1): 0.45; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta \) 8.70 (1H, s), 7.95–7.88 (2H, m), 2.66 (3H, s); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \( \delta \) 199.2, 151.9, 150.2, 139.6, 125.4, 123.0, 25.8. The spectroscopic data matched that reported in the literature.\(^{306}\)

5-bromopicolinaldehyde (46b).

This compound was prepared according to **general procedure H** by treating 2,5-dibromopyridine 45 (237 mg, 1 mmol) with DMF (115 µL, 1.5 mmol) in dry toluene. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as an off-brown solid (149 mg, 80%); \( R_f \) (hexane/ethyl acetate 8:1): 0.45; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 10.03 (1H, s), 8.85 (1H, dd, \( J = 0.8, 2.0 \) Hz), 8.02 (1H, ddd, \( J = 0.8, 2.0, 8.0 \) Hz), 7.85 (1H, dd, \( J = 0.8, 8.0 \) Hz); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 192.4, 151.7, 151.3, 140.0, 126.3, 122.8. The spectroscopic data matched that reported in the literature.\(^{308}\)

**General procedure I for benzyl protection**

The compounds were prepared following a literature procedure.\(^{309}\) To a solution of hydroxyaryl substrate (1.0 equiv., 1 mmol) in DMF (0.05 M) was added K\(_2\)CO\(_3\) (1.5 equiv., 1.5 mmol), followed by the dropwise addition of benzyl bromide (1.2 equiv., 1.2 mmol) at RT, and the mixture was stirred at RT for 12 h. After completion
monitored by TLC, the solvent was removed under reduced pressure, and the residue was washed with H$_2$O (30 mL) and extracted with ethyl acetate (3 x 20 mL), the organic layers were dried over MgSO$_4$ and concentrated in vacuo.

**Methyl 4-(benzyloxy)benzoate (47a)**

![Methyl 4-(benzyloxy)benzoate](image)

This compound was prepared according to **general procedure I** by treating methyl 4-hydroxybenzoate (1.52 g, 0.01 mol) with K$_2$CO$_3$ (2.07 g, 0.015 mol) and benzyl bromide (1.42 ml, 0.012 mol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as a white solid (2.37 g, 98%); $R_f$ (hexane/ethyl acetate 4:1): 0.50; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.01 (2H, d, $J = 8.1$ Hz), 7.43–7.34 (5H, m), 6.98 (2H, d, $J = 8.1$ Hz), 5.08 (2H, s), 3.87 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 166.7, 162.5, 136.3, 131.6, 128.6, 128.2, 127.5, 122.8, 114.5, 70.0, 51.8. The spectroscopic data matched that reported in the literature.$^{310}$

**4-(benzyloxy)benzaldehyde (47b)**

![4-(benzyloxy)benzaldehyde](image)

The compounds were prepared according to **general procedure I** by treating 4-hydroxybenzaldehyde (1.22 g, 0.01 mol) with K$_2$CO$_3$ (2.07 g, 0.015 mol) and benzyl bromide (1.42 ml, 0.012 mol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as a white solid (1.97 g, 93%). $R_f$ (hexane/ethyl acetate 4:1): 0.35; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 9.89 (1H, s), 7.84 (2H, d, $J = 7.8$ Hz), 7.43–7.35 (5H, m), 7.08 (2H, d, $J = 7.8$ Hz), 5.15 (2H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 190.9, 163.8, 136.0, 132.1, 130.2, 128.8, 128.4, 127.6, 115.3, 70.4. The spectroscopic data matched that reported in the literature.$^{311}$
1-(4-(benzyloxy)phenyl)ethan-1-one (47c)

This compound was prepared according to general procedure I by treating 1-(4-hydroxyphenyl)ethan-1-one (1.36 g, 0.01 mol) with K₂CO₃ (2.07 g, 0.015 mol) and benzyl bromide (1.42 ml, 0.012 mol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as a white solid (2.17 g, 96%); R₇ (hexane/ethyl acetate 4:1): 0.50; ¹H NMR (400 MHz, CDCl₃): δ 7.94 (2H, d, J = 2.0, 6.8 Hz), 7.45–7.33 (5H, m), 7.01 (2H, dd, J = 2.0, 6.8 Hz), 5.12 (2H, s), 2.55 (3H, s); ¹³C NMR (100 MHz, CDCl₃): δ 196.8, 162.7, 136.3, 130.7, 130.6, 128.7, 128.3, 127.5, 114.6, 70.2, 26.4. The spectroscopic data matched that reported in the literature.³¹²
General procedure J for aldol condensation

Table 18. Conditions for screening aldol condensation reactions.\(^{238-239}\)

<table>
<thead>
<tr>
<th>Base</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>(t)-BuOK</td>
<td>THF</td>
<td>25 to 70 °C</td>
<td>48b (85%)</td>
</tr>
<tr>
<td>NaOMe</td>
<td>MeOH</td>
<td>25 to 70 °C</td>
<td>48b (80%)</td>
</tr>
<tr>
<td>LDA(^a)</td>
<td>THF</td>
<td>-78 to 25 °C</td>
<td>complex mixture</td>
</tr>
</tbody>
</table>

\(^a\)LDA was freshly made from \(n\)-BuLi (1.0 equiv.) and \(N,N\)-diisopropylethylamine (1.2 equiv.) at \(-78\) °C.

LDA formation: To a solution of \(N,N\)-diisopropylethylamine (1.3 equiv.) in dry THF was added \(n\)-BuLi (1.2 equiv.) dropwise at \(-78\) °C. After addition, the resulting mixture was stirred for 30 min for using.

To a solution of base (0.6 mmol) in solvent (10 mL) was added 27a (110 mg, 0.55 mmol) at 25 °C or \(-78\) °C, and the resulting mixture was stirred for 30 min. To the mixture above was added a solution of 28a (121 mg, 0.5 mmol) in the corresponding solvent (0.1 M) dropwise, after addition, the resulting mixture was slowly warmed up to 70 °C or 25 °C. The reactions were monitored by TLC for 48 hours.
4-(benzyloxy)benzoic acid (48b)

\[
\text{HO} \quad \overset{\text{O}}{\text{Bn}}
\]

This byproduct was prepared according to general procedure J. After 1 h, the mixture was cooled to RT and poured into aq. \(\text{H}_3\text{PO}_4\) (1%, 10 mL), the off-white precipitate was obtained and filtered, washed by cold water and dried under reduced pressure to give the product as a white solid without further purification (97 mg, 85%); \(R_f\) (hexane/ethyl acetate 4:1): 0.25; \(^1\text{H NMR}\) (400 MHz, \(d\)-DMSO): \(\delta\) 12.64 (1H, br. s), 7.90 (2H, d, \(J = 9.2\) Hz), 7.48–7.33 (5H, m), 7.10 (2H, d, \(J = 9.2\) Hz), 5.19 (2H, s); \(^{13}\text{C NMR}\) (100 MHz, \(d\)-DMSO): \(\delta\) 166.9, 161.9, 136.5, 131.3, 128.5, 128.0, 127.8, 123.2, 114.6, 69.4. The spectroscopic data matched that reported in the literature.\(^{313}\)

1-(4-(benzyloxy)phenyl)-3-(5-bromopyridin-2-yl)-3-hydroxypropan-1-one (48d)

\[
\begin{array}{c}
\text{Br} \\
\text{HO} \\
\overset{\text{O}}{\text{Bn}}
\end{array}
\]

This compound was prepared according to general procedure J by treating 46b (186 mg, 1.0 mmol) with LDA (1.2 mmol) and 47c (249 mg, 1.1 mmol) in dry THF. After completion monitored by TLC, the reaction was quenched with sat. \(\text{NH}_4\text{Cl}\) (aq.) and extracted with ethyl acetate (3 x 15 mL). The organic layer was dried over \(\text{MgSO}_4\) and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 \(\rightarrow\) 3:1) to give the product as a white solid (322 mg, 78%); \text{mp.} 133–134 °C; \(R_f\) (hexane/ethyl acetate 3:1): 0.25; \(^1\text{H NMR}\) (400 MHz, \(\text{CDCl}_3\)): \(\delta\) 8.58 (1H, d, \(J = 2.4\) Hz), 7.93 (2H, d, \(J = 9.0\) Hz), 7.82 (1H, dd, \(J = 2.4, 8.4\) Hz), 7.51 (1H, d, \(J = 8.0\) Hz), 7.43–7.32 (5H, m), 6.99 (2H, d, \(J = 9.0\) Hz), 5.31 (1H, p, \(J = 4.0\) Hz), 5.12 (2H, s), 4.31 (1H, d, \(J = 4.8\) Hz), 3.60 (1H, dd, \(J = 3.6, 17.6\) Hz), 3.36 (1H, dd, \(J = 8.0, 17.6\) Hz); \(^{13}\text{C NMR}\) (100 MHz, \(\text{CDCl}_3\)): \(\delta\) 198.6, 163.2, 160.7, 149.7, 139.4, 136.1, 130.7, 129.9, 128.8, 128.4, 127.5, 122.1, 119.2, 114.8, 70.3, 70.2, 45.0; \text{HRMS (ESI+)} \quad \text{Calc. for } C_{21}H_{18}BrNO_3 [M + Na]^+ 434.0362/436.0342, \text{found } 434.0365/436.0345. \text{IR (neat, cm}^{-1}\): \(\tilde{\nu}\) 3257, 2890, 1672, 1601, 1571, 1253, 1170, 1011, 828, 699, 543.
**General procedure K** for the oxidation of alcohols

To a solution of alcohol substrate (1.0 equiv.) in CH$_2$Cl$_2$ (0.05 M) was added Dess-Martin periodinane (DMP) (1.5 equiv.), and the mixture was stirred at RT for 30 min. After completion monitored by TLC, the mixture was quenched with sat. NaHCO$_3$ (aq.) (5 mL) and the resulted mixture was extracted with CH$_2$Cl$_2$ (3 x 10 mL), the organic layers were dried over MgSO$_4$ and concentrated in vacuo.

1-(4-(benzyloxy)phenyl)-3-(5-bromopyridin-2-yl)propane-1,3-dione (48a)

![Chemical structure](image)

This compound was prepared according to **general procedure K** by treating 48d (206 mg, 0.5 mmol) with DMP (318 mg, 0.75 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as a yellow solid (195 mg, 95%). m.p. 162–163 °C; $R_f$ (hexane/ethyl acetate 8:1): 0.50; $^1$H NMR (400 MHz, CDCl$_3$): δ 16.54 (1H, br.s), 8.73 (1H, d, $J = 2.0$ Hz), 8.04 (2H, d, $J = 8.8$ Hz), 8.01–7.96 (2H, m), 7.46–7.35 (6H, m), 7.05 (2H, d, $J = 8.8$ Hz), 5.15 (2H, s); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 186.9, 180.9, 162.8, 151.2, 150.5, 139.8, 136.3, 129.9, 128.8, 128.4, 128.3, 127.6, 124.0, 123.2, 115.0, 93.1, 70.3; **HRMS** (ESI+) Calcd. for C$_{21}$H$_{16}$BrNO$_3$ [M + Na]$^+$ 432.0206/434.0185, found 432.0205/434.0185. **IR** (neat, cm$^{-1}$): ν 3031, 2944, 1588, 1510, 1492, 1451, 1242, 1170, 999, 826, 800, 624, 518.

**General procedure L** for the formation of pyrazoles

![Chemical structure](image)
The compounds were prepared following a literature procedure.\textsuperscript{240} To a solution of 1,3-dione (1.0 equiv.) in MeOH (0.05 M) was added N\textsubscript{2}H\textsubscript{4}•H\textsubscript{2}O (64%, 2.0 equiv.), and the resulting mixture was stirred at RT for 48 h under nitrogen. After completion monitored by TLC, the solvent was removed under reduced pressure, and the residue was treated with H\textsubscript{2}O (10 mL) and extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried over MgSO\textsubscript{4} and concentrated \textit{in vacuo}.

\textbf{2-(3-(4-(benzyloxy)phenyl)-1\textit{H}-pyrazol-5-yl)-5-bromopyridine (49)}

\begin{center}
\includegraphics[width=0.3\textwidth]{compound_49}
\end{center}

This compound was prepared according to \textbf{general procedure L} by treating 48a (150 mg, 0.37 mmol) with N\textsubscript{2}H\textsubscript{4}•H\textsubscript{2}O (58 µL, 0.74 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 \textrightarrow 2:1) to give the product as a pale yellow solid (113 mg, 75%). \textbf{m.p.} 241–243 °C; \textbf{R}\textsubscript{f} (hexane/ethyl acetate 1:1): 0.30; \textbf{\textsuperscript{1}H NMR} (400 MHz, \textit{d}-DMSO): \textbf{δ} 13.45 (1H, br.s), 8.72 (1H, s), 8.17–8.07 (1H, m), 7.97–7.87 (1H, m), 7.78–7.76 (2H, m), 7.48–7.32 (5H, m), 7.16–7.11 (3H, m), 5.16 (2H, s); \textbf{\textsuperscript{13}C NMR} (100 MHz, \textit{d}-DMSO): \textbf{δ} 158.8, 151.5, 150.3, 144.1, 139.8, 137.4, 128.9, 128.3, 128.2, 127.1, 122.3, 121.4, 119.0, 115.8, 115.5, 100.5, 69.8; \textbf{HRMS (ESI+)} Calc. for C\textsubscript{21}H\textsubscript{16}BrN\textsubscript{3}O [M + Na]\textsuperscript{+} 428.0369/430.0348, found 428.0367/430.0346. \textbf{IR} (neat, cm\textsuperscript{-1}): \textbf{ν} 3201, 3061, 2888, 1613, 1590, 1448, 1242, 798, 734, 518, 498.

\textbf{General procedure M for the protection of pyrazole-N}

\begin{center}
\includegraphics[width=0.3\textwidth]{general_procedure_M}
\end{center}

To a solution of pyrazole substrate (1.0 equiv.) in CH\textsubscript{2}Cl\textsubscript{2} (0.05 M) was added NaH (60% in mineral oil, 2.0 equiv.) slowly at 0 °C, after 10 min, benzyl carbonobromidate (CbzBr) or TsCl (1.1 equiv.) was added, and the resulting mixture was allowed to warm to RT for 30 min. After completion monitored by TLC, the
mixture was quenched with H₂O (5 mL) and extracted with CH₂Cl₂ (3 x 5 mL), the organic layers were dried over MgSO₄ and concentrated in vacuo.

**Benzyl-3-(4-(benzyloxy)phenyl)-5-(5-bromopyridin-2-yl)-1H-pyrazole-1-carboxylate (50a)**

This compound was prepared according to **general procedure M** by treating 49 (101 mg, 0.25 mmol) with CbzBr (59 mg, 0.27 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as a white solid (121 mg, 90%). **m.p.** 142–143 °C; **Rf** (hexane/ethyl acetate 6:1): 0.55; **¹H NMR** (300 MHz, CDCl₃): δ 8.69 (1H, d, J = 2.1 Hz), 8.10 (1H, d, J = 8.7 Hz), 7.89 (1H, dd, J = 2.1, 8.7 Hz), 7.48–7.27 (12H, m), 6.99 (1H, s), 6.96 (2H, d, J = 8.7 Hz), 5.35 (2H, s), 5.09 (2H, s); **¹³C NMR** (75 MHz, CDCl₃): δ 159.5, 153.9, 150.7, 150.1, 149.4, 148.6, 139.4, 136.8, 134.4, 130.6, 128.8, 128.7, 128.3, 127.8, 127.1, 123.3, 122.3, 121.0, 114.4, 110.0, 70.2, 69.9; **HRMS** (ESI⁺) Calc. for C₂₉H₂₂BrN₃O₃ [M + H]⁺ 540.0917/542.0897, found 540.0917/542.0897. **IR** (neat, cm⁻¹): ν 2923, 1760, 1612, 1516, 1332, 1294, 1090, 831, 734, 697.

**2-(3-(4-(benzyloxy)phenyl)-1-tosyl-1H-pyrazol-5-yl)-5-bromopyridine (50b)**

This compound was prepared according to **general procedure M** by treating 49 (101 mg, 0.25 mmol) with TsCl (52 mg, 0.27 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as a white solid (133 mg, 95%). **m.p.** 152–154 °C; **Rf** (hexane/ethyl acetate 6:1): 0.55; **¹H NMR** (300 MHz, CDCl₃): δ 8.64 (1H, d, J = 2.1 Hz), 8.06 (1H, d, J = 8.4 Hz), 7.88 (1H, dd, J = 2.1, 8.4 Hz), 7.57 (2H, d, J = 8.4 Hz), 7.49–7.36 (7H, m), 7.18 (2H, d, J = 8.4 Hz), 7.03 (2H, d, J = 8.4 Hz), 6.89 (1H, s), 5.14 (2H, s), 2.36 (3H, s); **¹³C NMR** (75 MHz, CDCl₃): δ 159.8, 154.4, 150.4, 149.5, 149.1, 145.5, 139.3, 136.6, 134.7,
This compound was prepared according to general procedure F by treating 50b (112 mg, 0.2 mmol) with (4-hydroxyphenyl)boronic acid (41 mg, 0.3 mmol) in 1,4-dioxane at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 2:1) to afford the product as a white solid (97 mg, 85%). m.p. 192–193 °C; Rf (hexane/ethyl acetate 2:1): 0.25; 1H NMR (400 MHz, CDCl3): δ 8.77 (1H, dd, J = 0.8, 2.4 Hz), 8.20 (1H, dd, J = 0.8, 8.4 Hz), 7.90 (1H, dd, J = 2.4, 8.4 Hz), 7.59 (2H, d, J = 8.4 Hz), 7.49–7.47 (4H, m), 7.44–7.34 (5H, m), 7.17 (2H, d, J = 8.0 Hz), 7.03 (2H, d, J = 8.8 Hz), 6.96–6.94 (3H, m), 5.88 (1H, br.s), 5.14 (2H, s), 2.35 (3H, s); 13C NMR (100 MHz, CDCl3): δ 159.9, 156.5, 155.3, 149.7, 148.7, 147.3, 145.5, 136.8, 136.5, 134.9, 134.7, 131.6, 129.9, 129.8, 128.8, 128.5, 128.3, 128.1, 127.7, 122.0, 121.3, 116.4, 114.3, 110.4, 70.3, 21.8; HRMS (ESI+) Calc. for C34H27N3O4S [M + Na]+ 596.1614, found 596.1613. IR (neat, cm⁻¹): ν 3061, 2926, 1608, 1378, 1242, 1174, 1112, 826, 809, 662, 591, 568, 543.

4-(6-(3-(4-hydroxyphenyl)-1H-pyrazol-5-yl)pyridin-3-yl)phenol (43)

To a solution of 51b (46 mg, 0.08 mmol) in MeOH was added KOH (22 mg, 0.4 mmol) at RT, and the resulting mixture was stirred at 75 °C for 1 h. After completion monitored by TLC, the solvent was removed and the residue was diluted with H2O
and extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with brine (5 mL) and dried over MgSO₄ and concentrated in vacuo to get the crude compound without further purification. To the crude intermediate obtained above in MeOH (5 mL) was added Pd/C (4 mg, 10 wt. %) under nitrogen, and the mixture was stirred at room temperature for 5 h under 1 atm of hydrogen. After completion monitored by TLC, the Pd-C was filtered through Celite®, and the filtrate was concentrated under reduced pressure. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 50:1 → 15:1) to give the product as a pale yellow solid (12 mg, 45% over two steps). m.p. 249–252 °C; Rf (CH₂Cl₂/MeOH 10:1): 0.25; ¹H NMR (400 MHz, d-DMSO): δ 13.30 (1H, br.s), 9.68 (1H, br.s), 8.84 (1H, s), 8.07–8.06 (1H, m), 7.97–7.96 (1H, m), 7.67 (2H, d, J = 8.4 Hz), 7.62 (2H, d, J = 8.8 Hz), 7.14 (1H, s), 6.91 (2H, d, J = 8.8 Hz), 6.85 (2H, d, J = 8.4 Hz); ¹³C NMR (100 MHz, d-DMSO): δ 158.2, 157.9, 147.0, 134.7, 134.6, 134.4 (two overlapping signals), 134.3, 128.3, 127.9, 127.0, 119.8, 116.5, 116.0, 100.1, 100.0; HRMS (ESI+) Calc. for (C₂₀H₁₅N₃O₂)[M + Na]⁺ 681.2221, found 681.2217. IR (neat, cm⁻¹): ν 3292, 2921, 2851, 1610, 1598, 1449, 1247, 824, 793, 506. HPLC: 98.0% (HPLC Method A), RT: 16.1 min.

**1-(benzyloxy)-4-bromobenzene (55)**

![1-(benzyloxy)-4-bromobenzene](image)

This compound was prepared according to general procedure I by treating 4-bromophenol (519 mg, 3 mmol) with benzyl bromide (427 µL, 3.6 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as a white solid (726 mg, 92%); Rf (hexane/ethyl acetate 10:1): 0.50; ¹H NMR (300 MHz, CDCl₃): δ 7.43–7.31 (7H, m), 6.86 (2H, d, J = 9.0 Hz), 5.04 (2H, s); ¹³C NMR (75 MHz, CDCl₃): δ 158.0, 136.7, 132.4, 128.8, 128.3, 127.6, 116.9, 113.3, 70.4. The spectroscopic data matched that reported in the literature.³¹⁴
4-(4-(benzyloxy)phenyl)pyridine (56)

This compound was prepared according to **general procedure F** by treating 55 (263 mg, 1 mmol) with pyridin-4-ylboronic acid 54 (185 mg, 1.5 mmol) in 1,4-dioxane at 100 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 3:1) to afford the product as a white solid (209 mg, 80%). **m.p.** 136–137 °C; **Rf** (hexane/ethyl acetate 3:1): 0.20; **1H NMR (300 MHz, CDCl3)**: δ 8.62 (2H, dd, J = 1.8, 4.5 Hz), 7.60 (2H, dd, J = 2.1, 6.9 Hz), 7.47–7.34 (7H, m), 7.08 (2H, dd, J = 2.1, 6.9 Hz), 5.13 (2H, s); **13C NMR (75 MHz, CDCl3)**: δ 159.9, 150.4, 147.9, 136.8, 130.8, 128.8, 128.3 (two overlapping signals), 127.6, 121.2, 115.7, 70.3; **HRMS (ESI+)** Calc. for C_{18}H_{15}NO [M + H]^{+} 262.1226, found 262.1224. **IR** (neat, cm\(^{-1}\)): ̃ν 3022, 2888, 1605, 1591, 1518, 1487, 1249, 1223, 1183, 1044, 991, 808, 747, 622, 511.

4-(4-(benzyloxy)phenyl)pyridine 1-oxide (57)

This compound was prepared following a literature procedure.\(^{245}\) To a solution of 56 (200 mg, 0.77 mmol) in acetic acid (10 mL) was added hydrogen peroxide (397 μL, 3.8 mmol, 30% (w/w) in H\(_2\)O) dropwise at RT, and the resulting mixture was heated to 70 °C and stirred for 15 h. After completion monitored by TLC, the volatiles were removed under reduced pressure and the residue was diluted with sat. NaHCO\(_3\) (aq.) (10 mL) and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were washed with brine (5 mL) and dried over MgSO\(_4\) and concentrated **in vacuo**. The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH
200:1 → 80:1) to afford the product as a white solid (171 mg, 80%). **m.p.** 179–180 °C; \( R_f \) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.35; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 8.22 (2H, dd, \( J = 2.0, 5.2 \) Hz), 7.75–7.71 (4H, m), 7.48–7.32 (5H, m), 7.13 (2H, dd, \( J = 2.0, 6.8 \) Hz), 5.18 (2H, s); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 158.9, 138.7, 136.8, 135.9, 128.4, 128.1, 127.9, 127.7, 127.6, 122.9, 115.5, 69.3; **HRMS** (ESI+) Calc. for C\(_{18}\)H\(_{15}\)NO\(_2\) [M + Na\(^+\)] 300.0995, found 300.1000. **IR** (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3032, 3012, 1602, 1475, 1467, 1237, 1222, 1177, 1026, 864, 827, 759, 706, 607, 533.

4-(4-((tert-butyldimethylsilyl)oxy)phenyl)pyridin-2-amine (60)

![Chemical Structure](image)

This compound was prepared according to **general procedure F** by treating 4-bromopyridin-2-amine 59 (346 mg, 2 mmol) and boronic acid 40 (756 mg, 3 mmol) in 1,4-dioxane at 100 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 3:1) to afford the product as a white solid (522 mg, 87%). **m.p.** 117–119 °C; \( R_f \) (hexane/ethyl acetate 2:1): 0.20; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 8.08 (1H, dd, \( J = 0.8, 5.6 \) Hz), 7.47 (2H, dd, \( J = 2.0, 6.4 \) Hz), 6.90 (2H, dd, \( J = 2.0, 6.4 \) Hz), 6.85 (1H, dd, \( J = 1.6, 5.6 \) Hz), 6.66 (1H, dd, \( J = 0.8, 1.6 \) Hz), 4.46 (2H, s), 1.00 (9H, s), 0.23 (6H, s); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 159.0, 156.7, 150.0, 148.6, 131.8, 128.1, 120.6, 112.6, 105.8, 148.8, 131.8, 128.1, 120.6, 112.6, 105.8, 25.8, 18.4, −4.2; **HRMS** (ESI+) Calc. for C\(_{17}\)H\(_{24}\)N\(_2\)OSi [M + H\(^+\)] 301.1731, found 301.1728. **IR** (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3467, 3297, 3139, 2956, 2928, 2854, 1633, 1596, 1518, 1471, 1253, 1239, 1180, 912, 901, 835, 821, 801, 784, 693, 666, 468.
General procedure N for the Sandmeyer reaction

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<th>Temperature</th>
<th>Product</th>
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<td>25 to 80 °C</td>
<td>NR</td>
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<tr>
<td>CuBr₂</td>
<td>MeCN</td>
<td>25 to 80 °C</td>
<td>NR</td>
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<tr>
<td>CuBr/CuBr₂ (1:1)</td>
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<td>25 to 80 °C</td>
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<td>CH₂I₂</td>
<td>25 °C</td>
<td>25%</td>
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</table>

<sup>a</sup>Catalytic amount of HI (57%, 2 mol%) was used.

To a solution of 60 (1.0 equiv.) in MeCN (0.05 M) was added isopentyl nitrite (1.5 equiv.) and the resulting mixture was stirred at RT. After 30 min, CuBr or CuBr₂ (1.2 equiv.) was added and heated to 80 °C and monitored by TLC for 24 h. The results are shown in Table 19.

4-(4-((<i>tert</i>-butyldimethylsilyl)oxy)phenyl)-2-iodopyridine (61b)

This compound was prepared following a literature procedure.<sup>242</sup> To a solution of 60 (255 mg, 0.85 mmol) in CH₂I₂ (5 mL) was added isoamyl nitrite (1.75 mL, 17 mmol)
at RT under nitrogen. After 30 min, aq. HI (57%, 2.5 µL, 0.017 mmol) was added, and the resulting mixture was stirred at RT for 24 h. After completion monitored by TLC, the mixture was poured into a mixture of NH₄OH/H₂O (1:1 (v/v), 50 mL) and extracted with CHCl₃ (2 x 25 mL). The combined organic layers were dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 100:1 → 40:1) to afford the product as colorless oil (85 mg, 25%). Rf (hexane/ethyl acetate 10:1): 0.40; ¹H NMR (400 MHz, CDCl₃): δ 8.34 (1H, dd, J = 0.4, 5.2 Hz), 7.90 (1H, dd, J = 0.8, 1.6 Hz), 7.48 (2H, dd, J = 2.0, 6.4 Hz), 7.42 (1H, dd, J = 1.6, 5.2 Hz), 6.93 (2H, dd, J = 2.0, 6.4 Hz), 1.00 (9H, s), 0.23 (6H, s); ¹³C NMR (100 MHz, CDCl₃): δ 157.5, 150.8, 150.0, 132.2, 129.4, 128.4, 121.0, 120.8, 119.0, 25.8, 18.4, −4.2; HRMS (ESI+) Calc. for C₁₇H₂₂NO₂Si [M + H]⁺ 412.0588, found 412.0586. IR (neat, cm⁻¹): ν 2954, 2929, 2857, 1604, 1579, 1513, 1470, 1263, 911, 828.

4-(4-((tert-butyldimethylsilyl)oxy)phenyl)picolinaldehyde (52)

This compound was prepared according to general procedure H by treating 61b (85 mg, 0.2 mmol) with DMF (23 µL, 0.3 mmol) in dry toluene. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as pale yellow oil (31 mg, 50%); Rf (hexane/ethyl acetate 10:1): 0.45; ¹H NMR (300 MHz, CDCl₃): δ 10.13 (1H, s), 8.78 (1H, d, J = 5.1 Hz), 8.16 (1H, d, J = 1.2 Hz), 7.69 (1H, dd, J = 1.8, 5.1 Hz), 7.60 (2H, d, J = 8.7 Hz), 6.96 (2H, d, J = 8.7 Hz), 1.00 (9H, s), 0.24 (6H, s); ¹³C NMR (75 MHz, CDCl₃): δ 193.8, 157.7, 153.5, 150.7, 149.4, 129.9, 128.4, 125.0, 121.1, 119.0, 25.8, 18.4, −4.2; HRMS (ESI+) Calc. for C₁₈H₂₃NO₂Si [M + Na]⁺ 336.1390, found 336.1338. IR (neat, cm⁻¹): ν 3331, 2957, 2927, 2858, 1714, 1604, 1515, 1462, 1263, 1037, 912, 830, 781.
This compound was prepared according to general procedure J by treating 52 (62 mg, 0.2 mmol) with 47c (50 mg, 0.22 mmol) and LDA (0.24 mmol) in dry THF. After completion monitored by TLC, the reaction was quenched with sat. NH₄Cl (aq.) and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 8:1) to give the product as pale yellow semi-solid (65 mg, 60%); 

$R_f$ (hexane/ethyl acetate 10:1): 0.25; $^1$H NMR (400 MHz, CDCl₃): δ 8.53 (1H, dd, $J = 0.4, 5.2$ Hz), 7.96 (2H, d, $J = 9.2$ Hz), 7.76–7.75 (1H, m), 7.56 (2H, d, $J = 8.8$ Hz), 7.43–7.32 (6H, m), 6.99 (2H, d, $J = 9.2$ Hz), 6.93 (2H, d, $J = 8.8$ Hz), 5.42 (1H, dd, $J = 3.6, 8.4$ Hz), 5.12 (2H, s), 4.41 (1H br.s), 3.63 (1H, dd, $J = 8.4, 17.2$ Hz), 3.53 (1H, dd, $J = 3.6, 17.2$ Hz), 3.41 (1H, dd, $J = 8.4, 17.2$ Hz), 1.01 (9H, s), 0.24 (6H, s); $^{13}$C NMR (100 MHz, CDCl₃): δ 198.8, 163.1, 162.2, 157.1, 149.1, 149.0, 136.2, 131.1, 130.8, 130.2, 128.8, 128.6, 128.4, 127.6, 120.8, 120.0, 118.0, 114.8, 70.7, 70.3, 45.6, 25.8, 18.4, –4.2; HRMS (ESI+) Calc. for C₃₃H₃₇NO₄Si [M + Na]$^{+}$ 562.2384, found 562.2380. IR (neat, cm⁻¹): ν 3487, 2955, 2929, 2857, 1660, 1599, 1514, 1253, 1172, 912, 900, 850, 833, 779, 613.

1-(4-(benzyl ox y)phenyl)-3-(4-(4-((tert-butyldimethylsilyl)oxy)phenyl)pyridin-2-yl)-3-hydroxypropan-1-one (62)

1-(4-(benzyl oxy)phenyl)-3-(4-((tert-butyldimethylsilyl)oxy)phenyl)pyridin-2-yl)propane-1,3-dione (53)
This compound was prepared according to general procedure K by treating 62 (54 mg, 0.1 mmol) with DMP (64 mg, 0.15 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 3:1) to give the product as a yellow solid (40 mg, 75%). m.p. 134–135 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.55; \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) 16.67 (1H, br.s), 8.70 (1H, d, \(J = 5.2\) Hz), 8.35 (1H, d, \(J = 8.8\) Hz), 7.63 (2H, d, \(J = 8.8\) Hz), 7.60 (1H, dd, \(J = 2.0, 5.2\) Hz), 7.54 (1H, s), 7.46–7.33 (5H, m), 7.06 (2H, d, \(J = 8.8\) Hz), 6.97 (2H, d, \(J = 8.4\) Hz), 5.15 (2H, s), 1.01 (9H, s), 0.25 (6H, s); \(^1\)C NMR (100 MHz, CDCl₃): \(\delta\) 187.0, 182.0, 162.8, 157.4, 153.2, 149.7, 149.3, 136.4, 130.5, 129.9, 128.8, 128.5, 128.4 (two overlapping signals), 127.7, 123.4, 121.0, 119.6, 115.0, 93.3, 70.3, 25.8, 18.4, −4.2; HRMS (ESI+) Calc. for C\(_{33}\)H\(_{35}\)N\(_2\)O\(_4\)Si [M + H]+ 538.2408, found 538.2414. IR (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 2953, 2928, 2857, 1604, 1510, 1453, 1248, 1173, 911, 832, 805, 733, 659.

2-(3-(4-(benzyloxy)phenyl)-1H-pyrazol-5-yl)-4-(4-((tert-butyldimethylsilyl)oxy)phenyl)pyridine (63)

This compound was prepared according to general procedure L by treating 53 (53.8 mg, 0.1 mmol) and \(\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}\) (7.8 µL, 0.2 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 50:1 → 20:1) to give the product as a pale yellow semi-solid (37 mg, 70%). Rf (CH₂Cl₂/MeOH 15:1): 0.25; \(^1\)H NMR (400 MHz, \(d\)-MeOD): \(\delta\) 8.59 (1H, s), 8.24 (1H, s), 7.78–7.74 (4H, m), 7.62 (1H, s), 7.49–7.31 (5H, m), 7.20 (1H, s), 7.11 (2H, d, \(J = 8.8\) Hz), 7.03 (2H, d, \(J = 8.8\) Hz), 5.16 (2H, s), 1.05 (9H, s), 0.28 (6H, s); \(^1\)C NMR (100 MHz, \(d\)-MeOD): \(\delta\) 160.5, 158.6, 150.7, 150.6, 144.6, 138.6, 132.0, 130.7, 129.5 (two overlapping signals), 129.4, 129.2, 128.9, 128.6, 128.1, 121.9, 118.6, 116.4, 102.8, 101.6, 71.1, 26.1, 19.1, −4.3; HRMS (ESI+) Calc. for C\(_{33}\)H\(_{35}\)N\(_3\)O\(_2\)Si [M + H]+ 534.2571, found 534.2568. IR (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 2952, 2928, 2856, 1606, 1515, 1471, 1454, 1254, 1175, 913, 835.
4-(2-(3-(4-hydroxyphenyl)-1H-pyrazol-5-yl)pyridin-4-yl)phenol (44)

To a solution of 63 (53 mg, 0.1 mmol) in MeOH was added Pd/C (5 mg, 10 wt. %) under nitrogen atmosphere, and the mixture was stirred at room temperature for 4 h under 1 atm of hydrogen. After completion monitored by TLC, the Pd-C was filtered through Celite®, and the filtrate was concentrated under reduced pressure to get the crude compound without further purification. To the crude intermediate obtained above in THF (3 mL) was added TBAF (0.2 mL, 0.2 mmol, 1 M in THF). The resulting mixture was stirred at RT for 30 min. After completion monitored by TLC, the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 20:1 → 10:1) to give the product as an off-white solid (18 mg, 55% over two steps). m.p. 159–161 °C; R{sub}f (CH₂Cl₂/MeOH 10:1): 0.15; ¹H NMR (500 MHz, d-DMSO): δ 13.29 (1H, br.s), 9.88 (1H, br.s), 9.60 (1H, br.s), 8.57 (1H, d, J = 5.0 Hz), 8.16 (1H, d, J = 1.0 Hz), 7.74 (2H, d, J = 7.0 Hz), 7.67 (2H, d, J = 8.5 Hz), 7.56 (1H, d, J = 4.0 Hz), 7.23 (1H, s), 6.93 (2H, d, J = 8.5 Hz), 6.84 (2H, d, J = 8.5 Hz); ¹³C NMR (125 MHz, d-DMSO): δ 159.5, 157.9, 150.4, 148.5, 133.0, 128.8 (two overlapping signals), 127.3, 127.2, 124.8, 123.7, 122.7, 119.8, 116.6, 116.2, 100.6; HRMS (ESI+) Calc. for C₂₀H₁₅N₃O₂ [M + H]⁺ 330.1237, found 330.1242. IR (neat, cm⁻¹): ν 2953, 2921, 2852, 1601, 1515, 1451, 1373, 1253, 1172, 823, 799, 776, 567. HPLC: 98.4% (HPLC Method A), RT: 15.9 min.

[2,2'-bipyridine] 1,1'-dioxide (66)

This compound was prepared following a literature procedure.²⁴⁵ To a solution of 2,2'-bipyridine 65 (1.0 g, 6.4 mmol) in Acetic acid (15 mL) was added hydrogen peroxide (3.3 mL, 32 mmol, 30% (w/w) in H₂O) dropwise at RT, and the resulting mixture was heated to 70 °C and stirred for 15 h. After completion, the cooled
The mixture was added acetone (20 mL) forming a white precipitate, filtered and dried. The crude white solid was recrystallised from H₂O to get the desired pure compound (1.08 g, 90%). \( R_f \) (CH₂Cl₂/MeOH 15:1): 0.35; \(^1\)H NMR (300 MHz, D₂O): \( \delta \) 8.46 (2H, d, \( J = 6.3 \) Hz), 7.88–7.83 (2H, m), 7.77–7.30 (4H, m); \(^{13}\)C NMR (75 MHz, D₂O): \( \delta \) 142.3, 140.4, 132.4, 129.5, 129.1. The spectroscopic data matched that reported in the literature.³¹⁵

**4,4'-dinitro-[2,2'-bipyridine] 1,1'-dioxide (67)**

![4,4'-dinitro-[2,2'-bipyridine] 1,1'-dioxide (67)](image)

This compound was prepared following a literature procedure.²⁴⁶ To a suspension of 66 (1.0 g, 5.3 mmol) in sulphuric acid (6 mL, 111 mmol) was added nitric acid (2.5 mL, 44 mmol) dropwise at 0 °C, and the resulting mixture was heated to 100 °C and stirred for 20 h. After completion monitored by TLC, the mixture was cooled to RT and ice (20 mL) was added to get yellow precipitate, filtered and washed with cold water and dried to get the desired compound without further purification (295 mg, 20%). \( R_f \) (CH₂Cl₂/MeOH 15:1): 0.40; \(^1\)H NMR (400 MHz, CDCl₃): \( \delta \) 8.69 (2H, d, \( J = 3.2 \) Hz), 8.59 (2H, d, \( J = 7.2 \) Hz), 8.38 (2H, dd, \( J = 3.2, 7.2 \) Hz); \(^{13}\)C NMR (100 MHz, CDCl₃): \( \delta \) 142.1, 141.2, 140.5, 123.8, 122.0. The spectroscopic data matched that reported in the literature.³¹⁵

**4,4'-dichloro-[2,2'-bipyridine] 1,1'-dioxide (68)**

![4,4'-dichloro-[2,2'-bipyridine] 1,1'-dioxide (68)](image)

This compound was prepared following a literature procedure.²⁴⁶ To a suspension of 67 (100 mg, 0.36 mmol) in acetic acid (2 mL) was added acetyl chloride (0.13 mL, 1.8 mmol) dropwise at RT under nitrogen atmosphere, the resulting mixture was stirred for 2 h, and then heated to 100 °C for 12 h. After completion monitored by TLC, the volatile was removed under reduced pressure and the residue was treated with sat. NaHCO₃ (aq.) (2 mL) and CH₂Cl₂ (5 mL), and the off-white precipitate was
obtained, filtered and washed with CH₂Cl₂ and dried to get the desired compound without further purification (79 mg, 85%). \( R_f \) (CH₂Cl₂/MeOH 10:1): 0.20; \(^1\)H NMR (400 MHz, \( d \)-DMSO+TFA): \( \delta \) 8.42 (2H, d, \( J = 6.8 \) Hz), 7.93 (2H, d, \( J = 3.2 \) Hz), 7.68 (2H, dd, \( J = 3.2, 6.8 \) Hz); \(^{13}\)C NMR (100 MHz, \( d \)-DMSO+TFA): \( \delta \) 143.0, 141.4, 132.3, 129.6, 128.5. The spectroscopic data matched that reported in the literature.³¹⁵

4,4'-(2,2'-bipyridine-4,4'-diyl)diphenol (64)

\[\text{HO} \quad \text{N} \quad \text{N} \quad \text{Cl} \quad \text{N} \quad \text{N} \quad \text{HO}\]

A mixture of 69 (40 mg, 0.18 mmol), (4-hydroxyphenyl)boronic acid (62 mg, 0.45 mmol) and aq. K₂CO₃ (2 M, 450 µL, 0.9 mmol) in 1,4-dioxane (5 mL) was degassed, and then [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (6.5 mg, 0.009 mmol) was added. The resulting mixture was degassed and back-filled with nitrogen, and then heated at reflux for 4 h. After completion monitored by TLC, the reaction
mixture was concentrated under reduced pressure, and then diluted with H₂O (5 mL), neutralised with sat. NH₄Cl (aq.) and extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with H₂O (5 mL) and dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (CH₂Cl₂: MeOH = 50:1 → 10:1) to afford the product as a white solid (25 mg, 40%).

**m.p.** 328–330 °C; **Rf** (CH₂Cl₂/MeOH 15:1): 0.30; **¹H NMR** (400 MHz, d-DMSO): δ 9.88 (2H, br.s), 8.70 (2H, d, J = 5.2 Hz), 8.64 (2H, d, J = 1.6 Hz), 7.74 (4H, d, J = 8.8 Hz), 7.71 (2H, dd, J = 1.6, 5.2 Hz), 6.94 (4H, d, J = 8.8 Hz); **¹³C NMR** (100 MHz, d-DMSO): δ 158.8, 155.8, 149.8, 148.0, 128.1, 120.7, 116.8, 116.1. **HRMS** (ESI+) Calc. for C₂₂H₁₆N₂O₂ [M + Na]⁺ 363.1104, found 363.1110. **IR** (neat, cm⁻¹): ν 3084, 2922, 1586, 1514, 1234, 826, 567. **HPLC**: 96.1% (HPLC Method A), RT: 18.7 min.

**2-amino-5-bromonicotinic acid (72)**

This compound was prepared following a literature procedure.²⁴⁸ To a suspension of 2-aminonicotinic acid 71 (3.75 g, 27 mmol) in glacial acetic acid (15 mL) was added a solution of bromine (1.8 mL, 35 mmol) in glacial acetic acid (3 mL) and the mixture was stirred at RT for 20 h. After completion monitored by TLC, the resulting precipitate was filtered and washed with glacial acetic acid 3 times. The remaining solid was air dried and then recrystallised from boiling methanol to afford the product as white crystalline needles (5.2 g, 90%). **¹H NMR** (400 MHz, d-DMSO): δ 8.36 (1H, d, J = 2.6 Hz), 8.25 (1H, d, J = 2.6 Hz), 7.95–7.79 (2H, br.s); **¹³C NMR** (100 MHz, d-DMSO): δ 166.6, 156.5, 149.9, 143.8, 109.3, 103.7. The spectroscopic data matched that reported in the literature.²⁴⁸

**Methyl 2-amino-5-bromonicotinate (73)**

To a solution of 2-amino-5-bromonicotinic acid 72 (1.0 g, 4.6 mmol) in MeOH (10 mL) was added sulphuric acid (2.0 mL, 36.8 mmol) dropwise and the mixture was
stirred at RT for 18 h. After completion monitored by TLC, the solvent was removed under reduced pressure. The residue was neutralised with sat. NaHCO₃ (aq.) and extracted with ethyl acetate (3 x 20 mL). The combined organic layers were dried over MgSO₄ and concentrated in vacuo to afford the product as a white powder (1.0 g, 94%) without further purification. m.p. 148–149 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.55; ^1H NMR (400 MHz, d-DMSO): δ 8.24 (1H, d, J = 2.4 Hz), 8.21 (1H, d, J = 2.4 Hz), 6.68–6.19 (2H, br.s), 3.89 (3H, s); ^13C NMR (100 MHz, d-DMSO): δ 166.6, 158.0, 154.4, 142.0, 107.5, 106.1, 52.4. HRMS (ESI+) Calc. for C₇H₇N₂O₂Br [M + H]^+ 230.9764/232.9743, found 230.9765/232.9745. IR (neat, cm⁻¹): v 3425, 3131, 2918, 1704, 1620, 1223, 796, 526.

6-bromo-4-hydroxy-1,8-naphthyridine-3-carbonitrile (74)

This compound was prepared following a literature procedure.²⁴⁷ To a sealed tube was added a mixture of methyl 2-amino-5-bromonicotinate 73 (500 mg, 2.16 mmol) and N,N-dimethylformamide dimethyl acetal (3 mL, 21.6 mmol), and stirred at 110 °C for 3 h. The volatiles were concentrated under reduced pressure, and the crude intermediate was dissolved in dry THF (30 mL), which would be used immediately in the following step. To a solution of n-BuLi (2.5 M in hexane, 1.9 mL, 4.75 mmol) in dry THF (10 mL) was added dry acetonitrile (248 µL, 4.75 mmol) dropwise at −78 °C under nitrogen atmosphere. The resulting reaction mixture was stirred at −78 °C for 20 min. To the resulting white suspension was added dropwise the intermediate solution described above, and the reaction mixture was stirred at −78 °C for 30 min, then at −40 °C for 2 h. Acetic acid (370 µL, 6.48 mmol) was added and the resulting yellow suspension was warmed to RT and stirred for 10 h. After completion monitored by TLC, the mixture was diluted with H₂O (20 mL) and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were washed with H₂O (2 x 15 mL) and brine, dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate = 5:1 → 1:1) to afford the product as an orange solid (190 mg, 35%). ^1H NMR (400 MHz, d-DMSO): δ 13.43–13.41 (1H, br.s), 8.97
(1H, d, J = 2.4 Hz), 8.85 (1H, s), 8.61 (1H, d, J = 2.4 Hz); $^{13}$C NMR (100 MHz, d-DMSO): $\delta$ 174.0, 154.8, 148.6, 148.4, 136.5, 121.2, 116.5, 115.9, 95.3. The spectroscopic data matched that reported in the literature.247

6-bromo-1,8-naphthyridin-4-ol (75)

This compound was prepared following a literature procedure.247 To a sealed tube was added a mixture of 6-bromo-4-hydroxy-1,8-naphthyridine-3-carbonitrile 74 (100 mg, 0.40 mmol) in aq. HCl (32%, 2 mL) and stirred at 150 °C for 3 h. After cooling to room temperature, the reaction mixture was neutralised with ammonia solution, extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with H$_2$O (2 x 10 mL), dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography (CH$_2$Cl$_2$: MeOH = 50:1 → 20:1) to afford the product as a pale yellow solid (30 mg, 33%). $^1$H NMR (300 MHz, d-DMSO): $\delta$ 12.4 (1H, br.s), 8.84 (1H, s), 8.53 (1H, s), 7.98 (1H, d, $J$ = 7.5 Hz), 6.15 (1H, d, $J$ = 7.5 Hz); $^{13}$C NMR (75 MHz, d-DMSO): $\delta$ 176.5, 153.6, 149.1, 140.9, 136.3, 121.3, 114.3, 110.0. The spectroscopic data matched that reported in the literature.247

3-bromo-5-chloro-1,8-naphthyridine (76)

This compound was prepared following a literature procedure.247 To a sealed tube was added a mixture of 6-bromo-1,8-naphthyridin-4-ol 75 (30 mg, 0.13 mmol) in POCl$_3$(1 mL) and stirred at 105 °C for 3 h. After cooling to RT, the reaction mixture was poured into ice, and the resulting mixture was neutralised with sat. NaHCO$_3$ (aq.) and extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with H$_2$O (2 x 5 mL), dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate = 5:1 → 3:1) to afford the product as a white solid (29 mg, 93%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 9.16
A mixture of 3-bromo-5-chloro-1,8-naphthyridine 76 (15 mg, 0.06 mmol), (4-hydroxyphenyl)boronic acid (21 mg, 0.15 mmol) and aq. K$_2$CO$_3$ (2 M, 180 µL, 0.36 mmol) in 1,4-dioxane (1 mL) was degassed, and then PdCl$_2$(dpff) (9 mg, 0.012 mmol) was added. The resulting mixture was degassed and back-filled with nitrogen, and then stirred at 130 °C (100 W) in microwave under nitrogen atmosphere for 2 h. After completion monitored by TLC, the reaction mixture was concentrated under reduced pressure, and diluted with H$_2$O (5 mL), neutralised with sat. NH$_4$Cl (aq.) and extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with H$_2$O (5 mL), dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography (CH$_2$Cl$_2$: MeOH = 50:1 → 10:1) to afford the product as a yellow solid (16 mg, 85%). m.p. 211–213 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 15:1): 0.30; $^1$H NMR (400 MHz, d-DMSO): δ 9.90 (1H, s), 9.78 (1H, s), 9.38 (1H, s), 9.04 (1H, br.s), 8.37 (1H, d, $J$ = 2.4 Hz), 7.62 (2H, d, $J$ = 8.4 Hz), 7.54 (1H, d, $J$ = 4.4 Hz), 7.48 (2H, d, $J$ = 8.4 Hz), 7.00 (2H, d, $J$ = 8.4 Hz), 6.92 (2H, d, $J$ = 8.4 Hz); $^{13}$C NMR (100 MHz, d-DMSO): δ 158.7, 158.5, 155.4, 152.8, 152.5, 149.6, 134.2, 131.5, 130.7, 129.0, 127.6, 127.3, 122.7, 121.1, 116.7, 116.3. HRMS (ESI+) Calc. for C$_{20}$H$_{14}$N$_2$O$_2$ [M + Na]$^+$ 337.0947, found 337.0950. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3363, 2921, 1606, 1512, 1265, 831, 559, 527. HPLC: 98.5% (HPLC Method A), RT: 15.5 min.
4,5-diiodo-1H-imidazole (79)

This compound was prepared following a literature procedure. To a solution of imidazole 78 (1.11 g, 16 mmol) in NaOH (4 M, 60 mL) was added a solution of KI (13.3 g, 80 mmol) and I₂ (8.88 g, 35 mmol) in H₂O (50 mL) dropwise. The resulting mixture stirred at RT for 10 h. After completion monitored by TLC, the mixture was reduced to pH = 8 with acetic acid, and the resulting white precipitate was filtered and washed with cold water. The remaining solid was air dried to afford the product as a white creamy solid (4.1 g, 80%). m.p. 188–190 °C; R_f (hexane/ethyl acetate 1:1): 0.15; ¹H NMR (300 MHz, d-DMSO): δ 7.77 (1H, s); ¹³C NMR (75 MHz, d-DMSO): δ 141.8, 86.9. The spectroscopic data matched that reported in the literature.

4,4’-(1H-imidazole-4,5-diyl)diphenol (77)

This compound was prepared following a literature procedure. The mixture of 4,5-diiodo-1H-imidazole 79 (0.11 g, 0.34 mmol), (4-hydroxyphenyl)boronic acid (141 mg, 1.02 mmol), TBAB (11 mg, 0.034 mmol), K₂HPO₄ (226 mg, 1.30 mmol) and Pd(PPh₃)₄ (25 mg, 6 mol%) were transferred to a microwave reactor tube equipped with a magnetic stirring bar, followed by the addition of a mixture solvent of MeOH/H₂O (5 mL, 4:1), and the resulting mixture was degassed and back-filled with nitrogen and then stirred at 100 °C (100 W) with microwave irradiation for 60 min. After completion monitored by TLC, the reaction mixture was dissolved in aq. HCl (10%, 10 mL) and stirred vigorously. The mixture was washed with ethyl acetate (2 x 10 mL), the combined aqueous layers were neutralised with sat. NaHCO₃ (aq.) and extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with H₂O, dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (CH₂Cl₂: MeOH = 50:1 → 10:1) to afford the product as a white solid (30 mg, 35%). R_f (CH₂Cl₂/MeOH 10:1): 0.25; ¹H NMR (400
MHz, \textit{d}-DMSO): \(\delta\) 9.44 (2H, Br.s), 7.67 (1H, s), 7.25 (4H, d, \(J = 8.4\) Hz), 6.72 (4H, d, \(J = 8.4\) Hz); \textsuperscript{13}C NMR (100 MHz, \textit{d}-DMSO): \(\delta\) 156.3, 134.4, 128.6, 125.6, 124.3, 115.2. \textbf{HRMS} (ESI+) Calc. for C\textsubscript{13}H\textsubscript{12}N\textsubscript{2}O\textsubscript{2} [M + Na]\textsuperscript{+} 275.0791, found 275.0794. \textbf{HPLC}: 97.7\% (HPLC Method A), RT: 13.3 min. The spectroscopic data matched that reported in the literature.\textsuperscript{317}

\textbf{General procedure O for the synthesis of hydrazones}

\begin{equation}
\text{R} \begin{array}{c}
\text{N} \\
\text{Cl}
\end{array} \rightarrow \text{R} \begin{array}{c}
\text{N} \\
\text{Cl}
\end{array} + \text{N}_2\text{H}_4\cdot\text{H}_2\text{O}, \text{ethanol} \rightarrow \text{R} \begin{array}{c}
\text{N} \\
\text{Cl}
\end{array} \begin{array}{c}
\text{N} \\
\text{OPG}
\end{array} \begin{array}{c}
\text{OHC} \\
\text{OPG}
\end{array}
\end{equation}

The compounds were prepared following a literature procedure.\textsuperscript{252} To a solution of chloro-pyrazine substrate (1.0 equiv.) in ethanol (0.05 M) was added hydrazine monohydrate (1.1 equiv.). The resulting mixture was stirred at 80 °C for 8 h. After completion monitored by TLC, the reaction mixture was cooled to RT, and the precipitate obtained filtered, washed by ethanol and dried to get a pale yellow solid. To a solution of the crude intermediate (1.0 equiv.) in ethanol (0.05 M) was added aryl-aldehyde (1.1 equiv.), and the resulting mixture was stirred at 80 °C for 10 h. After completion monitored by TLC, the reaction mixture was cooled to RT, and the precipitate was obtained and filtered, washed by ethanol and dried to get the products.

\textbf{2-(2-(4-(benzyloxy)benzylidene)hydrazinyl)-6-chloropyrazine (83)}

This compound was prepared according to \textbf{general procedure O} by treating 82 (1.49 0.01 mol) with 47b (2.33 g, 0.011 mol). Off-white solid (2.37 g, 70\%). \textbf{m.p.} 213–214 °C; \(R_f\) (hexane/ethyl acetate 2:1): 0.30; \textsuperscript{1}H NMR (300 MHz, \textit{d}-DMSO): \(\delta\) 11.40 (1H, br.s), 8.52 (1H, s), 8.01–7.99 (2H, m), 7.66 (2H, d, \(J = 8.1\) Hz), 7.46–7.30 (5H, m), 7.06 (2H, d, \(J = 8.1\) Hz), 5.14 (2H, s); \textsuperscript{13}C NMR (75 MHz, \textit{d}-DMSO): \(\delta\) 159.5, 152.4, 145.5, 142.7, 136.8, 131.9, 128.6, 128.4, 128.2, 127.9, 127.7, 127.3, 115.1, 69.3; \textbf{HRMS} (ESI+) Calc. for C\textsubscript{18}H\textsubscript{15}ClN\textsubscript{4}O [M + Na]\textsuperscript{+} 361.0827, found
361.0832. **IR** (neat, cm$^{-1}$): $\tilde{\nu}$ 3167, 3030, 2860, 1608, 1569, 1511, 1380, 1245, 822, 720, 508.

**General procedure P for the oxidative heterocyclisation**

The compounds were prepared following a literature procedure.$^{253}$ To a solution of hydrazone (1.0 equiv.) in CH$_2$Cl$_2$ (0.05 M) was added PhI(OAc)$_2$ (1.0 equiv.). The resulting mixture was stirred at RT for 12 h. After completion monitored by TLC, the solvent was removed under reduced pressure.

3-(4-(benzyloxy)phenyl)-5-chloro-[1,2,4]triazolo[4,3-a]pyrazine (84)

This compound was prepared according to **general procedure P** by treating 83 (2.37 g, 7 mmol) with PhI(OAc)$_2$ (2.25 g, 7 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 1:1) to give the product as a white solid (2.00 g, 85%). **m.p.** 216–217 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.30; $^1$H **NMR** (400 MHz, d-DMSO): $\delta$ 9.46 (1H, s), 8.06 (1H, s), 7.65 (2H, ddd, $J = 2.8, 4.8, 9.6$ Hz), 7.52–7.50 (2H, m), 7.45–7.41 (2H, m), 7.39–7.34 (1H, m), 7.19 (2H, ddd, $J = 2.8, 4.8, 9.6$ Hz), 5.22 (2H, s); $^{13}$C **NMR** (100 MHz, d-DMSO): $\delta$ 160.3, 147.8, 147.4, 143.2, 137.1, 133.3, 129.5, 128.9, 128.4, 128.3, 122.2, 119.8, 114.4, 69.9; **HRMS** (ESI+) Calc. for C$_{18}$H$_{13}$ClN$_3$O [M + Na]$^+$ 359.0670, found 359.0668. **IR** (neat, cm$^{-1}$): $\tilde{\nu}$ 3087, 2879, 1607, 1453, 1233, 750, 554.
2-(4-(benzyl oxy)phenyl)ethan-1-ol (85a)

**Step 1:** The formation of 4-(2-hydroxyethyl)phenol. This compound was prepared following a literature procedure.\(^{318}\) To a solution of 2-(4-hydroxyphenyl)acetic acid (760 mg, 5 mmol) in ethanol (20 mL) was added sulfuric acid (27 µL, 0.5 mmol), and the resulting mixture was heated at reflux for 3 h. After completion monitored by TLC, the solvent was removed under reduced pressure, and the crude product was dissolved in dry THF (20 mL), followed by the addition of LiAlH\(_4\) (228 mg, 6 mmol) slowly at 0 °C, and the resulting mixture was allowed to warm to RT for 12 h. After completion monitored by TLC, the solution was cooled to 0 °C, and quenched by Na\(_2\)SO\(_4\)•10H\(_2\)O solid and filtered through Celite®. The solvent was removed under reduced pressure and the residue was diluted with H\(_2\)O (20 mL) and extracted with ethyl acetate (3 x 20 mL). The combined organic layers were dried over MgSO\(_4\) and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 2:1) to give the product as an off-white solid (600 mg, 87%). \(R_f\) (hexane/ethyl acetate 2:1): 0.30; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.10 (2H, dd, \(J = 2.0, 6.4\) Hz), 6.78 (2H, dd, \(J = 2.0, 6.4\) Hz), 4.83 (1H, br.s), 3.83 (2H, dd, \(J = 6.4, 15.0\) Hz), 2.80 (2H, t, \(J = 6.4\)Hz); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 154.4, 130.6, 130.3, 115.6, 64.0, 38.4. The spectroscopic data matched that reported in the literature.\(^{318}\)

**Step 2:** The benzyl protection of 4-(2-hydroxyethyl)phenol was realised according to [general procedure I](#) by treating 4-(2-hydroxyethyl)phenol (600 mg, 4.3 mmol) with benzyl bromide (613 µL, 5.2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product 67a as a white solid (882 mg, 90%). \(R_f\) (hexane/ethyl acetate 4:1): 0.30; \(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 7.44−7.42 (2H, m), 7.40-7.37 (2H, m), 7.33−7.31 (1H, m), 7.14 (2H, d, \(J = 8.6\) Hz), 6.93 (2H, d, \(J = 8.6\) Hz), 5.05 (2H, s), 3.82 (2H, t, \(J = 6.5\) Hz), 2.81 (2H, t, \(J = 6.5\) Hz), 1.43 (1H, br.s); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)): \(\delta\) 157.6, 137.2, 130.8, 130.1, 128.7, 128.0, 127.5, 115.1, 70.1, 63.9, 38.4. The spectroscopic data matched that reported in the literature.\(^{309}\)
2-(3-(benzyloxy)phenyl)ethan-1-ol (85b)

This compound was prepared according to general procedure I by treating 3-(2-hydroxyethyl)phenol (600 mg, 4.3 mmol) with benzyl bromide (613 µL, 5.2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1→5:1) to give the product as a white solid (912 mg, 93%). $R_f$ (hexane/ethyl acetate 4:1): 0.35; $^1$H NMR (300 MHz, CDCl$_3$): δ 7.37–7.22 (5H, m), 7.18–7.13 (1H, m), 6.79–6.74 (3H, m), 4.98 (2H, s), 3.77 (2H, t, $J$ = 6.6 Hz), 2.76 (2H, t, $J$ = 6.6 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 159.1, 140.3, 137.1, 129.7, 128.7, 128.1, 127.6, 121.8, 115.9, 112.8, 70.1, 63.7, 39.3. The spectroscopic data matched that reported in the literature.$^{319}$

**General procedure Q for the synthesis ethers**

![Diagram](image)

To a solution of 84 (1.0 equiv.) in toluene (0.05 M) was added KOH (3.0 equiv.) and 18-crown-6 (0.07 equiv.), followed by the addition of 85a–b (1.1 equiv.). The resulting mixture was stirred at 40 °C for 4 h. After completion monitored by TLC, the reaction mixture was washed with H$_2$O and extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried over MgSO$_4$ and concentrated in vacuo.

5-(4-(benzyloxy)phenethoxy)-3-(4-(benzyloxy)phenyl)-[1,2,4]triazolo[4,3-a]pyrazine (86a)
This compound was prepared according to general procedure Q by treating 84 (674 mg, 2 mmol) with 85a (502 mg, 2.2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 1:1 → 1:1.5) to give the product as a white solid (772 mg, 73%). m.p. 156–158 °C; Rf (hexane/ethyl acetate 1:1): 0.15; 1H NMR (300 MHz, CDCl₃): δ 8.94 (1H, s), 7.52 (2H, d, J = 7.8 Hz), 7.38–7.19 (10H, m), 6.97 (2H, d, J = 7.8 Hz), 6.72 (2H, d, J = 8.1 Hz), 6.65 (2H, d, J = 8.1 Hz), 5.07 (2H, s), 4.93 (2H, s), 4.27 (2H, t, J = 5.4 Hz), 2.77 (2H, t, J = 5.4 Hz); 13C NMR (75 MHz, CDCl₃): δ 160.2, 157.7, 136.9, 136.4, 136.2, 132.3, 129.7, 128.7, 128.6, 128.1, 128.0, 127.4, 120.2, 115.0, 114.2, 108.0, 106.1, 71.6, 70.1, 70.0, 33.8; HRMS (ESI+) Calc. for C₃₃H₂₈N₄O₃ [M + Na]⁺ 551.2054, found 551.2050. IR (neat, cm⁻¹): ν 3033, 2951, 2857, 1608, 1505, 1247, 830, 695.

5-(3-(benzyloxy)phenethoxy)-3-(4-(benzyloxy)phenyl)-[1,2,4]triazolo[4,3-a]pyrazine (86b)

This compound was prepared according to general procedure Q by treating 84 (674 mg, 2 mmol) with 85b (502 mg, 2.2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 1:1 → 1:1.5) to give the product as a white solid (730 mg, 69%). m.p. 104–106 °C; Rf (hexane/ethyl acetate 1:1): 0.15; 1H NMR (300 MHz, CDCl₃): δ 8.93 (1H, s), 7.49 (2H, d, J = 8.1 Hz), 7.35–7.18 (10H, m), 7.05 (1H, t, J = 7.8 Hz), 6.92 (2H, d, J = 8.1 Hz), 6.75 (1H, d, J = 7.8 Hz), 6.46 (1H, s), 6.40 (1H, d, J = 7.2 Hz), 5.01 (2H, s), 4.91 (2H, s), 4.30 (2H, t, J = 6.0 Hz), 2.81 (2H, t, J = 6.0 Hz); 13C NMR (75 MHz, CDCl₃): δ 160.2, 159.1, 144.2, 138.1, 136.9, 136.6, 136.5, 132.3, 129.8, 128.8, 128.7, 128.2, 128.1, 127.6, 127.5, 121.4, 120.2, 115.8, 114.3, 112.9, 108.2, 71.2, 70.2, 70.1, 34.6; HRMS (ESI+) Calc. for C₃₃H₂₈N₄O₃ [M + Na]⁺ 551.2054, found 551.2052. IR (neat, cm⁻¹): ν 3032, 2917, 2853, 1607, 1506, 1243, 694.
**General procedure R** for the debenzylation

To a solution of benzyl protected substrates (1.0 equiv.) in MeOH (0.05 M) was added Pd/C (10 wt. %) under nitrogen atmosphere, and the mixture was stirred at RT for 2 h under 1 atm of hydrogen. After completion monitored by TLC, the Pd-C was filtered through Celite®, and the solvent was removed under reduced pressure.

**4-(5-(4-hydroxyphenethoxy)-1,2,4-triazolo[4,3-a]pyrazin-3-yl)phenol (80a)**

This compound was prepared according to **general procedure R** by treating 86a (700 mg, 1.3 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 50:1 → 10:1) to give the product as a pale yellow solid (317 mg, 70%). **m.p.** 235–237 °C; *R*<sub>f</sub> (CH₂Cl₂/MeOH 10:1): 0.25; **¹H NMR** (400 MHz, d-DMSO): δ 9.90 (1H, br.s), 9.17 (1H, br.s), 8.98 (1H, s), 7.53 (2H, dd, *J* = 2.0, 6.4 Hz), 7.50 (1H, s), 6.90 (2H, dd, *J* = 2.0, 6.8 Hz), 6.71 (2H, dd, *J* = 2.0, 6.4 Hz), 6.56 (2H, dd, *J* = 2.0, 6.8 Hz), 4.38 (2H, t, *J* = 6.4 Hz), 2.77 (2H, t, *J* = 6.4 Hz); **¹³C NMR** (100 MHz, d-DMSO): δ 158.8, 155.9, 147.2, 146.6, 143.9, 135.0, 132.3, 129.8, 127.3, 118.4, 115.0, 114.4, 108.5, 71.7, 33.1; **HRMS** (ESI+) Calc. for C<sub>19</sub>H<sub>16</sub>N<sub>4</sub>O<sub>3</sub> [M + Na]<sup>+</sup> 371.1115, found 371.1112. **IR** (neat, cm⁻¹): ν 3098, 2919, 1608, 1489, 1237, 826. **HPLC**: 98.8% (HPLC Method A), RT: 16.6 min.
Chapter 6

3-(2-((3-(4-hydroxyphenyl)-[1,2,4]triazolo[4,3-a]pyrazin-5-yl)oxy)ethyl)phenol (80b)

This compound was prepared according to general procedure R by treating 86b (700 mg, 1.3 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 50:1 → 10:1) to give the product as a pale yellow solid (303 mg, 67%). m.p. 245–246 °C; R_f (CH₂Cl₂/MeOH 10:1): 0.25; ¹H NMR (500 MHz, d-DMSO): δ 9.89–9.84 (1H, br.s), 9.26 (1H, br.s), 8.99 (1H, s), 7.54 (1H, s), 7.50 (2H, d, J = 9.0 Hz), 6.98 (1H, t, J = 8.0 Hz), 6.86 (2H, d, J = 9.0 Hz), 6.58 (1H, d, J = 8.0 Hz), 6.54 (1H, s), 6.34 (1H, d, J = 8.0 Hz), 4.43 (2H, t, J = 7.0 Hz), 2.80 (2H, t, J = 7.0 Hz); ¹³C NMR (125 MHz, d-DMSO): δ 159.4, 158.0, 147.9, 147.3, 144.5, 139.1, 135.7, 132.9, 129.8, 120.2, 118.9, 116.2, 115.0, 114.1, 109.3, 71.9, 34.5; HRMS (ESI⁺) Calc. for C₁₉H₁₆N₄O₃ [M + H]⁺ 349.1295, found 349.1295. IR (neat, cm⁻¹): ν 3102, 2956, 1608, 1510, 1439, 1242, 566. HPLC: 96.1% (HPLC Method A), RT: 16.8 min.

6-chloropyrazine-2-carboxylic acid (88)

This compound was prepared following a literature procedure.²⁵⁴ To a solution of Na₂WO₄•2H₂O (0.025 equiv., 0.24 mmol) in H₂O (0.05 M) was added aq. H₂O₂ (30%, 1.1 equiv., 1 mL) at RT, followed by the addition of H₂SO₄ (1 M) to make pH = 2, then pyrazine-2-carboxylic acid 87 (1.0 equiv., 8 mmol) was added, and the resulting suspension was stirred at 80 °C for 2 h. After completion monitored by TLC, the mixture was settled at RT until the precipitate was obtained, filtered and washed by H₂O and dried under reduced pressure to get white solid compound 3-carboxypyrazine 1-oxide without further purification. A solution of 3-
carboxypyrazine 1-oxide (1.0 equiv., 7 mmol) in POCl₃ (3 mL) in sealed tube was heated to 120 °C and stirred for 2 h. After completion monitored by TLC, the reaction mixture was cooled to RT and poured to ice water (20 mL), neutralised with sat. Na₂CO₃ (aq.) and extracted with ethyl acetate (3 x 20 mL). The combined organic layers were dried over MgSO₄ and concentrated in vacuo. The crude product was recrystallised from ethanol, filtered and washed by hexane, dried to get the product as a white solid (880 mg, 70%). m.p. 158–160 °C; Rf (hexane/ethyl acetate 1:1): 0.25; ¹H NMR (500 MHz, d-DMSO): δ 9.13 (1H, s), 9.00 (1H, s); ¹³C NMR (125 MHz, d-DMSO): δ 163.8, 148.0, 147.8, 143.7, 143.3. The spectroscopic data matched that reported in the literature.

3-((tert-butyldimethylsilyl)oxy)aniline (34b)

This compound was prepared according to general procedure E by treating 3-aminophenol (1.09 g, 0.01 mol), imidazole (1.60 g, 0.025 mol) and TBSCl (2.27 g, 0.015 mol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as pale yellow oil (2.07 g, 93%). Rf (hexane/ethyl acetate 2:1): 0.45; ¹H NMR (500 MHz, CDCl₃): δ 6.99 (1H, t, J = 8.0 Hz), 6.31–6.26 (2H, m), 6.21–6.20 (1H, m), 3.46 (2H, br.s), 0.99 (9H, s), 0.20 (6H, s); ¹³C NMR (125 MHz, CDCl₃): δ 156.9, 147.8,130.1, 110.7, 108.7, 107.3, 25.9, 18.3, −4.2. The spectroscopic data matched that reported in the literature.

General procedure S for amide coupling reactions

The compounds were prepared following a literature procedure. To a solution of carboxylic acid (1.0 equiv.) in dry CH₂Cl₂ (0.1 M) was added oxalyl chloride (1.5
equiv.) and DMF (0.1 equiv.) at 0 °C, and the resulting mixture was allowed to warm to RT for 2 h. Then the volatiles were concentrated under reduced pressure, and the residue was dissolved in dry CH₂Cl₂ (0.1 M), followed by the addition of aniline (1.0 equiv.) and triethylamine (1.1 equiv.), and the resulting mixture was stirred at RT for another 2 h. After completion monitored by TLC, the reaction mixture was quenched with H₂O and extracted with CH₂Cl₂ (3 x 20 mL), and washed with 1% HCl solution, and then sat. Na₂CO₃ (aq.). The combined organic layers were dried over MgSO₄ and concentrated in vacuo.

\[ \text{N-} (4-((\text{tert-butyldimethylsilyl})\text{oxy})\text{phenyl}) - 6-\text{chloropyrazine-2-carboxamide (89a)} \]

This compound was prepared according to general procedure S by treating 88 (316 mg, 2 mmol) with 34a (446 mg, 2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as an off-white solid (582 mg, 80%). m.p. 115–116 °C; \( R_f \) (hexane/ethyl acetate 4:1): 0.55; \(^1\)H NMR (500 MHz, CDCl₃): \( \delta \) 9.37 (1H, s), 9.30 (1H, br.s), 8.78 (1H, s), 7.59 (2H, dddd, \( J = 3.0, 5.5, 10.0 \) Hz), 6.86 (2H, dddd, \( J = 3.0, 5.5, 10.0 \) Hz), 0.98 (9H, s), 0.20 (6H, s); \(^{13}\)C NMR (125 MHz, CDCl₃): \( \delta \) 159.2, 153.1, 147.5, 147.4, 144.3, 142.3, 130.7, 121.6, 120.6, 25.8, 18.3, -4.3; HRMS (ESI+) Calc. for C₁₇H₂₂ClN₃O₂Si [M + Na]⁺ 386.1062, found 386.1061. IR (neat, cm⁻¹): \( \tilde{\nu} \) 3374, 2950, 2930, 2855, 1686, 1591, 1261, 845.

\[ \text{N-(3-((}\text{tert-butyldimethylsilyl})\text{oxy})\text{phenyl}) - 6-\text{chloropyrazine-2-carboxamide (89b)} \]
This compound was prepared according to **general procedure S** by treating **88** (316 mg, 2 mmol) with **34b** (446 mg, 2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as yellow oil (619 mg, 85%). $R_f$ (hexane/ethyl acetate 4:1): 0.55; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 9.41 (1H, s), 9.35 (1H, br.s), 8.81 (1H, s), 7.44 (1H, s), 7.28–7.21 (2H, m), 6.70–6.68 (1H, m), 1.02 (9H, s), 0.26 (6H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 159.4, 156.5, 147.6, 147.5, 144.2, 142.3, 138.0, 129.9, 117.0, 113.0, 112.1, 25.8, 18.3, −4.3; HRMS (ESI+) Calc. for C$_{17}$H$_{22}$Cl$_3$N$_3$O$_2$Si [M + Na]$^+$ 386.1062, found 386.1060. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3374, 2954, 2929, 2857, 1686, 1598, 1534, 1166, 779.

4-((tert-butyldimethylsilyloxy)benzaldehyde (90)

![4-((tert-butyldimethylsilyloxy)benzaldehyde](image)

This compound was prepared according to **general procedure E** by treating 4-hydroxybenzaldehyde (488 mg, 4 mmol), imidazole (640 mg, 10 mmol) and TBSCl (906 mg, 6 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as colorless oil (802 mg, 85%). $R_f$ (hexane/ethyl acetate 2:1): 0.45; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 9.88 (1H, s), 7.79 (2H, d, $J = 7.8$ Hz), 6.94 (2H, d, $J = 7.8$ Hz), 0.99 (9H, s), 0.24 (6H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 191.0, 161.6, 132.0, 130.5, 120.6, 25.7, 18.4, −4.2. The spectroscopic data matched that reported in the literature.$^{323}$

6-(2-(4-((tert-butyldimethylsilyl)oxy)benzylidene)hydrazinyl)-N-(4-((tert-butyldimethylsilyl)oxy)phenyl)pyrazine-2-carboxamide (91a)

![6-(2-(4-((tert-butyldimethylsilyl)oxy)benzylidene)hydrazinyl)-N-(4-((tert-butyldimethylsilyl)oxy)phenyl)pyrazine-2-carboxamide](image)

This compound was prepared according to **general procedure O** by treating **89a** (364 mg, 1 mmol) with **90** (236 mg, 1 mmol). Pale yellow solid (491 mg, 85%). m.p.
230–232 °C; \( R_f \) (hexane/ethyl acetate 1:1): 0.60; \(^1\)H NMR (400 MHz, CDCl₃): \( \delta \) 9.32 (1H, s), 8.94 (1H, s), 8.92 (1H, s), 8.31 (1H, s), 7.80 (1H, s), 7.62-7.55 (4H, m), 6.90 (2H, dd, \( J = 2.0, 6.8 \) Hz), 6.85 (2H, dd, \( J = 2.0, 6.8 \) Hz), 1.00 (9H, s), 0.99 (9H, s), 0.23 (6H, s), 0.20 (6H, s); \(^{13}\)C NMR (100 MHz, CDCl₃): \( \delta \) 160.9, 157.7, 152.8, 150.5, 142.4, 141.3, 134.9, 134.8, 131.2, 128.5, 127.2, 121.4, 120.7, 120.6, 25.8 (two overlapping signals), 18.4, 18.3, –4.2, –4.3; HRMS (ESI+) Calc. for C\(_{30}\)H\(_{43}\)N\(_5\)O\(_3\)Si\(_2\) [M + Na]\(^+\) 600.2797, found 600.2794. IR (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3374, 2953, 2928, 2856, 1675, 1582, 1505, 1253, 911.

6-(2-(4-((tert-butyldimethylsilyl)oxy)benzylidene)hydrazinyl)-N-(3-((tert-butyldimethylsilyl)oxy)phenyl)pyrazine-2-carboxamide (91b)

This compound was prepared according to general procedure O by treating \( 89b \) (364 mg, 1 mmol) with \( 90 \) (236 mg, 1 mmol). Off-white solid (451 mg, 78%). m.p. 212–214 °C; \( R_f \) (hexane/ethyl acetate 1:1): 0.60; \(^1\)H NMR (400 MHz, CDCl₃): \( \delta \) 9.36 (1H, s), 8.95 (1H, s), 8.93 (1H, s), 8.27 (1H, s), 7.81 (1H, s), 7.60 (2H, d, \( J = 8.8 \) Hz), 7.41 (1H, s), 7.23–7.22 (2H, m), 6.90 (2H, d, \( J = 8.8 \) Hz), 6.67–6.64 (1H, m), 1.01 (9H, s), 1.00 (9H, s), 0.25 (6H, s), 0.24 (6H, s); \(^{13}\)C NMR (100 MHz, CDCl₃): \( \delta \) 161.1, 157.7, 156.5, 150.5, 142.5, 138.6, 135.0, 132.4, 129.9, 128.5, 127.2, 120.7, 120.1, 116.6, 112.8, 111.9, 25.8 (two overlapping signals), 18.4 (two overlapping signals), –4.2 (two overlapping signals); HRMS (ESI+) Calc. for C\(_{30}\)H\(_{43}\)N\(_5\)O\(_3\)Si\(_2\) [M + Na]\(^+\) 600.2797, found 600.2794. IR (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3374, 2956, 2928, 2856, 1687, 1598, 1531, 1258, 828.
This compound was prepared according to **general procedure P** by treating 91a (289 mg, 0.5 mmol) with PhI(OAc)$_2$ (161 mg, 0.5 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 1:1) to give the product as a pale yellow solid (250 mg, 87%). **m.p.** 99–101 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.25; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 9.17 (1H, s), 9.08 (1H, s), 8.09 (1H, s), 7.43 (2H, d, $J = 8.4$ Hz), 7.18 (2H, d, $J = 8.8$ Hz), 6.80 (2H, d, $J = 8.4$ Hz), 6.70 (2H, d, $J = 8.8$ Hz), 0.97 (9H, s), 0.96 (9H, s), 0.17 (6H, s), 0.14 (6H, s); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 158.2, 156.8, 153.4, 145.7, 145.6, 130.6, 130.3, 130.0, 127.9, 125.4, 121.7, 120.5, 120.4, 119.1, 25.8, 25.7, 18.3 (two overlapping signals), −4.3, −4.4; HRMS (ESI+) Calc. for C$_{30}$H$_{41}$N$_5$O$_3$Si$_2$ [M + Na]$^+$ 598.2640, found 598.2639. IR (neat, cm$^{-1}$): $\tilde{v}$ 2956, 2929, 2857, 1676, 1607, 1535, 1251, 912, 778.

This compound was prepared according to **general procedure P** by treating 91b (289 mg, 0.5 mmol) with PhI(OAc)$_2$ (161 mg, 0.5 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 1:1) to give the product as a pale yellow solid (230 mg, 80%). **m.p.** 95–97 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.25; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 9.26 (1H, s), 9.10 (1H, s), 8.11 (1H, s), 7.42 (2H, d, $J =$
8.4 Hz), 7.07–7.03 (2H, m), 6.88 (1H, d, J = 8.4 Hz), 6.77 (2H, d, J = 8.4 Hz), 6.59 (1H, dd, J = 2.4, 8.4 Hz), 0.95 (9H, s), 0.94 (9H, s), 0.16 (6H, s), 0.10 (6H, s); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 158.1, 156.8, 156.1, 148.4, 145.4, 145.3, 137.8, 130.7, 129.8, 129.6, 125.3, 120.3, 118.7, 117.1, 112.7, 111.8, 25.7, 25.6, 18.2, 18.1, −4.4, −4.5; HRMS (ESI+) Calc. for C$_{30}$H$_{41}$N$_5$O$_3$Si$_2$ [M + Na]$^+$ 598.2640, found 598.2639.

IR (neat, cm$^{-1}$): $\tilde{\nu}$ 2955, 2929, 2857, 1683, 1607, 1254, 837, 778.

$N$-3-bis(4-hydroxyphenyl)-[1,2,4]triazolo[4,3-a]pyrazine-5-carboxamide (81a)

\[
\text{OH} \\
\text{HN} \\
\text{O} \\
\text{OH} \\
\text{HN} \\
\text{N} \quad \text{N} \\
\text{N} \\
\text{OH} \\
\text{HN}
\]

The compound was prepared according to general procedure G by treating 92a (200 mg, 0.35 mmol) with TBAF (0.7 mL, 0.70 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 $\rightarrow$ 20:1) to give the product as a pale yellow solid (118 mg, 97%). m.p. 289–291 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.20; $^1$H NMR (400 MHz, $d$-DMSO): $\delta$ 10.54 (1H, s), 9.80–9.79 (1H, br.s), 9.55 (1H, s), 9.36–9.30 (1H, br.s), 8.19 (1H, s), 7.40 (2H, dd, J = 2.0, 6.8 Hz), 7.11 (2H, dd, J = 2.0, 6.8 Hz), 6.72 (2H, dd, J = 2.0, 6.8 Hz), 6.66 (2H, dd, J = 2.0, 6.8 Hz); $^{13}$C NMR (100 MHz, $d$-DMSO): $\delta$ 159.4, 157.2, 154.7, 148.4, 146.1, 146.0, 130.2, 129.5, 125.6, 122.2, 118.1, 115.6, 115.4, 115.2; HRMS (ESI+) Calc. for C$_{18}$H$_{13}$N$_3$O$_3$ [M + H]$^+$ 348.1091, found 348.1091. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3184, 2920, 1668, 1512, 1276, 818. HPLC: 95.5% (HPLC Method A), RT: 13.6 min.
The compound was prepared according to **general procedure G** by treating 92b (200 mg, 0.35 mmol) with TBAF (0.7 mL, 0.70 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 → 20:1) to give the product as a pale yellow solid (115 mg, 95%). **m.p.** 239–242 °C; **R$_f$** (CH$_2$Cl$_2$/MeOH 20:1): 0.20; **$^1$H NMR** (500 MHz, d-DMSO): $\delta$ 10.68 (1H, br.s), 9.57 (1H, s), 8.22 (1H, s), 7.40 (2H, d, $J = 2.0$, 6.5 Hz), 7.05 (1H, t, $J = 8.0$ Hz), 6.94 (1H, t, $J = 2.0$ Hz), 6.77–6.75 (1H, m), 6.71 (2H, dd, $J = 2.0$, 6.5 Hz), 6.50–6.48 (1H, m); **$^{13}$C NMR** (125 MHz, d-DMSO): $\delta$ 159.6, 158.0, 157.8, 148.6, 146.4, 146.3, 139.2, 130.6, 130.2, 129.8, 125.5, 118.1, 115.7, 112.2, 111.2, 107.6; **HRMS** (ESI+) Calc. for C$_{19}$H$_{13}$N$_5$O$_3$ [M + Na]$^+$ 384.0911, found 384.0911. **IR** (neat, cm$^{-1}$): $\tilde{\nu}$ 3245, 2922, 2852, 1622, 1602, 1459, 1222, 739. **HPLC**: 95.6% (HPLC Method A), RT: 14.6 min.

**2-(2-bromo-5-methoxyphenyl)-1,3-dioxolane (95)**

This compound was prepared following a literature procedure.$^{324}$ To a solution of 2-bromo-5-methoxybenzaldehyde 94 (1.0 g, 4.6 mmol) in toluene (15 mL) was added ethylene glycol (1.26 mL, 23 mmol) and $p$-toluenesulfonic acid (9.5 mg, 0.05 mmol), and the resulting mixture was heated at reflux under a Dean-Stark water separator for 24 h. After completion monitored by TLC, the cooled mixture was diluted with ethyl acetate (10 mL) and washed with H$_2$O and brine. The organic layer was dried over MgSO$_4$ and concentrated *in vacuo*. The crude product was purified by flash chromatography (hexane/ethyl acetate 20:1) to give the product as colourless oil (1.1 g, 95%); **R$_f$** (hexane/ethyl acetate 10:1): 0.35; **$^1$H NMR** (400 MHz, CDCl$_3$): $\delta$ 7.50 (1H, d, $J = 8.8$ Hz), 7.10 (1H, d, $J = 2.4$ Hz), 6.87 (1H, dd, $J = 2.4$, 8.8 Hz), 6.04 (1H,
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s), 4.15–4.13 (2H, m), 4.06–4.04 (2H, m), 3.79 (3H, s); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 160.6, 128.7, 128.6, 123.4, 118.0, 113.5, 102.6, 65.4, 55.4. The spectroscopic data matched that reported in the literature.$^{324}$

N-(2-(1,3-dioxolan-2-yl)-4-methoxyphenyl)-4-methoxy-2-nitroaniline (96)

This compound was prepared following a literature procedure.$^{325}$ To a mixture of 95 (518 mg, 2 mmol), 4-methoxy-2-nitroaniline (336 mg, 2 mmol), Pd(OAc)$_2$ (45 mg, 0.2 mmol), Cs$_2$CO$_3$ (978 mg, 3 mmol) and (+)-BINAP (62.3 mg, 0.1 mmol) in toluene (15 mL) was purged with nitrogen, and the resulting mixture was stirred at 120 °C for 3 h. After completion monitored by TLC, the cooled mixture was washed with H$_2$O and brine. The organic layer was dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 8:1) to give the product as orange oil (616 mg, 89%); $R_f$ (hexane/ethyl acetate 4:1): 0.4; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 9.76 (1H, s), 7.59 (1H, d, $J = 3.0$ Hz), 7.38 (1H, d, $J = 8.5$ Hz), 7.35 (1H, d, $J = 9.0$ Hz), 7.04 (1H, dd, $J = 3.0$, 9.0 Hz), 6.89 (1H, d, $J = 2.5$ Hz), 6.63 (1H, dd, $J = 2.5$, 8.5 Hz), 5.75 (1H, s), 4.14–4.11 (2H, m), 4.00–3.98 (2H, m), 3.78 (3H, s), 3.75 (3H, s); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 161.1, 151.7, 140.1, 136.6, 134.2, 130.3, 125.7, 121.8, 119.0, 108.9, 108.5, 107.4, 103.3, 65.3, 56.0, 55.5. HRMS (ESI+) Calc. for C$_{17}$H$_{18}$N$_2$O$_6$ [M + Na]$^+$ 369.1057, found 369.1060. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3349, 2954, 2890, 1613, 1584, 1505, 1279, 1215, 1032, 794.

5-methoxy-2-((4-methoxy-2-nitrophenyl)amino)benzaldehyde (97)
This compound was prepared following a literature procedure. To a solution of 96 (346 mg, 1 mmol) in CHCl$_3$ (15 mL) was added TFA (459 µL, 6 mmol) dropwise, and the resulting mixture was stirred at RT for 30 min. After completion monitored by TLC, the mixture was quenched with H$_2$O and extracted with CH$_2$Cl$_2$ (3 x 10 mL) and brine. The combined organic layers were dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 4:1) to give the product as an orange solid (272 mg, 90%); m.p. 161–163 °C; $R_f$ (hexane/ethyl acetate 4:1): 0.35; $^1$H NMR (400 MHz, CDCl$_3$): δ 11.17 (1H, s), 9.83 (1H, s), 7.64 (1H, d, $J$ = 9.2 Hz), 7.59 (1H, d, $J$ = 3.2 Hz), 7.55 (1H, d, $J$ = 8.8 Hz), 7.15 (1H, dd, $J$ = 3.2, 9.2 Hz), 6.70 (1H, d, $J$ = 2.0 Hz), 6.52 (1H, dd, $J$ = 2.0, 8.8 Hz), 3.87 (3H, s), 3.80 (3H, s); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 192.0, 165.4, 154.9, 147.0, 141.3, 139.0, 129.9, 124.6, 122.4, 116.7, 109.0, 106.3, 99.1, 56.1, 55.7. HRMS (ESI+) Calc. for C$_{15}$H$_{14}$N$_2$O$_5$ [M + Na]$^+$ 325.0795, found 325.0799. IR (neat, cm$^{-1}$): ν 3282, 2842, 1644, 1611, 1564, 1470, 1413, 139.0, 129.9, 124.6, 122.4, 116.7, 109.0, 106.3, 99.1, 56.1, 55.7.

2,8-dimethoxy-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepine (98)

This compound was prepared according to general procedure R by treating 97 (302 mg, 1 mmol) with Pd/C (30 mg) and H$_2$. The crude product was purified by flash chromatography (hexane/ethyl acetate 1:1) to get the desired compound as a pale yellow solid (192 mg, 75%). m.p. 170–172 °C; $R_f$ (hexane/ethyl acetate 2:1): 0.30; $^1$H NMR (400 MHz, d$_2$-DMSO): δ 7.84 (1H, br.s), 6.82 (1H, d, $J$ = 7.6 Hz), 6.72 (1H, d, $J$ = 8.8 Hz), 6.41 (1H, s), 6.29 (1H, d, $J$ = 2.4 Hz), 6.23 (1H, s), 6.13 (1H, s), 5.58 (1H, br.s), 4.00 (2H, s), 3.68 (3H, s), 3.62 (3H, s); $^{13}$C NMR (100 MHz, d$_2$-DMSO): δ 159.4, 152.9, 146.1, 141.0, 129.6, 127.5, 119.6, 119.2, 105.5, 104.5, 102.9, 102.0, 55.4, 55.2, 50.8; HRMS (ESI+) Calc. for C$_{15}$H$_{16}$N$_2$O$_2$ [M + Na]$^+$ 279.1104, found 279.1109. IR (neat, cm$^{-1}$): ν 3333, 3278, 2992, 2833, 1617, 1598, 1505, 1451, 1219, 1036, 825, 795, 519.
10,11-dihydro-5H-dibenzo[b,e][1,4]diazepine-2,8-diol (93)

This compound was prepared according to **general procedure D** by treating 98 (128 me, 0.5 mmol) with BBr₃ (3 mL, 3 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 20:1) to give the product as a brown solid (57 mg, 50%). **m.p.** 256–258 °C; **Rf** (CH₂Cl₂/MeOH 10:1): 0.40; **¹H NMR** (500 MHz, d-DMSO): δ 10.99-10.90 (1H, br.s), 9.42 (1H, br.s), 8.64 (1H, s), 7.10 (1H, d, J = 9.0 Hz), 7.03 (1H, d, J = 8.0 Hz), 6.85 (1H, s), 6.75 (1H, d, J = 8.0 Hz), 6.50 (1H, s), 6.22 (1H, d, J = 8.0 Hz), 4.38 (2H, s); **¹³C NMR** (125 MHz, d-DMSO): δ 158.4, 150.0, 143.6, 131.6, 129.8, 124.0, 121.1, 117.0, 110.0, 109.4, 106.5, 103.5, 51.8; **HRMS** (ESI+) Calc. for C₁₃H₁₂N₂O₂ [M + Na]⁺ 251.0791, found 251.0792. **IR** (neat, cm⁻¹): ν 3385, 3312, 3286, 1612, 1517, 1470, 1222, 835, 806, 524. **HPLC**: 97.6% (HPLC Method C), RT: 12.4 min.

### 6.3 Synthesis of compounds from Chapter 3

5-bromo-N-(4-((tert-butyldimethylsilyl)oxy)phenyl)pyridin-2-amine (103)

To a solution of 5-bromopyridin-2-amine 102b (1.0 equiv., 1 mmol) in MeOH (0.05 M) was added boronic acid 40 (1.2 equiv., 1.2 mmol) at RT, followed by the addition of Cu(OAc)₂ (0.1 equiv., 0.1 mmol). The reaction mixture was stirred for 12 h in the air atmosphere at RT. After completion monitored by TLC, the metal solid was filtered through Celite®, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as a white solid (227 mg, 60%). **m.p.** 131–132 °C; **Rf** (hexane/ethyl acetate 10:1): 0.40; **¹H NMR** (400 MHz, CDCl₃): δ 8.16 (1H, d, J = 2.0 Hz), 7.51 (1H, dd, J = 2.0, 8.8 Hz), 7.14 (2H, dd, J = 2.0, 6.4 Hz), 6.83 (2H, dd, J = 2.0, 6.4 Hz), 6.60 (1H, d, J = 8.8 Hz), 6.55 (1H, s), 0.99 (9H, s), 0.20 (6H, s); **¹³C NMR** (100
MHz, CDCl$_3$): $\delta$ 155.8, 152.8, 148.4, 140.5, 133.0, 124.2, 121.0, 109.0, 108.4, 25.8, 18.4, −4.3; HRMS (ESI+) Calc. for C$_{17}$H$_{23}$BrN$_2$OSi [M + Na]$^+$ 401.0655/403.0635, found 401.0659/403.0639. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3244, 2947, 2855, 1605, 1525, 1251, 904, 778.

$N_{,5}$-bis(4-((tert-butyldimethylsilyl)oxy)phenyl)pyridin-2-amine (104)

This compound was prepared according to general procedure F by treating 103 (190 mg, 0.5 mmol) with boronic acid 40 (189 mg, 0.75 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the product as a white solid (203 mg, 80%), m.p. 135–136 °C; $R_f$ (hexane/ethyl acetate 5:1): 0.45; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.26 (1H, s), 7.71 (1H, d, $J = 9.0$ Hz), 7.37–7.34 (3H, m), 7.18 (2H, d, $J = 8.1$ Hz), 6.90 (2H, d, $J = 8.1$ Hz), 6.86–6.79 (3H, m), 1.00 (18H, s), 0.22 (6H, s), 0.21 (6H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 155.6, 155.1, 153.0, 142.9, 137.8, 132.7, 130.5, 127.5, 127.4, 124.4, 121.1, 120.8, 108.3, 25.8 (two overlapping signals), 18.4 (two overlapping signals), −4.2 (two overlapping signals); HRMS (ESI+) Calc. for C$_{29}$H$_{42}$N$_2$O$_2$Si$_2$ [M + H]$^+$ 507.2858, found 507.2861. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3232, 2962, 2856, 1603, 1508, 1249, 911, 822, 775.

4-(6-((4-hydroxyphenyl)amino)pyridin-3-yl)phenol (99a)

This compound was prepared according to general procedure G by treating 104 (114 mg, 0.2 mmol) with TBAF (0.4 mL, 0.4 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as a pale yellow solid (53 mg, 95%). m.p. 199–200 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.30; $^1$H NMR (400 MHz, d-MeOD): $\delta$ 8.18 (1H, dd, $J = 0.8$, 2.8 Hz), 7.70 (1H, dd, $J = 2.8$, 8.8 Hz), 7.37 (2H, ddd, $J = 2.8$, 4.8, 9.6 Hz), 7.21 (2H, ddd, $J = 3.2$, 5.6, 10.0 Hz), 171
6.84 (2H, ddd, J = 2.8, 4.8, 9.6 Hz), 6.78 (2H, ddd, J = 3.2, 5.6, 10.0 Hz), 6.74 (1H, dd, J = 0.8, 8.8 Hz); \(^{13}\text{C} \text{NMR} \ (100 \text{ MHz, } d\text{-MeOD}): \delta \ 157.9, 157.4, 154.5, 145.5, 137.3, 134.1, 130.8, 128.4, 128.1, 124.5, 116.8, 116.7, 110.1; \ \text{HRMS (ESI+)} \ \text{Calc. for} \ C_{17}H_{14}N_{2}O_{2} \ [M + H]^+ \ 279.1128, \ \text{found} \ 279.1131. \ \text{IR (neat, cm}^{-1}) : \tilde{\nu} \ 3217, 2953, 1607, 1490, 1218, 815. \ \text{HPLC: >99.9% (HPLC Method A), RT: 16.2 min.}

**General procedure T for reductive aminations**

![Diagram of reaction](image)

To a solution of aldehyde (1.0 equiv.) in CF\(_3\)CH\(_2\)OH (0.04 M) was added amine (1.0 equiv.), and the mixture was stirred at RT. After 30 min, NaBH(OAc)\(_3\) (1.5 equiv.) was added and stirred for 12 h at RT. After completion monitored by TLC, the solvent was removed under reduced pressure.

**4-(((5-bromopyridin-2-yl)amino)methyl)phenol (105)**

![Structural formula of compound](image)

This compound was prepared according to **general procedure T** by treating 5-bromopyridin-2-amine 102b (173 mg, 1 mmol) with 4-hydroxybenzaldehyde (122 mg, 1 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 \(\rightarrow\) 3:1) to give the product as a white solid (190 mg, 68%). **m.p.** 125–128 °C; \(R_f\) (hexane/ethyl acetate 4:1): 0.30; \(^{1}\text{H} \text{NMR} \ (400 \text{ MHz, } d\text{-MeOD}): \delta \ 7.98 \ (1\text{H, d, } J = 2.4 \text{ Hz}), \ 7.49 \ (1\text{H, dd, } J = 2.4, 8.8 \text{ Hz}), \ 7.17 \ (2\text{H, dd, } J = 2.0, 6.8 \text{ Hz}), \ 6.75 \ (2\text{H, dd, } J = 2.0, 6.8 \text{ Hz}), \ 6.48 \ (1\text{H, d, } J = 8.8 \text{ Hz}), \ 4.36 \ (2\text{H, s}); \ ^{13}\text{C} \text{NMR} \ (100 \text{ MHz, } d\text{-MeOD}): \delta \ 159.0, 157.6, 148.5, 140.9, 131.3, 129.8, 116.2, 111.4, 106.9, 46.0; \ \text{HRMS (ESI+)} \ \text{Calc. for} \ C_{12}H_{11}BrN_{2}O \ [M + Na]^+ \ 300.9947/302.9926, \ \text{found} \ 300.9950/302.9930. \ \text{IR (neat, cm}^{-1}) : \tilde{\nu} \ 3218, 1594, 1343, 815.
4-(6-((4-hydroxybenzyl)amino)pyridin-3-yl)phenol (99b)

This compound was prepared according to general procedure F by treating 105 (70 mg, 0.25 mmol) with (4-hydroxyphenyl)boronic acid (52 mg, 0.375 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as a pale yellow solid (51 mg, 70%), m.p. 193–196 °C; Rf (hexane/ethyl acetate 1:1): 0.35; \(^1\)H NMR (300 MHz, d-MeOD): \(\delta\) 8.13 (1H, s), 7.65 (1H, d, \(J = 8.7\) Hz), 7.34 (2H, d, \(J = 8.1\) Hz), 7.20 (2H, d, \(J = 8.1\) Hz), 6.85 (2H, d, \(J = 8.1\) Hz), 6.76 (2H, d, \(J = 8.1\) Hz), 6.58 (1H, d, \(J = 8.7\) Hz), 4.41 (2H, s); \(^{13}\)C NMR (75 MHz, d-MeOD): \(\delta\) 158.9, 157.7, 157.5, 145.2, 137.2, 131.6, 131.0, 129.7, 129.1, 127.1, 116.7, 116.2, 109.6, 46.3; HRMS (ESI+) Calc. for (C\(_{18}\)H\(_{16}\)N\(_2\)O\(_2\))\(_2\) [M + Na\(^+\)] 607.2316, found 607.2321. IR (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3211, 3025, 1613, 1508, 1247, 822, 529. HPLC: >99.9% (HPLC Method B), RT: 16.2 min.

3-(4-(benzyloxy)phenyl)propanal (106)

This compound was prepared in two steps. **Step 1:** The formation of 3-(4-(benzyloxy)phenyl)propan-1-ol. This compound was prepared according to general procedure I by treating 4-(3-hydroxypropyl)phenol (608 mg, 4 mmol) with benzyl bromide (570 µL, 6 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 5:1) to give the intermediate as a white solid (920 mg, 95%). Rf (hexane/ethyl acetate 4:1): 0.30; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.48–7.33 (5H, m), 7.15 (2H, ddd, \(J = 2.8, 5.2, 9.6\) Hz), 6.95 (2H, ddd, \(J = 2.8, 5.2, 9.6\) Hz), 5.06 (2H, s), 3.66 (2H, t, \(J = 6.4\) Hz), 2.70–2.66 (2H, m), 2.19 (1H, s), 1.92–1.85 (2H, m); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 157.1, 137.3, 134.3, 129.4, 128.6, 127.9, 127.5, 114.9, 70.1, 62.1, 34.4, 31.2. The spectroscopic data matched that reported in the literature.\(^{327}\)
Step 2: The formation of product 106. This compound was prepared according to general procedure K by treating 3-(4-(benzyloxy)phenyl)propan-1-ol (920 mg, 3.8 mmol) with DMP (2.42 g, 5.7 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as a white solid (866 mg, 95%). $R_f$ (hexane/ethyl acetate 10:1): 0.50; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 9.81 (1H, s), 7.44–7.32 (5H, m), 7.11 (2H, d, $J = 8.1$ Hz), 6.91 (2H, d, $J = 8.1$ Hz), 5.05 (2H, s), 2.91 (2H, t, $J = 7.2$ Hz), 2.75 (2H, t, $J = 7.2$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 201.9, 157.5, 137.2, 132.8, 129.4, 128.7, 128.1, 127.6, 115.1, 70.2, 45.6, 27.4. The spectroscopic data matched that reported in the literature.  

$N$-($3$-(4-(benzyloxy)phenyl)propyl)-5-bromopyridin-2-amine (107)

This compound was prepared according to general procedure T by treating 5-bromopyridin-2-amine 102b (173 mg, 1 mmol) with aldehyde 106 (240 mg, 1 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1) to give the product as a white solid (353 mg, 89%). m.p. 97–98 °C; $R_f$ (hexane/ethyl acetate 10:1): 0.40; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.09 (1H, d, $J = 2.4$ Hz), 7.47–7.31 (6H, m), 7.10 (2H, ddd, $J = 2.8$, 5.2, 9.6 Hz), 6.91 (2H, ddd, $J = 2.8$, 5.2, 9.6 Hz), 6.23 (1H, d, $J = 8.8$ Hz), 5.05 (2H, s), 4.65 (1H, br.s), 3.24 (2H, t, $J = 6.4$ Hz), 2.66 (2H, t, $J = 7.6$ Hz), 1.91 (2H, tt, $J = 7.6$, 14.4 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 157.5, 157.3, 148.7, 139.9, 137.3, 133.9, 129.4, 128.7, 128.0, 127.6, 115.0, 108.0, 106.8, 70.2, 41.9, 32.4, 31.2; HRMS (ESI+) Calc. for C$_{21}$H$_{21}$BrN$_2$O [M + H]$^+$ 397.0910/399.0890, found 397.0915/399.0894. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3247, 2937, 1586, 1243, 815, 726.
4-(6-((3-(4-benzyloxy)phenyl)propyl)amino)pyridin-3-yl)phenol (108)

This compound was prepared according to general procedure F by treating 107 (199 mg, 0.5 mmol) with (4-hydroxyphenyl)boronic acid (103 mg, 0.75 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 3:1) to give the product as a pale yellow solid (154 mg, 75%). m.p. 138–140 °C; R_f (hexane/ethyl acetate 6:1): 0.30; 1H NMR (300 MHz, CDCl_3): δ 8.26 (1H, s), 7.63 (1H, d, J = 8.7 Hz), 7.44–7.34 (7H, m), 7.09 (2H, d, J = 8.1 Hz), 6.93–6.88 (4H, m), 6.41 (1H, d, J = 8.7 Hz), 5.03 (2H, s), 4.68 (1H, br.s), 3.30–3.28 (2H, m), 2.67 (2H, t, J = 7.2 Hz), 1.93 (2H, tt, J = 7.2, 13.8 Hz); 13C NMR (75 MHz, CDCl_3): δ 157.5, 157.3, 155.9, 145.3, 137.3, 136.6, 133.9, 130.5, 129.5, 128.7, 128.0, 127.6 (two overlapping signals), 126.3, 116.2, 115.0, 106.5, 70.2, 42.0, 32.4, 31.3; HRMS (ESI+) Calc. for C_{27}H_{26}N_{2}O_{2} [M + H]^+ 411.2067, found 411.2071. IR (neat, cm⁻¹): ν 3409, 3029, 2928, 2856, 1606, 1501, 1232, 811, 517.

4-(6-((3-(4-hydroxyphenyl)propyl)amino)pyridin-3-yl)phenol (99d)

This compound was prepared according to general procedure R by treating 108 (100 mg, 0.24 mmol) with Pd/C (10 mg) and H_2. The crude product was purified by flash chromatography (hexane/ethyl acetate 1:1) to give the product as a pale yellow solid (69 mg, 90%). m.p. 217–219 °C; R_f (hexane/ethyl acetate 1:1): 0.40; 1H NMR (400 MHz, d-DMSO): δ 9.37 (1H, br.s), 9.11 (1H, br.s), 8.18 (1H, d, J = 2.4 Hz), 7.57 (1H, dd, J = 2.4, 8.8 Hz), 7.34 (2H, dd, J = 2.0, 6.4 Hz), 7.00 (2H, dd, J = 2.0, 6.4 Hz), 6.79 (2H, dd, J = 2.0, 6.4 Hz), 6.67 (2H, dd, J = 2.0, 6.4 Hz), 6.52–6.48 (2H, m),
3.25–3.20 (2H, m), 2.54 (2H, t, J = 7.6 Hz), 1.77 (2H, tt, J = 7.6, 14.8 Hz); $^{13}$C NMR (100 MHz, $d$-DMSO): $\delta$ 157.7, 156.1, 155.2, 144.7, 134.5, 131.9, 129.1 (two overlapping signals), 126.5, 123.7, 115.7, 115.0, 107.9, 40.5, 31.9, 31.2; HRMS (ESI+) Calc. for C$_{20}$H$_{20}$N$_2$O$_2$ [M + H]$^+$ 321.1598, found 321.1601. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3403, 3150, 2922, 2852, 1609, 1508, 1450, 1234, 813. HPLC: 99.0% (HPLC Method B), RT: 18.3 min.

5-bromo-$N$-(4-methoxyphenethyl)pyridin-2-amine (110)

To a solution of 5-bromo-$N$-(4-methoxyphenethyl)pyridin-2-amine 109 (200 mg, 0.65 mmol) in DMF (15 ml) was added K$_2$CO$_3$ (270 mg, 1.95 mmol) and 2-(4-methoxyphenyl)ethan-1-amine (148 µl, 0.78 mmol), and the mixture was stirred at 120 °C for 8 h. After completion monitored by TLC, the mixture was quenched with H$_2$O and extracted with ethyl acetate (3 x 15 mL). The combined organic layers were dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 4:1) to give the product as a brown solid (125 mg, 63%). m.p. 154–156 °C; $R_f$ (hexane/ethyl acetate 2:1): 0.40; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.09 (1H, dd, J = 0.4, 2.4 Hz), 7.44 (1H, dd, J = 2.4, 8.8 Hz), 7.12 (2H, add, J = 2.0, 2.9, 8.6 Hz), 6.84 (2H, add, J = 2.0, 2.9, 8.6 Hz), 6.25 (1H, dd, J = 0.4, 8.8 Hz), 4.60 (1H, br.s), 3.78 (3H, s), 3.48 (2H, dd, J = 6.9, 12.8 Hz), 2.84 (2H, t, J = 6.9 Hz); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 158.4, 157.3, 148.8, 139.8, 131.0, 129.8, 114.2, 108.4, 107.0, 55.4, 43.6, 34.6; HRMS (ESI+) Calc. for C$_{14}$H$_{15}$N$_2$OBr [M + H]$^+$ 307.0441/309.0420, found 307.0443/309.0423. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3334, 2954, 2930, 2835, 1635, 1502, 1241, 1026, 816, 528.

$N$-(4-methoxyphenethyl)-5-(4-methoxyphenyl)pyridin-2-amine (111)
This compound was prepared according to **general procedure F** by treating 110 (100 mg, 0.33 mmol) with (4-methoxyphenyl)boronic acid (76 mg, 0.50 mmol) in toluene/ethanol 3:1 at 90 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as an off-white solid (99 mg, 90%). **m.p.** 236–240 °C; **Rf** (hexane/ethyl acetate 4:1): 0.40; **1H NMR** (400 MHz, CDCl_3): δ 8.30 (1H, d, J = 2.0 Hz), 7.61 (1H, dd, J = 2.5, 8.6 Hz), 7.42 (2H, dd, J = 2.1, 6.7 Hz), 7.16 (2H, d, J = 8.6 Hz), 6.96 (2H, dd, J = 2.1, 6.7 Hz), 6.86 (2H, dd, J = 2.1, 6.7 Hz), 6.43 (1H, d, J = 8.6 Hz), 4.57 (1H, t, J = 5.6 Hz), 3.84 (3H, s), 3.80 (3H, s), 3.56 (2H, dd, J = 6.9, 12.9 Hz), 2.89 (2H, t, J = 6.9 Hz); **13C NMR** (100 MHz, CDCl_3): δ 158.8, 158.4, 157.7, 146.1, 136.0, 131.3, 129.9, 127.3, 126.1, 114.5, 114.2, 106.8, 55.5, 55.4, 43.8, 34.9; **HRMS** (ESI+) Calc. for C_{21}H_{22}N_2O_2 [M + Na]^+ 357.1573, found 357.1575. **IR** (neat, cm\(^{-1}\)): ν 3204, 2978, 2956, 1610, 1478, 1098, 876, 789.

**4-(6-((4-hydroxyphenethyl)amino)pyridin-3-yl)phenol (99c)**

This compound was prepared according to **general procedure D** by treating 111 (100 mg, 0.3 mmol) with BBr_3 (1.8 mL, 1.8 mmol). The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 100:1 → 40:1) to give the product as a white solid (54 mg, 59%). **m.p.** 156–158 °C; **Rf** (CH_2Cl_2/MeOH 20:1): 0.45; **1H NMR** (500 MHz, d-DMSO): δ 9.70 (1H, s), 9.23 (1H, s), 8.69 (1H, br s), 8.18 (1H, dd, J = 2.1, 9.3 Hz), 8.01 (1H, s), 7.48 (2H, ddd, J = 2.0, 2.9, 8.6 Hz), 7.13 (1H, s), 7.10 (2H, d, J = 8.6 Hz), 6.87 (2H, ddd, J = 2.0, 2.9, 8.6 Hz), 6.71 (2H, ddd, J = 2.0, 2.9, 8.6 Hz), 3.55 (2H, dd, J = 7.4, 11.5 Hz), 2.82 (2H, t, J = 7.4 Hz); **13C NMR** (125 MHz, d-DMSO): δ 158.2, 156.4, 151.7, 141.8, 131.9, 130.2, 128.7, 127.8, 125.5, 125.2, 116.4, 115.6, 114.1, 43.9, 33.6; **HRMS** (ESI+) Calc. for C_{19}H_{18}N_2O_2 [M + Na]^+ 329.1260, found 329.1262. **IR** (neat, cm\(^{-1}\)): ν 3513, 3256, 3009, 1662, 1514, 833. **HPLC**: 98.4% (HPLC Method A), RT: 16.5 min.
General procedure U for the synthesis of secondary amines

The compounds were prepared following a literature procedure.\(^{329}\) To a solution of glyoxal (40\% in ethanol, 0.9 equiv.) in ethanol (0.1 M) was added amine (1.1 equiv.), and the mixture was stirred at room temperature for 4 h. After completion monitored by TLC, the resulting precipitate was filtered and washed by hexane, and dried to get the intermediate diimine as a yellow solid without further purification. To a solution of diimine (1.0 equiv.) in a mixture solvent of CH\(_2\)Cl\(_2\)/MeOH (1:1 (v/v), 0.03 M) was added NaBH\(_4\) (1.0 equiv.) at 0 °C, and allowed to warm to RT for 30 min. The solvent was removed under reduced pressure, and H\(_2\)O (10 mL) was added to afford a white precipitate, which was filtered and washed with hexane, recrystallised from hot ethanol and dried under reduced pressure to get the product without further purification.

4,4’-(ethane-1,2-diylbis(azanediyl))diphenol (100a)

This compound was prepared according to general procedure U by treating 4-aminophenol 112a (109 mg, 1 mmol) to give the product as an off-white solid (220 mg, 90\%). m.p. 170–172 °C; \(R_f\) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.35; \(^1\)H NMR (500 MHz, d-DMSO): \(\delta\) 8.38 (2H, s), 6.54 (4H, d, \(J = 8.8\) Hz), 6.44 (4H, d, \(J = 8.8\) Hz), 4.90 (2H, s), 3.09 (4H, t, \(J = 2.5\) Hz); \(^{13}\)C NMR (125 MHz, d-DMSO): \(\delta\) 148.3, 141.7, 115.7, 113.5, 43.4; HRMS (ESI\(^+\)) Calc. for C\(_{14}\)H\(_{16}\)N\(_2\)O\(_2\) [M + Na]\(^+\) 267.1104, found 267.1104. \(\nu\) (neat, cm\(^{-1}\)): 3283, 3066, 2970, 1507, 641, 533. HPLC: 96.5\% (HPLC Method A), RT: 10.9 min.
4,4'-(ethane-1,2-diylbis(azanediyl))bis(ethane-2,1-diyl)diphenol (100c)

This compound was prepared according to general procedure U by treating 4-(2-aminoethyl)phenol 112b (68 mg, 0.5 mmol) to give the product as an off-white solid (129 mg, 86%). m.p. 205–209 °C; \( R_f \) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.30; \(^1\)H NMR (500 MHz, \( d \)-DMSO): \( \delta \) 6.96 (4H, d, \( J = 8.1 \) Hz), 6.66 (4H, d, \( J = 8.1 \) Hz), 2.65–2.62 (4H, m), 2.55–2.52 (8H, m), NH and OH signals not observed; \(^{13}\)C NMR (125 MHz, \( d \)-DMSO): \( \delta \) 155.9, 130.9, 129.8, 115.5, 51.9, 49.4, 35.6; HRMS (ESI+) Calc. for C\(_{18}\)H\(_{24}\)N\(_2\)O\(_2\) [M + H]\(^+\) 301.1910, found 301.1910. IR (neat, cm\(^{-1}\)): ν 3179, 3019, 2814, 1671, 1182, 1131. HPLC: >99.9% (HPLC Method C), RT: 16.2 min.

**General procedure V for methylation of secondary amines to tertiary amines**

To a solution of secondary amine (1.0 equiv.) in acetic acid (0.04 M) was added formaldehyde (37%, 10.0 equiv.), and the mixture was stirred at RT. After 30 min, NaBH\(_4\) (1.0 equiv.) was added and stirred for another 30 min. The solvent was removed under reduced pressure and taken up in sat. Na\(_2\)CO\(_3\) (aq.), extracted with ethyl acetate (3 x 15 mL) and the combined organic layers were dried over MgSO\(_4\) and concentrated in vacuo.

4,4'-(ethane-1,2-diylbis(methylazanediyl))diphenol (101a)

This compound was prepared according to general procedure V by treating 100a (61 mg, 0.25 mmol) with formaldehyde (37%, 249 µL, 2.5 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 100:1 → 80:1) to give the product as a white solid (60 mg, 88%). m.p. 174–176 °C; \( R_f \) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.45; \(^1\)H
NMR (400 MHz, d-MeOD): δ 6.70–6.34 (8H, m), 3.33 (4H, s), 2.81 (6H, s), OH signals not observed; ¹³C NMR (100 MHz, d-MeOD): δ 150.6, 144.7, 116.9, 116.8, 52.4, 40.2; HRMS (ESI+) Calc. for C₁₆H₂₀N₂O₂ [M + H]⁺ 273.1597, found 273.1597.

IR (neat, cm⁻¹): ν 3489, 3377, 3100, 1662, 1192, 545.

HPLC: 99.7% (HPLC Method C), RT: 16.7 min.

4,4'-(ethane-1,2-diylbis(methylazanediyl))bis(ethane-2,1-diyl)diphenol (101c)

This compound was prepared according to general procedure V by treating 100c (75 mg, 0.25 mmol) with formaldehyde (37%, 249 µL, 2.5 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 80:1) to give the product as a pale brown solid (73 mg, 89%). m.p. 229–230 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.40; ¹H NMR (400 MHz, d-DMSO): δ 9.07 (2H, br s), 6.96 (4H, d, J = 8.4 Hz), 6.62 (4H, d, J = 8.4 Hz), 2.56–2.52 (4H, m), 2.48–2.43 (4H, m), 2.40 (4H, s), 2.17 (6H, s); ¹³C NMR (100 MHz, d-DMSO): δ 155.8, 131.0, 129.9, 115.4, 60.2, 55.4, 42.6, 32.6; HRMS (ESI+) Calc. for C₂₀H₂₈N₂O₂ [M + H]⁺ 329.2223, found 329.2224. IR (neat, cm⁻¹): ν 3201, 2939, 2622, 1513, 1200, 830, 659. HPLC: 95.7% (HPLC Method A), RT: 12.3 min.

4,4'-(ethane-1,2-diylbis(azanediyl))bis(methylene)diphenol (100b)

This compound was prepared following a literature procedure. To a solution of ethylenediamine (55 µL, 0.82 mmol) in toluene (20 mL) was added 4-hydroxybenzaldehyde 113 (200 mg, 1.64 mmol), and the mixture was stirred at 110 °C for 20 min to form a yellow precipitate, which was filtered to get the crude intermediate without further purification. The obtained solid was dissolved in a mixture solvent of CH₂Cl₂/MeOH (1:1 (v/v), 20 mL) followed by the addition of NaBH₄ (15.9 mg, 0.42 mmol) at 0 °C, and the resulting mixture was allowed to warm
to RT for 30 min. After completion monitored by TLC, the mixture was quenched by sat. NH₄Cl (aq.) and extracted with ethyl acetate (3 x 15 mL), and the combined organic layers were dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 80:1) to give the product as an off-white solid (207 mg, 93%). m.p. 139–141 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.35; ¹H NMR (500 MHz, d-DMSO): δ 9.17 (2H, s), 7.07 (4H, d, J = 8.4 Hz), 6.67 (4H, d, J = 8.4 Hz), 3.52 (4H, s), 2.53 (4H, s); ¹³C NMR (125 MHz, d-DMSO): δ 155.9, 131.2, 128.9, 114.7, 52.5, 48.2; HRMS (ESI+) Calc. for C₁₆H₂₀N₂O₂ [M + H]+= 273.1597, found 273.1598.

IR (neat, cm⁻¹): ν 3254, 3018, 2856, 1611, 1512, 876.

HPLC: >99.9% (HPLC Method A), RT: 10.8 min.

4,4'-(ethane-1,2-diylbis(methylazanediyl))bis(methylene)diphenol (101b)

This compound was prepared according to general procedure V by treating 100b (68 mg, 0.25 mmol) with formaldehyde (37%, 249 µL, 2.5 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 80:1) to give the product as a white solid (67 mg, 90%). m.p. 158–159 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.45; ¹H NMR (500 MHz, d-DMSO): δ 9.21 (2H, s), 7.03 (4H, d, J = 8.3 Hz), 6.67 (4H, d, J = 8.3 Hz), 3.32 (4H, s), 2.42 (4H, s), 2.06 (6H, s); ¹³C NMR (100 MHz, d-MeOD): δ 158.0, 132.0, 129.3, 116.1, 62.8, 54.8, 42.7; HRMS (ESI+) Calc. for C₁₈H₂₄N₂O₂ [M + H]+= 301.1910, found 301.1910. IR (neat, cm⁻¹): ν 3269, 3018, 2856, 1611, 1512, 876. HPLC: >99.9% (HPLC Method C), RT: 14.3 min.

N¹,N²-bis(4-methoxyphenethyl)oxalamide (115)

This compound was prepared following a literature procedure.³³¹ To a solution of 2-(4-methoxyphenyl)ethan-1-amine 114 (1.03 g, 6.8 mmol) in toluene (20 mL) was
added diethyl oxalate (496 mg, 3.4 mmol), and the resulting mixture was heated to 110 °C and stirred for 12 h. After completion monitored by TLC, the reaction mixture was cooling down, hexane was added to get white precipitate, filtered, washed by hexane and collected after dried under reduced pressure. The product was given without further purification as a white solid (1.10 g, 91%). m.p. 191–192 °C; $^1$H NMR (500 MHz, d-DMSO): $\delta$ 8.47 (2H, s), 6.89 (4H, d, $J$ = 7.0 Hz), 6.63 (4H, d, $J$ = 7.0 Hz), 3.50 (6H, s), 3.10 (4H, s) (covered by solvent peak), 2.29 (4H, s); $^{13}$C NMR (125 MHz, d-DMSO): $\delta$ 159.8, 157.7, 130.9, 129.5, 113.7, 54.9, 40.5, 33.7; HRMS (ESI+) Calc. for C$_{20}$H$_{24}$N$_2$O$_4$ [M + H]$^+$ 357.1809, found 357.1806. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3306, 1654, 1544, 1270, 1234, 1190, 742, 698.

$N^1,N^2$-bis(4-methoxyphenethyl)ethane-1,2-diamine (116)

![Chemical structure](image)

This compound was prepared following a literature procedure. To a solution of 115 (370 mg, 1.04 mmol) in dry THF (20 mL) was added LiAlH$_4$ (400 mg, 10.4 mmol) at 0 °C under nitrogen atmosphere and the resulting mixture was allowed to warm to RT for 30 min. After that, the reaction was heated to 70 °C and stirred another 12 h. After completion monitored by TLC, the reaction mixture was quenched by NaSO$_4$•10H$_2$O at 0 °C until no bubble released any more. Filtered through Celite® and washed with ethyl acetate. The filtrate was concentrated under reduced pressure and the crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 100:1 → 80:1) to give the product as a colorless semisolid (314 mg, 92%). $^1$H NMR (400 MHz, d-MeOD): $\delta$ 7.19 (4H, d, $J$ = 8.4 Hz), 6.92 (4H, d, $J$ = 8.4 Hz), 3.79 (6H, s), 2.87–2.85 (4H, m), 2.81 (4H, dd, $J$ = 6.4 Hz, $J$ = 16.8 Hz), 2.76 (4H, s); $^{13}$C NMR (100 MHz, d-MeOD): $\delta$ 159.5, 132.8, 130.6, 114.9, 55.6, 52.1, 49.3, 35.9; HRMS (ESI+) Calc. for C$_{20}$H$_{28}$N$_2$O$_2$ [M + H]$^+$ 329.2224, found 329.2222. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3296, 3060, 2997, 1617, 1509, 1240, 1042, 811.
4,4'-(ethane-1,2-diylbis(azanediyl))bis(ethane-2,1-diyl)diphenol (100c)

This compound was prepared following a literature procedure. A solution of 116 (100 mg, 0.3 mmol) in aq. HBr (48%, 2 mL) was heated to 120 °C and stirred for 5 h. After completion monitored by TLC, the reaction mixture was diluted with H₂O and neutralised with solid KOH until the pH was adjusted to 8, and then extracted with ethyl acetate (3 x 20 mL), the combined organic layers were dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 80:1) to get the product as an off-white solid (72 mg, 80%). The spectrum data matched that shown as above.

6.4 Synthesis of compounds from Chapter 4

5-bromo-3-(4-nitrophenyl)-1-tosyl-1H-pyrrolo[2,3-b]pyridine (117a)

This compound was prepared according to general procedure F by treating 26a (119 mg, 0.25 mmol) with (4-nitrophenyl)boronic acid (50 mg, 0.3 mmol) in toluene/ethanol 3:1 at 90 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 8:1 → 5:1) to give the product as pale yellow solid (92 mg, 78%). m.p. 253–254 °C; Rf (hexane/ethyl acetate 4:1): 0.55; ¹H NMR (500 MHz, CDCl₃): δ 8.53 (1H, d, J = 2.1 Hz), 8.34 (2H, dd, J = 2.1, 6.9 Hz), 8.21 (1H, d, J = 2.1 Hz), 8.12 (2H, dd, J = 1.7, 6.7 Hz), 8.03 (1H, s), 7.73 (2H, dd, J = 2.1, 6.9 Hz), 7.32 (2H, d, J = 8.2 Hz), 2.40 (3H, s); ¹³C NMR (125 MHz, CDCl₃): δ 147.3, 146.5, 146.2, 145.8, 139.0, 134.8, 131.0, 130.1, 128.6, 127.9, 125.7, 124.7, 122.4, 117.4, 116.1, 21.9. HRMS (ESI+) Calc. for C₂₀H₁₄BrN₃O₄S [M + Na]⁺ 493.9781/495.9760, found 493.9789/495.9768. IR (neat, cm⁻¹): ν 3142, 1597, 1512, 1171, 692.
5-bromo-3-(1H-indol-5-yl)-1-tosyl-1H-pyrrolo[2,3-b]pyridine (117b)

This compound was prepared according to general procedure F by treating 26a (119 mg, 0.25 mmol) with (1H-indol-5-yl)boronic acid (48 mg, 0.3 mmol) in toluene/ethanol 3:1 at 90 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 8:1 → 5:1) to give the product as a colorless semisolid (93 mg, 80%). 

\[ R_f \text{ (hexane/ethyl acetate 5:1): } 0.40; \delta 8.40 (1H, d, J = 2.1 Hz), 8.31 (1H, d, J = 2.1 Hz), 8.03 (2H, d, J = 8.5 Hz), 7.93 (1H, s), 7.77 (1H, d, J = 1.1 Hz), 7.49 (1H, d, J = 8.4 Hz), 7.36 (2H, d, J = 8.2 Hz), 7.33 (1H, dd, J = 1.7, 8.4 Hz), 7.29 (1H, d, J = 3.1 Hz), 6.52 (1H, dd, J = 0.8, 3.1 Hz), 2.36 (3H, s), \]

NH signal not observed; 

\[ ^{13}C\text{ NMR (125 MHz, d-MeOD): } \delta 147.3, 147.2, 146.1, 137.5, 136.4, 132.6, 130.9, 130.0, 129.0, 126.7, 125.2, 124.6, 123.7, 123.2, 122.2, 120.4, 116.4, 112.9, 102.8, 21.6; \]

HRMS (ESI+) Calc. for C\(_{22}\)H\(_{16}\)N\(_3\)O\(_2\)BrS [M + Na]\(^{+}\) 488.0039/490.0018, found 488.0035/490.0015. 

IR (neat, cm\(^{-1}\)): \( \nu \) 3401, 2922, 1595, 1382, 1173, 582.

4-(5-bromo-1-tosyl-1H-pyrrolo[2,3-b]pyridin-3-yl)benzonitrile (117c)

This compound was prepared according to general procedure F by treating 26a (119 mg, 0.25 mmol) with (4-cyanophenyl)boronic acid (44 mg, 0.3 mmol) in toluene/ethanol 3:1 at 90 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 8:1 → 5:1) to give the product as an off-white solid (96 mg, 85%). 

\[ \text{m.p. } 219–220 °C; \delta 8.52 (1H, d, J = 2.1 Hz), 8.19 (1H, d, J = 2.1 Hz), 8.11 (2H, dd, J = 1.7, 6.7 \]
Hz), 7.98 (1H, s), 7.76 (2H, dd, J = 1.9, 6.6 Hz), 7.67 (2H, dd, J = 1.9, 6.6 Hz), 7.32 (2H, d, J = 8.2 Hz), 2.40 (3H, s); $^{13}$C NMR (125 MHz, CDCl$_3$): δ 146.4, 146.1, 145.8, 137.1, 134.8, 133.2, 130.9, 130.0, 128.5, 127.9, 125.4, 122.4, 118.7, 117.8, 116.0, 111.6, 21.9; HRMS (ESI+) Calc. for C$_{21}$H$_{14}$BrN$_3$O$_3$S [M + Na]$^+$ 473.9882/475.9862, found 473.9889/475.9869. IR (neat, cm$^{-1}$): ν 2219, 1607, 1178, 591.

4-(5-bromo-1-tosyl-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (117d)

This compound was prepared according to general procedure F by treating 26a (119 mg, 0.25 mmol) with (4-hydroxyphenyl)boronic acid (41 mg, 0.3 mmol) in toluene/ethanol 3:1 at 90 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 6:1 → 4:1) to give the product as a white solid (87 mg, 80%). m.p. 158–168 °C; R$_f$ (hexane/ethyl acetate 4:1): 0.40; $^1$H NMR (500 MHz, CDCl$_3$): δ 8.47 (1H, d, J = 2.1 Hz), 8.15 (1H, d, J = 2.1 Hz), 8.07 (2H, d, J = 8.4 Hz), 7.79 (1H, s), 7.42 (2H, dd, J = 2.1, 6.6 Hz), 7.29 (2H, d, J = 8.2 Hz), 6.95 (2H, dd, J = 2.1, 6.6 Hz), 2.38 (3H, s), OH signal not observed; $^{13}$C NMR (125 MHz, CDCl$_3$): δ 155.5, 145.7, 145.6, 145.5, 135.0, 131.0, 129.7, 128.8, 128.1, 125.1, 124.5, 123.3, 119.3, 116.1, 115.4, 21.6; HRMS (ESI+) Calc. for C$_{20}$H$_{15}$N$_2$O$_3$BrS [M + Na]$^+$ 464.9879/466.9858, found 464.9876/466.9855. IR (neat, cm$^{-1}$): ν 3370, 1595, 1386, 1175, 1160, 588.

4-(3-(4-nitrophenyl)-1-tosyl-1H-pyrrolo[2,3-b]pyridin-5-yl)phenol (118a)

This compound was prepared according to general procedure F by treating 117a (94
mg, 0.2 mmol) with (4-hydroxyphenyl)boronic acid (33 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 1:1) to give the product as a yellow solid (84 mg, 87%). **m.p.** 253–255 °C; *R*<sub>f</sub> (hexane/ethyl acetate 2:1): 0.40; **¹H NMR** (300 MHz, *d*-acetone): δ 8.65 (1H, s), 8.44 (1H, s), 8.35 (3H, d, *J* = 7.8 Hz), 8.20–8.15 (4H, m), 7.58 (2H, d, *J* = 7.9 Hz), 7.44 (2H, d, *J* = 7.9 Hz), 6.97 (2H, d, *J* = 8.0 Hz), 2.38 (3H, s); **¹³C NMR** (75 MHz, *d*-acetone): δ 158.5, 147.8, 147.3, 146.9, 144.9, 140.5, 136.1, 134.2, 130.8, 130.0, 129.6, 129.2, 129.1, 127.2, 126.5, 125.0, 121.4, 118.9, 116.8, 21.5; **HRMS** (ESI+) Calc. for C<sub>22</sub>H<sub>19</sub>N<sub>3</sub>O<sub>5</sub>S [M + Na]<sup>+</sup> 508.0938, found 508.0933. **IR** (neat, cm<sup>−1</sup>): ν 3132, 1594, 1517, 1232, 581.

4-(3-(1H-indol-5-yl)-1-tosyl-1H-pyrrolo[2,3-b]pyridin-5-yl)phenol (118b)

![Chemical Structure](image)

This compound was prepared according to **general procedure F** by treating 117b (93 mg, 0.2 mmol) with (4-hydroxyphenyl)boronic acid (33 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 1:1) to give the product as an off-white solid (77 mg, 80%). **m.p.** 142–145 °C; *R*<sub>f</sub> (hexane/ethyl acetate 2:1): 0.45; **¹H NMR** (500 MHz, CDCl<sub>3</sub>): δ 8.62 (1H, d, *J* = 2.1 Hz), 8.36 (1H, s), 8.22 (1H, d, *J* = 2.1 Hz), 8.12 (2H, d, *J* = 8.4 Hz), 7.86–7.85 (2H, m), 7.49 (1H, d, *J* = 8.4 Hz), 7.42 (3H, dd, *J* = 2.2, 8.5 Hz), 7.29–7.27 (3H, m), 6.92 (2H, d, *J* = 8.5 Hz), 6.61 (1H, t, *J* = 2.2 Hz), 5.46 (1H, br.s), 2.36 (3H, s); **¹³C NMR** (125 MHz, CDCl<sub>3</sub>): δ 155.8, 146.9, 145.3, 144.0, 135.7, 135.6, 132.7, 131.2, 129.9, 128.9, 128.6, 128.1, 127.2, 125.3, 124.3, 122.8, 122.5, 122.2, 119.9, 116.2, 111.9, 103.1, 21.8; **HRMS** (ESI+) Calc. for C<sub>28</sub>H<sub>21</sub>N<sub>3</sub>O<sub>3</sub>S [M + H]<sup>+</sup> 480.1376, found 480.1373. **IR** (neat, cm<sup>−1</sup>): ν 3410, 1703, 1611, 1318, 1230, 584.
4-(5-(4-hydroxyphenyl)-1-tosyl-1H-pyrrolo[2,3-b]pyridin-3-yl)benzonitrile (118c)

This compound was prepared according to **general procedure F** by treating 117c (90 mg, 0.2 mmol) with (4-hydroxyphenyl)boronic acid (33 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as an off-white solid (83 mg, 89%). **m.p.** 167–169 °C; **Rf** (hexane/ethyl acetate 3:1): 0.40; **1H NMR** (500 MHz, d-DMSO): δ 9.64 (1H, s), 8.66 (1H, d, J = 2.1 Hz), 8.45 (1H, s), 8.37 (1H, d, J = 2.1 Hz), 8.08 (4H, dd, J = 7.2, 8.4 Hz), 7.93 (2H, d, J = 8.3 Hz), 7.60 (2H, dd, J = 2.0, 6.6 Hz), 7.44 (2H, d, J = 8.4 Hz), 6.87 (2H, dd, J = 2.0, 6.6 Hz), 2.34 (3H, s); **13C NMR** (125 MHz, d-DMSO): δ 157.5, 145.8, 145.8, 143.6, 137.0, 134.4, 132.9, 132.7, 130.1, 128.6, 128.1, 127.9, 127.7, 126.3, 125.7, 120.1, 118.9, 117.9, 115.9, 109.8, 21.1; **HRMS** (ESI+) Calc. for C_{27}H_{19}O_{3}N_{3}S [M + Na]^+ 488.1039, found 488.1036. **IR** (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3483, 2226, 1607, 1159, 578.

**General procedure W** for the synthesis of tetrazole\(^{274}\)

To a solution of cyanated compound (1.0 equiv.) in a mixture solvent of \(t\)-BuOH/H\(_2\)O (1:1 (v/v), 0.125 M) was added TBAF (1 M in THF, 1.0 equiv.), trimethylsilyl azide (10.0 equiv.), CuSO\(_4\cdot5\)H\(_2\)O (0.01 equiv.) and sodium ascorbate (0.03 equiv.), and the mixture was stirred at 80 °C under microwave irradiation (100 W) for 2 h. After completion monitored by TLC, the mixture was quenched with H\(_2\)O and extracted with ethyl acetate (3 x 10 ml), the combined organic layers were dried over MgSO\(_4\) and concentrated *in vacuo*. 

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4-(3-(4-(1H-tetrazol-5-yl)phenyl)-1-tosyl-1H-pyrrolo[2,3-b]pyridin-5-yl)phenol (118d)

This compound was prepared according to general procedure W by treating 118c (46.5 mg, 0.1 mmol) and TMSN₃ (132 µL, 1 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/Methanol 100:1 → 50:1) to give the product as a white solid (42 mg, 83%). m.p. 233–235 °C; Rᶠ (CH₂Cl₂/Methanol 20:1): 0.35; ¹H NMR (500 MHz, d-MeOD): δ 8.41 (1H, d, J = 2.1 Hz), 8.12 (1H, d, J = 2.1 Hz), 8.03–8.01 (3H, m), 7.99 (2H, d, J = 8.7 Hz), 7.71 (2H, d, J = 8.4 Hz), 7.32 (2H, dd, J = 2.0, 6.6 Hz), 7.27 (2H, d, J = 8.2 Hz), 6.81 (2H, dd, J = 2.0, 6.6 Hz), 2.27 (3H, s), NH and OH signals not observed; ¹³C NMR (125 MHz, d-MeOD): δ 158.7, 157.3, 147.5, 147.2, 144.7, 136.9, 136.4, 134.6, 130.9, 130.2, 129.5, 129.1, 129.0, 128.8, 127.6, 125.5, 124.3, 122.3, 120.5, 116.9, 21.6; HRMS (ESI+) Calc. for C₂₇H₂₀N₆O₃S [M + H]⁺ 509.1390, found 509.1387. IR (neat, cm⁻¹): ν 3137, 2921, 2852, 1378, 1164, 582.

4-(5-(4-nitrophenyl)-1-tosyl-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (118e)

This compound was prepared according to general procedure F by treating 117d (89 mg, 0.2 mmol) with (4-nitrophenyl)boronic acid (40 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 1:1) to give the product as a pale yellow solid (82 mg, 85%). m.p. 136–138 °C; Rᶠ (hexane/ethyl acetate 1:1): 0.50; ¹H NMR (500 MHz, d-MeOD): δ 8.68 (1H, d, J = 2.2 Hz), 8.38 (1H, d, J = 2.2 Hz), 8.32 (2H,
dd, $J = 2.1, 6.9$ Hz), 8.06 (2H, d, $J = 8.5$ Hz), 7.94 (1H, s), 7.90 (2H, dd, $J = 2.1, 7.0$ Hz), 7.53 (2H, dd, $J = 2.1, 6.6$ Hz), 7.37 (2H, d, $J = 8.3$ Hz), 6.92 (2H, dd, $J = 2.1, 6.6$ Hz), 2.37 (3H, s), OH signal not observed; $^{13}$C NMR (125 MHz, $d$-MeOD): $\delta$ 158.7, 148.8, 148.7, 147.2, 146.0, 144.8, 136.5, 132.1, 130.9, 130.0, 129.5, 129.0, 128.9, 125.2, 124.5, 124.2, 123.4, 122.5, 117.0, 21.5; HRMS (ESI+) Calc. for $C_{26}H_{19}N_3O_5S$ [M + H]$^+$ 486.1118, found 486.1115. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 2919, 1597, 1515, 1346, 587.

4-(5-(1H-indol-5-yl)-1-tosyl-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (118f)

This compound was prepared according to general procedure F by treating 117d (89 mg, 0.2 mmol) with (1H-indol-5-yl)boronic acid (39 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 $\rightarrow$ 1:1) to give the product as a pale yellow solid (78 mg, 81%). m.p. 136–140 °C; $R_f$ (hexane/ethyl acetate 2:1): 0.45; $^1$H NMR (500 MHz, $d$-MeOD): $\delta$ 8.60 (1H, d, $J = 2.1$ Hz), 8.25 (1H, d, $J = 2.1$ Hz), 8.03 (2H, d, $J = 8.4$ Hz), 7.85 (1H, s), 7.76 (1H, d, $J = 1.2$ Hz), 7.50 (2H, dd, $J = 2.1, 6.6$ Hz), 7.46 (1H, d, $J = 8.4$ Hz), 7.34–7.32 (3H, m), 7.26 (1H, s), 6.92 (2H, dd, $J = 2.1, 6.6$ Hz), 6.50–6.49 (1H, m), 2.33 (3H, s), NH and OH signals not observed; $^{13}$C NMR (125 MHz, $d$-MeOD): $\delta$ 158.6, 147.6, 147.0, 145.0, 145.0, 137.4, 136.6, 136.3, 130.9, 130.3, 130.2, 129.9, 128.8, 128.4, 126.6, 124.9, 123.5, 123.4, 122.6, 122.1, 120.2, 117.0, 112.8, 102.7, 21.5; HRMS (ESI+) Calc. for $C_{28}H_{21}N_3O_5S$ [M + Na]$^+$ 502.1196, found 502.1193. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3416, 2963, 2926, 1616, 1385, 1172, 589.
4-(3-(4-hydroxyphenyl)-1-tosyl-1H-pyrrolo[2,3-b]pyridin-5-yl)benzonitrile (118g)

This compound was prepared according to general procedure F by treating 117d (89 mg, 0.2 mmol) with (4-cyanophenyl)boronic acid (35 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 1:1) to give the product as an off-white solid (87 mg, 93%). m.p. 203–205 °C; R_f (hexane/ethyl acetate 3:1): 0.40; ^1H NMR (400 MHz, CDCl_3): δ 8.67 (1H, d, J = 2.2 Hz), 8.18 (1H, d, J = 2.2 Hz), 8.13 (2H, d, J = 8.4 Hz), 7.85 (1H, s), 7.75 (2H, dd, J = 2.0, 6.6 Hz), 7.66 (2H, dd, J = 2.0, 6.6 Hz), 7.47 (2H, dd, J = 2.1, 6.6 Hz), 7.30 (2H, d, J = 8.1 Hz), 6.96 (2H, dd, J = 2.1, 6.6 Hz), 2.38 (3H, s), OH signal not observed; ^13C NMR (100 MHz, CDCl_3): δ 155.7, 147.5, 145.6, 144.1, 143.2, 135.4, 133.0, 131.0, 129.9, 129.1, 128.3, 128.2, 127.4, 125.0, 123.3, 122.1, 120.3, 118.8, 116.3, 111.6, 21.8; HRMS (ESI+) Calc. for C_{27}H_{19}N_{3}O_{3}S [M + Na]^+ 488.1039, found 488.1036. IR (neat, cm^{-1}): ν 3510, 3126, 2223, 1609, 1119, 666, 576.

4-(5-(4-(1H-tetrazol-5-yl)phenyl)-1-tosyl (118h)

This compound was prepared according to general procedure W by treating 118g (46.5 mg, 0.1 mmol) with TMSN_3 (132 µL, 1 mmol). The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 100:1 → 50:1) to give the product as a white solid (41 mg, 81%). m.p. 244–245 °C; R_f (CH_2Cl_2/MeOH 20:1): 0.35; ^1H NMR (300 MHz, d-DMSO): δ 9.66 (1H, br.s), 8.80 (1H, d, J = 1.8 Hz), 8.47 (1H, d, J = 1.8 Hz), 8.16–8.01 (7H, m), 7.67 (2H, d, J = 8.5 Hz), 7.42 (2H, d, J = 8.2 Hz), 6.92 (2H,
d, \( J = 8.5 \) Hz), 2.33 (3H, s), NH signal not observed; \(^{13}\text{C NMR}\) (75 MHz, \( d\)-DMSO): \( \delta \) 157.2, 155.1, 146.7, 145.6, 143.7, 140.1, 134.6, 131.0, 130.0, 128.8, 128.3, 127.6, 127.5, 127.4, 123.5, 122.8, 122.5, 121.0, 120.1, 115.9, 21.1; \textit{HRMS} (ESI+) Calc. for \( \text{C}_{27}\text{H}_{20}\text{N}_{6}\text{O}_{3} \) [M + H]\(^+\) 509.1390, found 509.1386. \textit{IR} (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3400, 3102, 2927, 1508, 1310, 577.

4-(3-(4-nitrophenyl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenol (119a)

This compound was prepared according to \textit{general procedure C} by treating 118a (97 mg, 0.2 mmol) with KOH (56 mg, 1 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 100:1 \( \rightarrow \) 50:1) to give the product as a yellow solid (62 mg, 95%). \textit{m.p.} 306–316 °C; \( R_f \) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.50; \(^1\text{H NMR}\) (500 MHz, \( d\)-DMSO): \( \delta \) 12.33–12.29 (1H, br.s), 9.59–9.55 (1H, br.s), 8.54 (1H, d, \( J = 2.1 \) Hz), 8.47 (1H, d, \( J = 2.1 \) Hz), 8.26 (2H, d, \( J = 1.9 \) Hz), 8.24 (1H, s), 8.10 (2H, d, \( J = 1.9 \) Hz), 7.61 (2H, dd, \( J = 2.1 \), 8.6 Hz), 6.90 (2H, dd, \( J = 2.1 \), 6.6 Hz; \(^{13}\text{C NMR}\) (125 MHz, \( d\)-DMSO): \( \delta \) 156.9, 148.4, 144.5, 142.4, 142.2, 129.8, 129.4, 128.3, 127.5, 126.3, 124.9, 124.3, 117.0, 115.8, 112.5; \textit{HRMS} (ESI+) Calc. for \( \text{C}_{19}\text{H}_{13}\text{N}_{3}\text{O}_{3} \) [M + Na]\(^+\) 354.0849, found 354.0848. \textit{IR} (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3217, 3173, 3024, 2921, 1591, 1341, 1263, 849.

4-(3-(1H-indol-5-yl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenol (119b)

This compound was prepared according to \textit{general procedure C} by treating 118b (48 mg, 0.1 mmol) with KOH (28 mg, 0.5 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 100:1 \( \rightarrow \) 50:1) to give the product as a yellow
solid (30 mg, 93%). m.p. 268–271 °C; \( R_f \) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.45; \(^1\)H NMR (500 MHz, \( d \)-MeOD): \( \delta \) 8.40 (2H, dd, \( J = 1.9, 10.2 \) Hz), 7.85 (1H, s), 7.56 (1H, s), 7.49 (3H, d, \( J = 8.6 \) Hz), 7.44 (1H, dd, \( J = 1.3, 8.4 \) Hz), 7.27 (1H, d, \( J = 3.0 \) Hz), 6.92 (2H, d, \( J = 8.5 \) Hz), 6.52 (1H, d, \( J = 3.0 \) Hz), NH and OH signals not observed; \(^{13}\)C NMR (125 MHz, \( d \)-MeOD): \( \delta \) 158.0, 148.9, 142.1, 136.7, 132.1, 131.0, 130.1, 129.4, 127.4, 127.1, 126.1, 123.9, 122.5, 120.6, 119.6, 119.1, 116.9, 112.6, 102.5; HRMS (ESI+) Calc. for C\(_{21}\)H\(_{15}\)N\(_3\)O [M + H]\(^+\) 326.1288, found 326.1288. IR (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3480, 3243, 2916, 1606, 1262, 1248, 690. HPLC: 99.1% (HPLC Method A), RT: 18.2 min.

4-(3-(4-(1H-tetrazol-5-yl)phenyl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenol (119c)

This compound was prepared according to general procedure C by treating 118d (51 mg, 0.1 mmol) with KOH (28 mg, 0.5 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 50:1 \( \rightarrow \) 10:1) to give the product as a pale yellow solid (32 mg, 90%). m.p. 282–284 °C; \( R_f \) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.20; \(^1\)H NMR (400 MHz, \( d \)-DMSO): \( \delta \) 12.08 (1H, br s), 9.51 (1H, s), 8.51 (1H, d, \( J = 2.0 \) Hz), 8.44 (1H, d, \( J = 2.0 \) Hz), 8.11 (2H, d, \( J = 8.4 \) Hz), 8.07 (1H, d, \( J = 2.6 \) Hz), 8.04 (2H, d, \( J = 8.4 \) Hz), 7.60 (2H, d, \( J = 8.6 \) Hz), 6.89 (2H, d, \( J = 8.6 \) Hz), NH of tetrazole and OH signals not observed; \(^{13}\)C NMR (100 MHz, \( d \)-DMSO): \( \delta \) 156.8, 155.3, 148.3, 141.9, 137.9, 129.6, 129.3, 128.3, 127.5, 126.8, 125.6, 124.8, 121.2, 117.1, 115.8, 113.5; HRMS (ESI+) Calc. for C\(_{20}\)H\(_{14}\)N\(_6\)O [M + H]\(^+\) 355.1302, found 355.1302. IR (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3477, 3263, 3230, 2919, 1612, 1531, 1326, 1262, 1243, 836. HPLC: 96.4% (HPLC Method A), RT: 17.1 min.
4-(5-(4-nitrophenyl)-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (119e)

This compound was prepared according to general procedure C by treating 118e (97 mg, 0.2 mmol) with KOH (56 mg, 1 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 50:1) to give the product as a yellow solid (62 mg, 93%). m.p. 178–180 °C; Rᶠ(CH₂Cl₂/MeOH 20:1): 0.50; ¹H NMR (400 MHz, d-DMSO): δ 11.98 (1H, s), 9.39 (1H, s), 8.68 (1H, d, J = 2.1 Hz), 8.52 (1H, d, J = 2.1 Hz), 8.32 (2H, dd, J = 1.9, 6.9 Hz), 8.11 (2H, dd, J = 1.9, 6.9 Hz), 7.78 (1H, d, J = 2.4 Hz), 7.60 (2H, dd, J = 2.0, 6.6 Hz), 6.87 (2H, dd, J = 2.0, 6.6 Hz; ¹³C NMR (100 MHz, d-DMSO): δ 155.8, 149.1, 146.2, 145.9, 142.0, 128.0, 127.8, 126.2, 126.1, 125.4, 124.0, 123.7, 117.5, 115.7, 115.4; HRMS (ESI+) Calc. for C₁₉H₁₃N₃O₃ [M + H]⁺ 332.1030, found 332.1032. IR (neat, cm⁻¹): ν 3404, 3119, 2858, 1593, 1336, 1267. HPLC: 96.8% (HPLC Method A), RT: 23.0 min.

4-(5-(1H-indol-5-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (119f)

This compound was prepared according to general procedure C by treating 118f (48 mg, 0.1 mmol) with KOH (28 mg, 0.5 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 50:1) to give the product as a yellow solid (31 mg, 94%). m.p. 241–244 °C; Rᶠ(CH₂Cl₂/MeOH 20:1): 0.45; ¹H NMR (500 MHz, d-DMSO): δ 11.72 (1H, s), 11.13 (1H, s), 9.37 (1H, br.s), 8.53 (1H, d, J = 2.1 Hz), 8.32 (1H, d, J = 2.1 Hz), 7.86 (1H, d, J = 1.6 Hz), 7.69 (1H, d, J = 2.4 Hz), 7.57 (2H, dd, J = 2.1, 6.6 Hz), 7.50 (1H, d, J = 3.4 Hz), 7.45 (1H, dd, J = 1.7, 8.4 Hz), 7.38 (1H, t, J = 2.7 Hz), 6.87 (2H, dd, J = 2.1, 6.6 Hz), 6.50 (1H, dt, J = 0.7, 2.7 Hz; ¹³C NMR (125 MHz, d-DMSO): δ 155.6, 148.0, 142.0, 135.2, 130.2, 130.1, 128.3, 127.6,
126.0, 125.9, 125.1, 122.9, 120.9, 118.4, 117.4, 115.7, 114.7, 111.8, 101.4; HRMS (ESI+) Calc. for C_{21}H_{15}N_{3}O [M + H]^+ 326.1288, found 326.1288. IR (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3262, 2917, 1459, 1257, 732. HPLC: 96.5% (HPLC Method A), RT: 18.0 min.

4-(5-(4-(1H-tetrazol-5-yl)phenyl)-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (119g)

This compound was prepared according to general procedure C by treating 118h (51 mg, 0.1 mmol) with KOH (28 mg, 0.5 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 50:1 \(\rightarrow\) 10:1) to give the product as a pale yellow solid (32 mg, 90%). m.p. 301–303 °C; R\(_f\) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.20; \(^1\)H NMR (400 MHz, d-DMSO): \(\delta\) 12.06 (1H, s), 8.68 (1H, s), 8.55 (1H, s), 8.19 (2H, d, \(J = 6.3\) Hz), 8.05 (2H, d, \(J = 6.3\) Hz), 7.78 (1H, s), 7.61 (2H, d, \(J = 6.5\) Hz), 6.88 (2H, d, \(J = 6.5\) Hz), NH of tetrazole and OH signals not observed; \(^{13}\)C NMR (100 MHz, d-DMSO): \(\delta\) 155.8, 148.8, 141.7, 141.5, 127.8, 127.7, 127.6, 127.4, 127.4, 127.3, 125.6, 125.5, 123.4, 117.4, 115.7, 115.2; HRMS (ESI+) Calc. for C\(_{20}\)H\(_{14}\)N\(_6\)O [M + H]^+ 355.1302, found 355.1301. IR (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3330, 2921, 1455, 829, 518. HPLC: 95.8% (HPLC Method A), RT: 17.1 min.

General procedure X for reduction of nitro compounds

To a solution of nitro substrate (1.0 equiv.) in aq. HCl (3 M, 0.03 M) was added iron powder (10.0 equiv.), and the reaction was heated to 110 °C for 3 h. After completion monitored by TLC, the mixture was cooling down and neutralised with sat. NaHCO\(_3\) (aq.), and extracted with ethyl acetate (3 x 10 mL), and the combined organic layers were dried over MgSO\(_4\) and concentrated \textit{in vacuo}.
4-(3-(4-aminophenyl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenol (119d)

This compound was prepared according to general procedure X by treating 119a (50 mg, 0.15 mmol) with Fe (84 mg, 1.5 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 20:1) to give the product as a pale yellow solid (38 mg, 84%). m.p. 113–115 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.40; ¹H NMR (500 MHz, d-DMSO): δ 9.47 (1H, s), 8.39 (1H, d, J = 1.7 Hz), 7.75 (1H, d, J = 1.7 Hz), 7.47 (2H, d, J = 8.4 Hz), 7.36 (2H, d, J = 8.3 Hz), 6.86 (1H, s), 6.80 (1H, d, J = 8.3 Hz), 6.61 (2H, d, J = 8.4 Hz), 6.09–6.08 (2H, m), OH signal not observed; ¹³C NMR (125 MHz, d-DMSO): δ 156.2, 148.5, 148.4, 141.8, 129.9, 128.2, 128.1, 127.1, 126.6, 124.4, 123.4, 118.0, 116.3, 115.2, 115.0; HRMS (ESI+) Calc. for C₁⁹H₁₅N₃O [M + H]+ 302.1288, found 302.1290. IR (neat, cm⁻¹): ̃ν 3481, 3444, 3359, 3338, 2918, 1552, 1231, 794. HPLC: 96.0% (HPLC Method A), RT: 14.4 min.

4-(5-(4-aminophenyl)-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (119h)

This compound was prepared according to general procedure X by treating 119e (50 mg, 0.15 mmol) with Fe (84 mg, 1.5 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 40:1) to give the product as a pale yellow solid (32 mg, 70%). m.p. 216–220 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.30; ¹H NMR (400 MHz, d-DMSO): δ 11.66 (1H, s), 9.34 (1H, s), 8.41 (1H, d, J = 2.1 Hz), 8.19 (1H, d, J = 2.1 Hz), 7.65 (1H, d, J = 2.1 Hz), 7.54 (2H, dd, J = 2.1, 6.6 Hz), 7.40 (2H, dd, J = 1.9, 6.6 Hz), 6.85 (2H, dd, J = 2.1, 6.6 Hz), 6.67 (2H, dd, J = 1.9, 6.6 Hz), 5.15 (2H, s); ¹³C NMR (100 MHz, d-DMSO): δ 155.6, 147.9, 147.8, 141.1, 129.2, 127.6, 127.5,
126.4, 125.9, 123.8, 122.8, 117.4, 115.7, 114.6, 114.4; **HRMS** (ESI+) Calc. for \( \text{C}_{19}\text{H}_{15}\text{N}_{3}\text{O} [\text{M} + \text{H}]^+ \) 302.1288, found 302.1288. **IR** (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3351, 3234, 3024, 2870, 1610, 1542, 1478, 1260, 821, 530. **HPLC:** >99.9% (HPLC Method A), RT: 13.7 min.

\[ N-(4-(3-(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenyl)acetamide \]

(120a)

To a solution of \( \text{119h} \) (50 mg, 0.17 mmol) in acetic anhydride (24 \( \mu \)l, 0.25 mmol), and the reaction was heated to 110 °C for 5 h. After completion monitored by TLC, the mixture was cooling and neutralised with sat. \( \text{Na}_2\text{CO}_3 \) (aq.), and extracted with ethyl acetate (3 x 10 mL), and the combined organic layers were dried over \( \text{MgSO}_4 \) and concentrated in vacuo. The crude product was purified by flash chromatography (\( \text{CH}_2\text{Cl}_2/\text{MeOH} \) 100:1 \( \rightarrow \) 50:1) to give the product as a pale yellow solid (45 mg, 77%). **m.p.** 272–275 °C; \( R_f \) (\( \text{CH}_2\text{Cl}_2/\text{MeOH} \) 20:1): 0.35; \( ^1\text{H NMR} \) (300 MHz, \( d-\text{DMSO} \)): \( \delta \) 11.78 (1H, s), 10.02 (1H, s), 9.42 (1H, s), 8.51 (1H, s), 8.31 (1H, s), 7.69–7.68 (5H, m), 7.56 (2H, d, \( J = 8.0 \text{ Hz} \)), 6.86 (2H, d, \( J = 8.0 \text{ Hz} \)), 2.07 (3H, s); \( ^{13}\text{C NMR} \) (75 MHz, \( d-\text{DMSO} \)): \( \delta \) 168.3, 155.7, 148.3, 141.5, 138.4, 133.7, 128.2, 127.7, 127.2, 125.7, 124.8, 123.1, 119.5, 117.4, 115.7, 114.9, 24.0; **HRMS** (ESI+) Calc. for \( \text{C}_{21}\text{H}_{17}\text{N}_{3}\text{O}_2 [\text{M} + \text{H}]^+ \) 344.1394, found 344.1396. **IR** (neat, cm\(^{-1}\)): \( \tilde{\nu} \) 3317, 3088, 2922, 1523, 1244, 886, 516. **HPLC:** 98.3% (HPLC Method A), RT: 16.3 min.
**N-(4-(3-(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenyl) methanesulfonamide (120b)**

To a solution of 119h (50 mg, 0.17 mmol) in H₂O (5 ml) was added methanesulfonyl chloride (20 µl, 0.25 mmol), and the mixture was stirred at room temperature for 10 h. After completion monitored by TLC, the mixture was extracted with ethyl acetate (3 x 10 mL), and the combined organic layers were dried over MgSO₄ and concentrated in vacuo. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 50:1) to give the product as a pale yellow solid (41 mg, 64%). m.p. 192–195 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.30; ¹H NMR (400 MHz, d-DMSO): δ 11.83 (1H, d, J = 2.2 Hz), 8.51 (1H, d, J = 2.1 Hz), 8.32 (1H, d, J = 2.1 Hz), 7.74 (1H, s), 7.72 (2H, d, J = 1.6 Hz), 7.56 (2H, d, J = 8.7 Hz), 7.31 (2H, d, J = 8.7 Hz), 6.85 (2H, d, J = 8.7 Hz), 5.76 (1H, s), 3.01 (3H, s), OH signal not observed; ¹³C NMR (100 MHz, d-DMSO): δ 156.1, 148.8, 142.0, 137.9, 135.1, 128.4, 128.1, 126.1, 125.5, 123.6, 120.8, 117.8, 116.2, 115.3, 109.0, 55.4; HRMS (ESI+) Calc. for C₂₁H₁₇N₃O₃S [M + H]⁺ 380.1063, found 380.1067. IR (neat, cm⁻¹): ν 3401, 2920, 2893, 1610, 1520, 1152. HPLC: 95.9% (HPLC Method A), RT: 17.5 min.

**1-(4-(3-(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenyl)urea (120c)**

To a mixture of 119h (50 mg, 0.17 mmol), citric acid (326 mg, 1.70 mmol), mannitol (310 mg, 1.70 mmol) and urea (153 mg, 2.55 mmol) was added I₂ (4.3 mg, 0.017 mmol), and the reaction was heated to 80 °C for 6 h. After completion monitored by TLC, the mixture was cooling, and H₂O (10 ml) was added and extracted with ethyl acetate (3 x 10 mL), and the combined organic layers were dried over MgSO₄ and
concentrated in vacuo. The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 → 10:1) to give the product as a pale yellow solid (41 mg, 70%). **m.p.** 236–239 °C; **$R_f$** (CH$_2$Cl$_2$/MeOH 20:1): 0.25; **$^1$H NMR** (500 MHz, d-DMSO): $\delta$ 11.75 (1H, s), 9.37 (1H, s), 8.66 (1H, s), 8.49 (1H, d, $J = 2.0$ Hz), 8.28 (1H, d, $J = 2.0$ Hz), 7.69 (1H, d, $J = 1.4$ Hz), 7.61 (2H, d, $J = 8.6$ Hz), 7.56 (2H, d, $J = 8.5$ Hz), 7.51 (2H, d, $J = 8.6$ Hz), 6.86 (2H, d, $J = 8.6$ Hz), 5.87 (2H, s); **$^{13}$C NMR** (125 MHz, d-DMSO): $\delta$ 156.1, 155.8, 148.3, 141.5, 139.8, 131.9, 128.5, 127.8, 127.3, 125.9, 124.7, 123.1, 118.3, 117.5, 115.8, 114.9; **HRMS** (ESI+) Calc. for C$_{20}$H$_{16}$N$_4$O$_2$ [M + H]$^+$ 345.1346, found 345.1345. **IR** (neat, cm$^{-1}$): $\tilde{\nu}$ 2954, 2921, 2852, 1535, 1242, 813. **HPLC**: >98.3% (HPLC Method A), RT: 13.3 min.

**5-bromo-3-(4-methoxyphenyl)-1-tosyl-1H-indole (117e)**

This compound was prepared according to **general procedure F** by treating 26b (238 mg, 0.5 mmol) with (4-methoxyphenyl)boronic acid (91 mg, 0.6 mmol) in toluene/ethanol 3:1 at 90 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 6:1) to give the product as a white solid (212 mg, 93%). **m.p.** 114–116 °C; **$R_f$** (hexane/ethyl acetate 5:1): 0.55; **$^1$H NMR** (300 MHz, CDCl$_3$): $\delta$ 7.92 (1H, d, $J = 8.4$ Hz), 7.84 (1H, s), 7.77 (2H, d, $J = 7.4$ Hz), 7.61 (1H, s), 7.48–7.43 (3H, m), 7.24 (2H, d, $J = 8.3$ Hz), 7.00 (2H, d, $J = 7.4$ Hz), 3.86 (3H, s), 2.35 (3H, s); **$^{13}$C NMR** (75 MHz, CDCl$_3$): $\delta$ 159.5, 145.4, 135.1, 134.3, 131.4, 130.1, 129.1, 127.8, 127.0, 124.8, 123.5, 123.3, 117.3, 116.8, 115.4, 114.6, 55.5, 21.7; **APCI** Calc. for C$_{22}$H$_{15}$N$_2$O$_2$BrS [M + H]$^+$, 456.0264/458.0243, found 456.0260/458.0234. **IR** (neat, cm$^{-1}$): $\tilde{\nu}$ 2926, 1614, 1250, 1175, 587.
4-(5-bromo-1-tosyl-1H-indol-3-yl)benzonitrile (117f)

This compound was prepared according to general procedure F by treating 26b (238 mg, 0.5 mmol) with (4-cyanophenyl)boronic acid (88 mg, 0.6 mmol) in toluene/ethanol 3:1 at 90 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 8:1 → 6:1) to give the product as an off-white solid (212 mg, 94%). m.p. 187–188 °C; Rf (hexane/ethyl acetate 6:1): 0.45; ¹H NMR (500 MHz, CDCl₃): δ 7.92 (1H, d, J = 8.8 Hz), 7.82 (2H, d, J = 11.5 Hz), 7.79 (2H, d, J = 4.8 Hz), 7.73 (2H, d, J = 8.0 Hz), 7.65 (2H, d, J = 8.1 Hz), 7.46 (1H, d, J = 8.8 Hz), 7.26 (2H, d, J = 8.0 Hz), 2.35 (3H, s); ¹³C NMR (125 MHz, CDCl₃): δ 145.8, 137.3, 134.7, 134.1, 132.9, 130.3, 128.3, 128.2, 127.0, 125.2, 122.8, 121.3, 118.7, 117.7, 115.4, 111.2, 21.7; APCI Calc. for C₂₂H₁₈N₂O₂BrS [M + H]⁺, 451.0110/453.0090, found 451.0112/453.0091. IR (neat, cm⁻¹): ν 3122, 2224, 1610, 1448, 1145, 534.

3-(4-methoxyphenyl)-1-tosyl-1'H,1'1'H,5,5'-biindole (118i)

This compound was prepared according to general procedure F by treating 117e (91 mg, 0.2 mmol) with (1H-indol-5-yl)boronic acid (39 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 3:1) to give the product as a pale brown solid (86 mg, 87%). m.p. 163–164 °C; Rf (hexane/ethyl acetate 3:1): 0.55; ¹H NMR (300 MHz, CDCl₃): δ 8.28 (1H, s), 8.09 (1H, d, J = 8.5 Hz), 7.94 (1H, s), 7.88 (1H, s), 7.83 (2H, d, J = 7.7 Hz), 7.67 (1H, s), 7.55–7.42 (5H, m), 7.25–7.21 (2H, m), 6.95 (2H, d, J = 7.9 Hz), 6.61 (1H, s), 3.83 (3H, s), 2.34 (3H, s), NH signal not observed;
**13C NMR** (125 MHz, CDCl₃): δ 159.0, 144.9, 136.7, 135.3, 134.6, 134.1, 130.6, 129.9, 128.4, 128.3, 126.9, 125.6, 125.0, 124.6, 124.1, 123.6, 123.0, 122.6, 120.1, 118.6, 114.2, 114.0, 111.5, 102.9, 55.4, 21.6; **HRMS** (ESI+) Calc. for C₃₀H₂₄N₂O₃S [M + H]⁺ 493.1580, found 493.1584. **IR** (neat, cm⁻¹): ν 3416, 2927, 2835, 1608, 1168, 584.

4-(5-(4-methoxyphenyl)-1-tosyl-1H-indol-3-yl)benzonitrile (118j)

This compound was prepared according to **general procedure F** by treating 117f (92 mg, 0.2 mmol) with (4-methoxyphenyl)boronic acid (36 mg, 0.24 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 → 3:1) to give the product as a white solid (86 mg, 90%). **m.p.** 220–221 °C; **R_f** (hexane/ethyl acetate 4:1): 0.40; **¹H NMR** (400 MHz, CDCl₃): δ 8.09 (1H, d, J = 8.6 Hz), 7.85 (2H, d, J = 8.4 Hz), 7.83 (1H, d, J = 1.7 Hz), 7.79 (1H, s), 7.77–7.72 (4H, m), 7.58 (1H, dd, J = 1.7, 8.6 Hz), 7.50 (2H, dd, J = 2.1, 6.7 Hz), 7.28–7.26 (2H, m), 6.98 (2H, dd, J = 2.1, 6.7 Hz), 3.85 (3H, s), 2.36 (3H, s); **¹³C NMR** (100 MHz, CDCl₃): δ 159.4, 145.6, 138.2, 137.5, 135.2, 134.6, 133.7, 132.9, 130.2, 129.0, 128.6, 128.5, 127.1, 124.9, 124.7, 122.3, 118.9, 118.0, 114.5, 114.3, 111.1, 55.5, 21.8; **APCI** Calc. for C₂₉H₂₂N₂O₃S [M + H]⁺, 479.1424, found 479.1419. **IR** (neat, cm⁻¹): ν 2934, 2835, 2222, 1609, 1246, 672, 577.

3-(4-(1H-tetrazol-5-yl)phenyl)-5-(4-methoxyphenyl)-1-tosyl-1H-indole (118k)
This compound was prepared according to **general procedure W** by treating 118j (60 mg, 0.12 mmol) and TMSN₃ (159 µL, 1.2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as an off-white solid (46 mg, 74%). **m.p.** 199–200 °C; *R*ᵣ/(CH₂Cl₂/MeOH 20:1): 0.45; **¹H NMR** (500 MHz, *d*-DMSO): δ 8.26 (1H, s), 8.17 (2H, d, *J* = 8.2 Hz), 8.07 (1H, d, *J* = 8.7 Hz), 8.04 (2H, d, *J* = 8.2 Hz), 8.00–7.99 (3H, m), 7.66 (1H, dd, *J* = 1.5, 8.7 Hz), 7.64 (2H, d, *J* = 8.7 Hz), 7.41 (2H, d, *J* = 8.4Hz), 7.01 (2H, d, *J* = 8.7 Hz), 3.78 (3H, s), 2.32 (3H, s), NH signal not observed; **¹³C NMR** (125 MHz, *d*-DMSO): δ 159.3, 155.8, 146.2, 136.9, 135.5, 134.4, 134.3, 133.1, 130.8, 129.2, 129.0, 128.7, 128.0, 127.4, 125.7, 124.6, 123.9, 122.7, 118.2, 114.8, 114.3, 55.6, 21.5; **HRMS** (ESI+) Calc. for C₂₉H₂₃N₅O₃S [M + Na]⁺ 544.1414, found 544.1416. **IR** (neat, cm⁻¹): ν 2924, 2853, 1610, 1458, 1173, 839, 585.

### 3-(4-methoxyphenyl)-1H,1'H-5,5'-biindole (119i)

![Image of compound 119i]

This compound was prepared according to **general procedure C** by treating 118i (60 mg, 0.12 mmol) with KOH (34 mg, 0.6 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as an off-white solid (38 mg, 94%). **m.p.** 193–195 °C; *R*ᵣ (hexane/ethyl acetate 1:1): 0.40; **¹H NMR** (500 MHz, CDCl₃): δ 8.15–8.12 (3H, m), 7.92 (1H, s), 7.65 (2H, d, *J* = 8.2 Hz), 7.55 (2H, dd, *J* = 8.4, 19.8 Hz), 7.45 (2H, t, *J* = 8.8 Hz), 7.29 (1H, d, *J* = 1.8 Hz), 7.22 (1H, t, *J* = 2.3 Hz), 7.03 (2H, d, *J* = 8.2 Hz), 6.62 (1H, d, *J* = 1.0 Hz), 3.87 (3H, s); **¹³C NMR** (125 MHz, CDCl₃): δ 158.2, 135.8, 135.3, 135.0, 134.9, 128.8, 128.5, 128.3, 126.5, 124.8, 122.9, 122.6, 121.8, 119.5, 118.4, 118.3, 114.4, 111.6, 111.2, 103.0, 55.5; **HRMS** (ESI+) Calc. for (C₂₉H₁₈N₂O₂)₂ [M + Na]⁺ 699.2731, found 699.2734. **IR** (neat, cm⁻¹): ν 3405, 2964, 1606, 1475, 1269, 837.
**3-(4-(1H-tetrazol-5-yl)phenyl)-5-(4-methoxyphenyl)-1H-indole (119j)**

![Chemical Structure]

This compound was prepared according to general procedure C by treating \textbf{118k} (60 mg, 0.11 mmol) with KOH (31 mg, 0.55 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 100:1 → 50:1) to give the product as an off-white solid (36 mg, 89%). **m.p.** 247–249 °C; \textit{R}$_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.30; \textit{\textsuperscript{1}H} NMR (500 MHz, \textit{d}-DMSO): \(\delta\) 11.57 (1H, d, \(J = 1.2\) Hz), 8.10 (2H, d, \(J = 8.2\) Hz), 8.08 (1H, s), 7.99 (2H, d, \(J = 8.2\) Hz), 7.90 (1H, d, \(J = 2.5\) Hz), 7.65 (2H, d, \(J = 8.7\) Hz), 7.53 (1H, d, \(J = 8.5\) Hz), 7.43 (1H, dd, \(J = 1.2, 8.5\) Hz), 7.03 (2H, d, \(J = 8.7\) Hz), 3.80 (3H, s), NH signal not observed; \textit{\textsuperscript{13}C} NMR (125 MHz, \textit{d}-DMSO): \(\delta\) 158.8, 155.9, 139.4, 136.9, 135.0, 133.1, 128.6, 128.1, 127.6, 126.0, 125.9, 121.7, 121.4, 117.2, 115.7, 114.9, 113.1, 55.8; \textbf{HRMS} (ESI+) Calc. for C$_{22}$H$_{17}$N$_{5}$O [M + Na]$^+$ 390.1325, found 390.1328. \textbf{IR} (neat, cm$^{-1}$): \(\tilde{v}\) 3412, 2928, 1612, 1244, 836.

**4-(1\textit{H},1'\textit{H}-[5,5'-biindol]-3-yl)phenol (120d)**

![Chemical Structure]

This compound was prepared according to general procedure D by treating \textbf{119i} (34 mg, 0.1 mmol) with BBr$_3$ (1 mL, 1 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 100:1 → 50:1) to give the product as a pale yellow solid (23 mg, 70%). **m.p.** 177–179 °C; \textit{R}$_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.45; \textit{\textsuperscript{1}H} NMR (400 MHz, \textit{d}-DMSO): \(\delta\) 11.18 (1H, s), 11.06 (1H, s), 9.30 (1H, s), 7.96 (1H, s), 7.79 (1H, s), 7.53–7.51 (3H, m), 7.47 (1H, s), 7.45–7.42 (2H, m), 7.40 (1H, dd, \(J = 1.7, 8.4\) Hz), 7.35 (1H, t, \(J = 2.7\) Hz), 6.87 (2H, dd, \(J = 2.0, 6.6\) Hz), 6.48 (1H, t, \(J = 2.1\) Hz); \textit{\textsuperscript{13}C
NMR (100 MHz, d-DMSO): $\delta$ 155.3, 135.7, 134.9, 133.7, 133.3, 128.3, 127.8, 126.7, 125.7 (3), 125.7 (0), 122.6, 121.1, 120.9, 118.0, 116.8, 116.2, 115.6, 112.0, 111.5, 101.3; HRMS (ESI+) Calc. for C$_{22}$H$_{16}$N$_2$O [M + H]$^+$ 325.1335, found 325.1333. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3392, 2921, 1500, 798.

HPLC: 97.4% (HPLC Method A), RT: 24.1 min.

4-(3-(4-(1H-tetrazol-5-yl)phenyl)-1H-indol-5-yl)phenol (120e)

This compound was prepared according to general procedure D by treating 119j (37 mg, 0.1 mmol) with BBr$_3$ (1 mL, 1 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 $\rightarrow$ 10:1) to give the product as a white solid (24 mg, 67%). m.p. 285–286 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.20; $^1$H NMR (500 MHz, d-DMSO): $\delta$ 11.43 (1H, s), 8.05 (2H, d, $J$ = 8.2 Hz), 8.01 (1H, s), 7.74 (2H, d, $J$ = 8.2 Hz), 7.71 (1H, d, $J$ = 2.1 Hz), 7.50 (2H, d, $J$ = 8.6 Hz), 7.47 (1H, s), 7.37 (1H, d, $J$ = 8.4 Hz), 6.86 (2H, d, $J$ = 8.4 Hz), NH of tetrazole and OH signals not observed; $^{13}$C NMR (125 MHz, d-DMSO): $\delta$ 160.7, 156.5, 136.1, 134.5, 132.8, 132.6, 129.8, 127.9, 126.6, 126.4, 125.7, 124.0, 120.7, 116.5, 116.2, 115.8, 112.3; HRMS (ESI+) Calc. for C$_{21}$H$_{15}$N$_3$O [M + Na]$^+$ 376.1169, found 376.1172. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3160, 2918, 2852, 1606, 1220, 758. HPLC: 95.7% (HPLC Method A), RT: 21.1 min.

5-bromo-1-tosyl-1H-indole-3-carbonitrile (117g)$_{279}$

To a solution of 5-bromo-1H-indole 25b (500 mg, 2.56 mmol) in MeCN (30 ml) was added chlorosulfonylisocyanate (245 $\mu$L, 2.82 mmol) dropwise at 0 °C. The reaction mixture was stirred at 0 °C for another 2 h, then triethylamine (372 $\mu$L, 2.69 mmol)
was added dropwise over 10 min. The reaction mixture was allowed to warm to RT for 12 h. After completion monitored by TLC, the mixture was poured into ice-water mixture (50 ml), and the aqueous layer was extracted with ethyl acetate (3 x 20 ml). The combined organic layers were dried over MgSO$_4$ and concentrated \textit{in vacuo}. The obtained crude intermediate was treated with TsCl (570 mg, 3.0 mmol) according to step 2 in \textit{general procedure A} without further purification. The crude product was purified by flash chromatography (hexane/ethyl acetate 10:1 → 8:1) to give the product as an off-white solid (864 mg, 90% over two steps). \textbf{m.p.} 174–175 °C; \textit{R}$_f$ (hexane/ethyl acetate 8:1): 0.50; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.08 (1H, s), 7.87 (1H, d, $J = 8.9$ Hz), 7.83 (1H, d, $J = 1.7$ Hz), 7.80 (2H, dd, $J = 1.7$, 6.7 Hz), 7.53 (1H, dd, $J = 1.9$, 8.9 Hz), 7.32 (2H, d, $J = 8.1$ Hz), 2.39 (3H, s); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 146.9, 134.2, 134.0, 132.6, 130.6, 130.1, 129.8, 127.3, 123.2, 118.7, 115.3, 112.9, 93.1, 21.8; HRMS (ESI+) Calc. for C$_{16}$H$_{11}$N$_2$O$_2$BrS $[$M + Na$]^+$ 396.9617/398.9596, found 396.9619/398.9598. \textbf{IR} (neat, cm$^{-1}$): $\tilde{\nu}$ 3132, 2230, 1399, 1190, 582.

5-(4-methoxyphenyl)-1-tosyl-1H-indole-3-carbonitrile (118I)

![5-(4-methoxyphenyl)-1-tosyl-1H-indole-3-carbonitrile (118I)](image)

This compound was prepared according to \textit{general procedure F} by treating 117g (100 mg, 0.27 mmol) with (4-methoxyphenyl)boronic acid (49 mg, 0.32 mmol) in toluene/ethanol 3:1 at 110 °C. The crude product was purified by flash chromatography (hexane/ethyl acetate 8:1 → 4:1) to give the product as a white solid (100 mg, 92%). \textbf{m.p.} 144–145 °C; \textit{R}$_f$ (hexane/ethyl acetate 6:1): 0.55; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.09 (1H, s), 8.01 (1H, d, $J = 8.7$ Hz), 7.86 (1H, s), 7.82 (2H, d, $J = 6.5$ Hz), 7.62 (1H, d, $J = 8.7$ Hz), 7.52 (2H, d, $J = 8.1$ Hz), 7.31 (2H, d, $J = 8.0$ Hz), 6.99 (2H, d, $J = 8.1$ Hz), 3.86 (3H, s), 2.39 (3H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 159.6, 146.5, 138.3, 134.3, 133.6, 132.8, 130.6, 129.1, 128.5, 127.4, 126.0, 118.0, 115.0, 114.6, 114.1, 113.7, 94.0, 55.5, 21.8; HRMS (ESI+) Calc. for C$_{23}$H$_{18}$N$_2$O$_3$S $[$M + H$]^+$ 403.1111, found 403.1107. \textbf{IR} (neat, cm$^{-1}$): $\tilde{\nu}$ 3141, 2937, 2227, 1383, 1173, 577.
5-(4-methoxyphenyl)-3-(1H-tetrazol-5-yl)-1-tosyl-1H-indole (118m)

This compound was prepared according to general procedure W by treating 118l (84 mg, 0.2 mmol) with TMSN₃ (264 µL, 2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 3:1 → 1:1) to give the product as a white solid (69 mg, 77%). m.p. 121–123 °C; Rₜ (hexane/ethyl acetate 1:1): 0.35; ¹H NMR (300 MHz, CDCl₃):  δ 8.47 (1H, s), 8.38 (1H, s), 7.97 (1H, d, J = 8.7 Hz), 7.79 (2H, d, J = 8.0 Hz), 7.54 (1H, d, J = 8.7 Hz), 7.46 (2H, d, J = 8.2 Hz), 7.14 (2H, d, J = 8.0 Hz), 6.88 (2H, d, J = 8.2 Hz), 3.77 (3H, s), 2.24 (3H, s); NH signal not observed; ¹³C NMR (75 MHz, CDCl₃): δ 158.9, 150.0, 146.2, 136.7, 133.5, 133.1, 132.2, 130.5, 128.1, 127.8, 127.4, 127.0, 124.7, 118.9, 114.4, 113.7, 107.0, 55.1, 21.0; HRMS (ESI+) Calc. for C₂₃H₁₉N₅O₃S [M + Na]⁺ 468.1101, found 468.1103. IR (neat, cm⁻¹): ν 3112, 2936, 1610, 1174, 584.

5-(4-methoxyphenyl)-3-(1H-tetrazol-5-yl)-1H-indole (119k)

This compound was prepared according to general procedure C by treating 118m (89 mg, 0.2 mmol) with KOH (56 mg, 1 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100:1 → 50:1) to give the product as a white solid (56 mg, 96%). m.p. 253–254 °C; Rₜ (CH₂Cl₂/MeOH 20:1): 0.40; ¹H NMR (500 MHz, d-DMSO): δ 11.88 (1H, s), 8.40 (1H, d, J = 1.4 Hz), 8.10 (1H, d, J = 2.8 Hz), 7.64 (2H, dd, J = 3.0, 11.8 Hz), 7.59 (1H, d, J = 8.2 Hz), 7.50 (1H, dd, J = 1.7, 8.5 Hz), 7.05 (2H, dd, J = 3.0, 11.8 Hz), 3.80 (3H, s); ¹³C NMR (125 MHz, d-DMSO): δ 158.4, 151.4, 135.7, 133.9, 133.3, 128.0, 127.7, 125.1, 121.9, 117.7, 114.4, 112.7, 99.9, 55.2; HRMS (ESI+) Calc. for C₁₆H₁₃N₃O [M + Na]⁺ 314.1012, found
4-(3-(1H-tetrazol-5-yl)-1H-indol-5-yl)phenol (120f)

This compound was prepared according to general procedure D by treating 119k (58 mg, 0.2 mmol) with BBr₃ (1.2 mL, 1.2 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 50:1 → 10:1) to give the product as a white solid (39 mg, 70%). m.p. 225 – 227 °C; R₇ (CH₂Cl₂/MeOH 20:1): 0.20; ¹H NMR (400 MHz, d-DMSO): δ 11.84 (1H, s), 9.48 (1H, s), 8.37 (1H, s), 8.07 (1H, d, J = 2.8 Hz), 7.57 – 7.52 (3H, m), 7.47 (1H, dd, J = 1.6, 8.5 Hz), 6.88 (2H, d, J = 8.6 Hz), NH of tetrazole signal not observed; ¹³C NMR (100 MHz, d-DMSO): δ 156.9, 152.1, 135.9, 133.9, 132.7, 128.3, 127.8, 125.5, 122.0, 117.9, 116.1, 113.0, 100.6; HRMS (ESI+) Calc. for C₁₅H₁₁N₅O [M + Na]⁺ 300.0856, found 300.0854. IR (neat, cm⁻¹): ν 3568, 3370, 3177, 2901, 2850, 1599, 1473, 1211, 806. HPLC: 97.7% (HPLC Method A), RT: 18.3 min.

5-bromo-1-tosyl-1H-pyrrolo[2,3-b]pyridine (121)

This compound was prepared according to step 2 in general procedure A by treating 25a (197 mg, 1.0 mmol) with TsCl (228 mg, 1.2 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 20:1 → 10:1) to give the product as a white solid (316 mg, 90%). m.p. 140 – 141 °C; R₇ (hexane/ethyl acetate 12:1): 0.50; ¹H NMR (300 MHz, CDCl₃): δ 8.41 (1H, d, J = 2.1 Hz), 8.05 (2H, d, J = 8.2 Hz), 7.87 (1H, d, J = 2.1 Hz), 7.73 (1H, d, J = 4.0 Hz), 7.23 (2H, d, J = 8.2Hz), 6.51 (1H, d, J = 4.0 Hz), 2.29 (3H, s); ¹³C NMR (75 MHz, CDCl₃): δ 145.3, 145.1, 145.0, 134.8, 131.5, 129.5, 127.8, 127.7, 124.2, 114.9, 104.4, 21.4; HRMS (ESI+) Calc. for C₁₄H₁₁N₂O₂BrS [M + Na]⁺ 372.9617/374.9596, found 372.9619/374.9599. IR (neat, cm⁻¹): ν 1592, 1438, 1153, 664.
1-tosyl-1H-pyrrolo[2,3-b]pyridine-5-carboxylic acid (122)

This compound was prepared following a literature procedure.\(^{281}\) To a solution of 121 (1.0 equiv., 500 mg), Pd(OAc)\(_2\) (5 mol%, 16 mg), dppf (10 mol%, 79 mg) and \(i\)-Pr\(_2\)NEt (5.0 equiv., 1.24 mL) in DMF (0.05 M) was added acetic-formic anhydride (5.0 equiv., 626 mg) which was freshly prepared by treating the Ac\(_2\)O (5.0 equiv., 673 µL) with formic acid (5.0 equiv., 251 µL) at 65 °C for 30 min.\(^{334}\) the resulting mixture was heated to 110 °C for 5 h. After completion monitored by TLC, the reaction mixture was cooled to RT and diluted with ethyl acetate (30 mL) and quenched with aq. KOH (20%, 10 mL) to adjust pH = 9, the aqueous layer was collected and acidified by aq. HCl (32%) to adjust pH 4–6, and the corresponding acid was precipitated and filtered to get the product as a white powder (374 mg, 83%) without further purification. **m.p.** 175–176 °C; **R\(_f\)** (hexane/ethyl acetate 1:1): 0.25; \(^1\)H NMR (300 MHz, \(d\)-DMSO): \(\delta\) 13.28 (1H, s), 8.88 (1H, d, \(J = 2.0\) Hz), 8.56 (1H, d, \(J = 2.0\) Hz), 8.03–8.00 (3H, m), 7.42 (2H, d, \(J = 8.2\) Hz), 6.93 (1H, d, \(J = 4.0\) Hz), 2.33 (3H, s); \(^{13}\)C NMR (75 MHz, \(d\)-DMSO): \(\delta\) 166.3, 148.2, 145.9 (two overlapping signals), 134.3, 131.6, 130.1, 128.3, 127.6, 122.4, 122.1, 106.5, 21.1; **HRMS** (ESI+) Calc. for C\(_{15}\)H\(_{12}\)N\(_2\)O\(_4\)S [M + Na\(^+\)] 339.0410, found 339.0409. **IR** (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3146, 3102, 2854, 1680, 1528, 1192, 669, 589.

**General procedure Y** for amide coupling reactions by utilising coupling reagent

\[
\begin{align*}
\text{COOH} & \quad \text{H}_{2}N \quad \text{R} \quad \text{Het} \quad \text{HBTU, \(i\)-Pr\(_2\)NEt, DMSO, RT} \\
\text{RO} & \quad \text{Het} \quad \text{R} \quad \text{RO} \\
\end{align*}
\]

To a solution of carboxylic acid (1.0 equiv.) in DMSO (0.05 M) was added HBTU (1.2 equiv.) under nitrogen at 0 °C, followed by the addition of \(i\)-Pr\(_2\)NEt (2.5 equiv.) and aniline (1.1 equiv.). The reaction mixture was allowed to warm to RT for 2 h. after completion monitored by TLC, the mixture was extracted with ethyl acetate (3 x 10 mL) and H\(_2\)O (15 mL), and the combined organic layers were dried over MgSO\(_4\)
and concentrated in vacuo.

\textit{N-}(4-((\textit{tert}-butyldimethylsilyl)oxy)phenyl)-1-tosyl-\textit{1H}-pyrrolo[2,3-\textit{b}]pyridine-5-carboxamide (123a)

This compound was prepared according to \textit{general procedure Y} by treating 122 (158 mg, 0.5 mmol) with 34a (123 mg, 0.55 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 $\rightarrow$ 3:1) to give the product as a white solid (159 mg, 61%). \textit{m.p.} 190–192 °C; $R_f$ (hexane/ethyl acetate 2:1): 0.35; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.81 (1H, d, $J = 2.1$ Hz), 8.26 (1H, d, $J = 2.1$ Hz), 8.06–8.03 (3H, m), 7.72 (1H, d, $J = 4.0$ Hz), 7.48 (2H, d, $J = 8.8$ Hz), 7.26 (2H, d, $J = 8.1$ Hz), 6.82 (2H, dd, $J = 2.1$, 6.7 Hz), 6.55 (1H, d, $J = 4.0$ Hz), 2.36 (3H, s), 0.98 (9H, s), 0.19 (6H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 164.5, 152.9, 148.3, 145.8, 143.7, 135.1, 131.5, 129.9, 129.1, 128.3, 127.9, 126.7, 122.4, 122.1, 120.6, 105.6, 25.8, 21.8, 18.3, −4.3; HRMS (ESI+) Calc. for C$_{27}$H$_{31}$N$_3$O$_4$Si $[M + Na]^+$ 544.1697, found 544.1693. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3363, 2954, 2855, 1672, 1508, 1155, 997.

\textit{N-}(3-((\textit{tert}-butyldimethylsilyl)oxy)phenyl)-1-tosyl-\textit{1H}-pyrrolo[2,3-\textit{b}]pyridine-5-carboxamide (123b)

This compound was prepared according to \textit{general procedure Y} by treating 122 (158 mg, 0.5 mmol) with 34b (123 mg, 0.55 mmol). The crude product was purified by flash chromatography (hexane/ethyl acetate 5:1 $\rightarrow$ 3:1) to give the product as a white solid (206 mg, 79%). \textit{m.p.} 105–107 °C; $R_f$ (hexane/ethyl acetate 2:1): 0.35; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.82 (1H, d, $J = 1.7$ Hz), 8.62 (1H, s), 8.22 (1H, d, $J = 1.7$ Hz), 8.06 (2H, d, $J = 8.2$ Hz), 7.65 (1H, d, $J = 4.0$ Hz), 7.41–7.39 (1H, m), 7.28–7.25 (3H,
m), 7.18 (1H, t, J = 8.0 Hz), 6.67–6.65 (1H, m), 6.46 (1H, d, J = 4.0 Hz), 2.38 (3H, s), 1.00 (9H, s), 0.23 (6H, s); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 164.9, 156.2, 148.0, 145.7, 144.0, 139.1, 134.8, 129.8, 129.6, 128.9, 128.1, 127.5, 126.6, 122.0, 116.4, 113.2, 112.3, 105.5, 25.7, 21.6, 18.2, –4.4; HRMS (ESI+) Calc. for C$_{27}$H$_{31}$N$_3$O$_4$Si [M + Na]$^+$ 544.1697, found 544.1694. IR (neat, cm$^{-1}$): ν 2952, 2929, 2857, 1596, 1374, 1191, 838, 578.

$N$-(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridine-5-carboxamide (124a)

This compound was prepared according to general procedure C by treating 123a (104 mg, 0.2 mmol) with KOH (56 mg, 1 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 → 30:1) to give the product, including TBS deprotection, as a pale yellow solid (49 mg, 96%). m.p. 295–297 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.20; $^1$H NMR (300 MHz, $d$-DMSO): δ 11.94 (1H, s), 10.04 (1H, s), 9.24 (1H, s), 8.81 (1H, s), 8.53 (1H, s), 7.57–7.54 (3H, m), 6.75 (2H, d, J = 8.4 Hz), 6.59 (1H, d, J = 3.0 Hz); $^{13}$C NMR (75 MHz, $d$-DMSO): δ 164.7, 153.6, 149.5, 142.7, 130.9, 127.8, 127.6, 122.9, 122.2, 118.7, 115.0, 100.9; HRMS (ESI+) Calc. for C$_{14}$H$_{11}$N$_3$O$_2$ [M + Na]$^+$ 276.0743, found 276.0742. IR (neat, cm$^{-1}$): ν 3334, 3114, 2863, 1636, 1535, 1235, 752.

$N$-(3-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridine-5-carboxamide (124b)

This compound was prepared according to general procedure C by treating 123b (104 mg, 0.2 mmol) with KOH (56 mg, 1 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 → 30:1) to give the product, including TBS deprotection, as a pale yellow solid (48 mg, 94%). m.p. 290–295 °C; $R_f$ (hexane/ethyl acetate 1:1): 0.20; $^1$H NMR (400 MHz, $d$-DMSO): δ 11.95 (1H, s),...
10.13 (1H, s), 9.38 (1H, s), 8.80 (1H, d, J = 2.2 Hz), 8.54 (1H, d, J = 1.8 Hz), 7.59 (1H, dd, J = 2.5, 3.4 Hz), 7.39 (1H, t, J = 2.1 Hz), 7.20–7.17 (1H, m), 7.11 (1H, t, J = 8.0 Hz), 6.60 (1H, dd, J = 1.8, 3.4 Hz), 6.50 (1H, ddd, J = 1.0, 2.4, 8.0 Hz); \(^{13}\)C NMR (100 MHz, \(d\)-DMSO): \(\delta\) 165.7, 158.0, 150.1, 143.3, 140.9, 129.7, 128.5, 123.3, 119.1, 111.5, 111.1, 107.9, 101.5; HRMS (ESI+) Calc. for C\(_{14}\)H\(_{11}\)N\(_3\)O\(_2\) [M + Na]\(^+\) 276.0743, found 276.0741. IR (neat, cm\(^{-1}\)): \(\bar{\nu}\) 3343, 3283, 3135, 2882, 1619, 1597, 1441, 1212, 760.

**General procedure Z for iodination**

To a solution of 124a–b (1.0 equiv.) in dry DMF (0.05 M) was added KOH (5.0 equiv.) under nitrogen atmosphere at RT, after 10 min, a solution of I\(_2\) (1.2 equiv.) in DMF (0.6 M) was added dropwise, and the resulting mixture was stirred at RT for 2 h. After completion monitored by TLC, the reaction was quenched with sat. Na\(_2\)S\(_2\)O\(_3\) (aq.), and extracted with ethyl acetate (3 x 10 mL), the organic layers were dried over MgSO\(_4\) and concentrated *in vacuo*.

**N-(4-hydroxyphenyl)-3-iodo-1H-pyrrolo[2,3-b]pyridine-5-carboxamide (125a)**

This compound was prepared according to **general procedure Z** by treating 124a (51 mg, 0.2 mmol) with I\(_2\) (61 mg, 0.24 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 50:1) to give the product as a white solid (65 mg, 86%). m.p. 209–211 °C; \(R_f\) (CH\(_2\)Cl\(_2\)/MeOH 50:1): 0.20; \(^1\)H NMR (400 MHz, \(d\)-DMSO): \(\delta\) 12.46–12.32 (1H, br.s), 10.17 (1H, s), 9.30–9.21 (1H, br.s), 8.83 (1H, d, J = 2.1 Hz), 8.29 (1H, d, J = 2.1 Hz), 7.83 (1H, s), 7.55 (2H, dd, J = 2.1, 6.8 Hz), 6.75 (2H, dd, J = 2.1, 6.8 Hz); \(^{13}\)C NMR (100 MHz, \(d\)-DMSO): \(\delta\) 164.1, 153.7, 149.2, 143.9, 132.1, 130.7, 127.7, 123.7, 122.4, 121.1, 115.0, 55.7; HRMS (ESI+) Calc. for
This compound was prepared according to general procedure Z by treating 124b (51 mg, 0.2 mmol) with I₂ (61 mg, 0.24 mmol). The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 50:1) to give the product as a white solid (61 mg, 80%). m.p. 205–208 °C; Rf (CH₂Cl₂/MeOH 50:1): 0.20; \(^{1}H\) NMR (300 MHz, d-DMSO): δ 12.40 (1H, br.s), 10.26 (1H, s), 9.41 (1H, br.s), 8.84 (1H, d, J = 1.8 Hz), 8.30 (1H, d, J = 1.8 Hz), 7.85 (1H, s), 7.39–7.38 (1H, m), 7.20–7.10 (2H, m), 6.52 (1H, d, J = 7.6 Hz); \(^{13}C\) NMR (75 MHz, d-DMSO): δ 164.6, 157.5, 149.3, 144.1, 140.2, 132.2, 129.2, 127.9, 123.6, 121.1, 111.2, 110.8, 107.6, 55.8; HRMS (ESI+) Calc. for C₁₄H₁₀N₃O₂I [M + Na]⁺ 401.9710, found 401.9709. IR (neat, cm⁻¹): ν 3276, 3108, 2921, 1534, 1292, 1167.

\[ \text{N-(3-hydroxyphenyl)-3-iodo-1H-pyrrolo[2,3-b]pyridine-5-carboxamide (125b)} \]

This compound was prepared according to general procedure F by treating 125a (25 mg, 0.1 mmol) with (4-hydroxyphenyl)boronic acid (17 mg, 0.12 mmol) in toluene/ethanol 3:1 at 100 °C. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 50:1→ 10:1) to give the product as a pale yellow solid (22 mg, 65%). m.p. 210–212 °C; Rf (CH₂Cl₂/MeOH 20:1): 0.30; \(^{1}H\) NMR (500 MHz, d-DMSO): δ 12.04 (1H, s), 10.11 (1H, s), 9.42 (1H, br.s), 9.25 (1H, br.s), 8.82 (1H, d, J = 2.0 Hz), 8.73 (1H, d, J = 2.0 Hz), 7.78 (1H, s), 7.57 (2H, d, J = 8.6 Hz), 7.57 (2H, d, J = 8.6 Hz), 7.20–7.10 (2H, m), 6.52 (1H, d, J = 7.6 Hz); \(^{13}C\) NMR (75 MHz, d-DMSO): δ 164.6, 157.5, 149.3, 144.1, 140.2, 132.2, 129.2, 127.9, 123.6, 121.1, 111.2, 110.8, 107.6, 55.8; HRMS (ESI+) Calc. for C₁₄H₁₀N₃O₂I [M + Na]⁺ 401.9710, found 401.9709. IR (neat, cm⁻¹): ν 3103, 3068, 2981, 2851, 1553, 1272, 773, 683.

\[ \text{N,3-bis(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridine-5-carboxamide (126a)} \]
7.53 (2H, d, J = 8.9 Hz), 6.88 (2H, d, J = 8.6 Hz), 6.75 (2H, d, J = 8.9 Hz); \(^{13}\text{C NMR}\) (125 MHz, d-DMSO): \(\delta\) 164.7, 156.0, 153.6, 150.0, 143.0, 130.7, 127.8, 127.2, 127.0, 125.3, 123.7, 122.9, 122.4, 116.4, 115.7, 115.0; \(\text{HRMS (ESI+)}\) Calc. for \(\text{C}_{20}\text{H}_{15}\text{N}_{3}\text{O}_{3}\) [M + Na]\(^{+}\) 368.1004, found 368.1006. IR (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3334, 3011, 1636, 1535, 1235. \(\text{HPLC}\): 98.8% (HPLC Method A), RT: 16.8 min.

\(N\)-(3-hydroxyphenyl)-3-(4-hydroxyphenyl)-1\(H\)-pyrrolo[2,3-b]pyridine-5-carboxamide (126b)

![Chemical structure](image)

This compound was prepared according to \textbf{general procedure F} by treating 125b (25 mg, 0.1 mmol) with (4-hydroxyphenyl)boronic acid (17 mg, 0.12 mmol) in toluene/ethanol 3:1 at 100 °C. The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 50:1 → 10:1) to give the product as a pale yellow solid (21 mg, 60%). m.p. 197–200 °C; \(R_f\) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.30; \(^{1}\text{H NMR}\) (500 MHz, d-DMSO): \(\delta\) 12.04 (1H, s), 10.18 (1H, s), 9.39 (2H, br.s), 8.80 (1H, d, J = 2.0 Hz), 8.71 (1H, d, J = 2.0 Hz), 7.76 (1H, s), 7.56 (2H, ddd, J = 2.0, 2.9, 6.6 Hz), 7.34 (1H, t, J = 2.1 Hz), 7.17–7.15 (1H, s), 7.10 (1H, t, J = 8.0 Hz), 6.87 (2H, ddd, J = 2.0, 2.9, 6.6 Hz), 6.50–6.48 (1H, m); \(^{13}\text{C NMR}\) (125 MHz, d-DMSO): \(\delta\) 165.2, 157.5, 156.0, 150.0, 143.1, 140.3, 129.2, 127.9, 127.2, 125.2, 123.7, 122.9, 116.3, 115.8, 115.7, 111.2, 110.7, 107.5; \(\text{HRMS (ESI+)}\) Calc. for \(\text{C}_{20}\text{H}_{15}\text{N}_{3}\text{O}_{3}\) [M + Na]\(^{+}\) 368.1004, found 368.1006. IR (neat, cm\(^{-1}\)): \(\tilde{\nu}\) 3214, 2920, 2851, 1609, 1494, 1312. \(\text{HPLC}\): 97.8% (HPLC Method A), RT: 17.7 min.

\(5\)-bromo-1\(H\)-pyrrolo[2,3-b]pyridine-3-carboxylic acid (127)

![Chemical structure](image)

To a solution of 25a (1 g, 5.0 mmol) in dry CH\(_2\)Cl\(_2\) (0.1 M) was added AlCl\(_3\) (1.67 g, 12.5 mmol) at 0 °C under nitrogen atmosphere. After 10 min, trichloroacetyl chloride
(670 µL, 6 mmol) was added dropwise, and the resulting mixture was allowed to warm to RT for 2 h. After completion monitored by TLC, the reaction was quenched with cold water (20 mL), and extracted with CH$_2$Cl$_2$ (3 x 10 mL), the organic layers were dried over MgSO$_4$ and concentrated in vacuo. The crude intermediate was treated with aq. NaOH (3 M, 30 mL), and stirred at RT for 3 h. After completion monitored by TLC, the resulting mixture was added aq. HCl (32%) dropwise at 0 °C to adjust pH = 4−6, and the resulting precipitate was filtered and washed by H$_2$O and hexane, dried in vacuo to give the product as a pale yellow solid (856 mg, 70% over two steps). $R_f$ (CH$_2$Cl$_2$/MeOH 50:1): 0.25; $^1$H NMR (500 MHz, d-DMSO): $\delta$ 12.65 (1H, s), 12.40 (1H, s), 8.41 (1H, d, $J = 2.8$ Hz), 8.39 (1H, d, $J = 2.8$ Hz), 8.20 (1H, d, $J = 3.7$ Hz); $^{13}$C NMR (125 MHz, d-DMSO): $\delta$ 164.9, 147.1, 143.8, 134.2, 130.5, 120.0, 112.8, 106.0. HRMS (ESI+) Calc. for C$_8$H$_5$N$_2$O$_2$Br $[M + H]^+$ 240.9607/242.9587, found 240.9606/242.9585. The spectroscopic data matched that reported in the literature.$^{335}$

5-bromo-N-(4-((tert-butyldimethylsilyloxy)phenyl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamide (128a)

This compound was prepared according to **general procedure Y** by treating 127 (120 mg, 0.5 mmol) with 34a (123 mg, 0.55 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 100:1 → 50:1) to give the product as a pale yellow solid (156 mg, 70%). m.p. 270−272 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.45; $^1$H NMR (500 MHz, d-DMSO): $\delta$ 12.47 (1H, br.s), 9.76 (1H, s), 8.61 (1H, d, $J = 2.9$ Hz), 8.43 (1H, d, $J = 1.7$ Hz), 8.38 (1H, d, $J = 2.9$ Hz), 7.60 (2H, dd, $J = 3.2$, 11.3 Hz), 6.83 (2H, ddd, $J = 2.6$, 4.2, 6.8 Hz), 0.96 (9H, s), 0.19 (6H, s); $^{13}$C NMR (125 MHz, d-DMSO): $\delta$ 161.8, 150.7, 146.8, 143.7, 133.0, 131.1, 130.3, 121.5, 120.4, 120.0, 112.4, 109.1, 25.6, 17.9, −4.5; HRMS (ESI+) Calc. for C$_{20}$H$_{24}$N$_3$O$_2$BrSi $[M + Na]^+$
5-bromo-\(N\)-(3-((\textit{tert}-butyldimethylsilyl)oxy)phenyl)-1\(H\)-pyrrolo[2,3-\(b\)]pyridine-3-carboxamide (128b)

This compound was prepared according to general procedure Y by treating 127 (120 mg, 0.5 mmol) with 34b (123 mg, 0.55 mmol). The crude product was purified by flash chromatography (CH\(_2\)Cl\(_2\)/MeOH 100:1 → 50:1) to give the product as a pale yellow solid (167 mg, 75%). m.p. 234–235 °C; \(R_f\) (CH\(_2\)Cl\(_2\)/MeOH 20:1): 0.45; \(^1\)H NMR (300 MHz, \textit{d}-DMSO): \(\delta\) 12.52 (1H, s), 9.80 (1H, s), 8.63 (1H, d, \(J = 2.1\) Hz), 8.48 (1H, s), 8.39 (1H, d, \(J = 2.1\) Hz), 7.41-7.40 (1H, m), 7.36 (1H, d, \(J = 8.2\) Hz), 7.19 (1H, t, \(J = 8.0\) Hz), 6.54 (1H, dd, \(J = 1.4, 8.0\) Hz), 0.97 (9H, s), 0.21 (6H, s); \(^{13}\)C NMR (75 MHz, \textit{d}-DMSO): \(\delta\) 162.1, 155.2, 146.9, 143.8, 140.5, 131.1, 130.7, 129.4, 120.5, 114.4, 112.9, 112.5, 111.3, 109.0, 25.5, 17.9, −4.5; HRMS (ESI+) Calc. for C\(_{20}\)H\(_{24}\)N\(_3\)O\(_2\)BrSi [M + Na]% 468.0713/470.0693, found 468.0710/470.0690. IR (neat, \(\text{cm}^{-1}\)): \(\tilde{\nu}\) 3362, 3091, 2927, 1637, 1536, 837.

\(N\)-(4-((\textit{tert}-butyldimethylsilyl)oxy)phenyl)-5-(4-hydroxyphenyl)-1\(H\)-pyrrolo[2,3-\(b\)]pyridine-3-carboxamide (129a)

This compound was prepared according to general procedure F by treating 128a (89 mg, 0.2 mmol) with (4-hydroxyphenyl)boronic acid (33 mg, 0.24 mmol) in toluene/ethanol 3:1 at 100 °C. The crude product was purified by flash
chromatography (CH$_2$Cl$_2$/MeOH 50:1 → 20:1) to give the product as a pale yellow solid (58 mg, 63%). **m.p.** 274–275 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.35; $^1$H NMR (400 MHz, $d$-DMSO): $\delta$ 12.24–12.23 (1H, br.s), 9.73 (1H, s), 9.55 (1H, s), 8.60 (1H, d, $J$ = 2.2 Hz), 8.52 (1H, d, $J$ = 2.2 Hz), 8.38 (1H, d, $J$ = 3.0 Hz), 7.62 (2H, dd, $J$ = 2.2, 6.8 Hz), 7.52 (2H, dd, $J$ = 2.0, 6.6 Hz), 6.89 (2H, dd, $J$ = 2.0, 6.6 Hz), 6.83 (2H, dd, $J$ = 2.2, 6.8 Hz), 0.96 (9H, s), 0.18 (6H, s); $^{13}$C NMR (100 MHz, $d$-DMSO): $\delta$ 162.4, 156.9, 150.6, 147.6, 142.3, 133.3, 129.9, 129.4, 129.3, 128.1, 126.4, 119.7, 118.7, 115.9, 109.5, 25.6, 17.9, −4.5; HRMS (ESI+) Calc. for C$_{26}$H$_{29}$N$_3$O$_3$Si [M + Na]$^+$ 482.1870, found 482.1867. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3231, 2954, 1508, 1262.

$N$-3-((tert-butyldimethylsilyloxy)phenyl)-5-(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamide (129b)

This compound was prepared according to **general procedure F** by treating 128b (89 mg, 0.2 mmol) with (4-hydroxyphenyl)boronic acid (33 mg, 0.24 mmol) in toluene/ethanol 3:1 at 100 °C. The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 50:1 → 20:1) to give the product as a pale yellow solid (56 mg, 61%). **m.p.** 242–244 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 20:1): 0.35; $^1$H NMR (400 MHz, $d$-DMSO): $\delta$ 12.29 (1H, s), 9.76 (1H, s), 9.55 (1H, s), 8.59 (1H, d, $J$ = 2.2 Hz), 8.53 (1H, d, $J$ = 2.3 Hz), 8.43 (1H, d, $J$ = 2.9 Hz), 7.53 (2H, d, $J$ = 8.6 Hz), 7.42 (1H, t, $J$ = 2.1 Hz), 7.40–7.38 (1H, m), 7.20 (1H, t, $J$ = 8.1 Hz), 6.90 (2H, d, $J$ = 8.6 Hz), 6.56–6.54 (1H, m), 0.98 (9H, s), 0.23 (6H, s); $^{13}$C NMR (100 MHz, $d$-DMSO): $\delta$ 162.6, 156.9, 155.2, 147.6, 142.4, 140.8, 130.0, 129.7, 129.4, 129.3, 128.1, 126.4, 118.7, 115.9, 114.2, 112.9, 111.2, 109.4, 25.6, 17.9, −4.5; HRMS (ESI+) Calc. for C$_{26}$H$_{29}$N$_3$O$_3$Si [M + Na]$^+$ 482.1870, found 482.1866. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3392, 2953, 2851, 1588, 1203, 830.
N,5-bis(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamide (130a)

This compound was prepared according to general procedure G by treating 129a (46 mg, 0.1 mmol) with TBAF (0.2 mL, 0.2 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 20:1 → 10:1) to give the product as a pale yellow solid (32 mg, 93%). m.p. 225–227 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 10:1): 0.30; $^1$H NMR (400 MHz, d-DMSO): $\delta$ 12.21–12.20 (1H, br.s), 9.64 (1H, s), 9.54 (1H, s), 9.17 (1H, s), 8.60 (1H, d, $J = 2.2$ Hz), 8.51 (1H, d, $J = 2.2$ Hz), 8.38 (1H, d, $J = 2.8$ Hz), 7.52 (4H, d, $J = 8.6$ Hz), 6.89 (2H, d, $J = 8.6$ Hz), 6.74 (2H, d, $J = 8.8$ Hz); $^{13}$C NMR (100 MHz, d-DMSO): $\delta$ 162.3, 156.9, 153.2, 147.6, 142.2, 131.0, 129.9, 129.5, 129.1, 128.1, 126.4, 121.9, 118.8, 115.9, 115.0, 109.6; HRMS (ESI$^+$) Calc. for C$_{20}$H$_{15}$N$_3$O$_3$ [M + Na]$^+$ 368.1006, found 368.1006. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3090, 2960, 2873, 1511, 820. HPLC: 99.7% (HPLC Method A), RT: 16.3 min.

N-(3-hydroxyphenyl)-5-(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamide (130b)

This compound was prepared according to general procedure G by treating 129b (46 mg, 0.1 mmol) with TBAF (0.2 mL, 0.2 mmol). The crude product was purified by flash chromatography (CH$_2$Cl$_2$/MeOH 20:1 → 10:1) to give the product as a pale yellow solid (33 mg, 95%). m.p. 280–284 °C; $R_f$ (CH$_2$Cl$_2$/MeOH 10:1): 0.30; $^1$H NMR (400 MHz, d-DMSO): $\delta$ 12.25 (1H, d, $J = 2.5$ Hz), 9.70 (1H, s), 9.55 (1H, s), 9.34 (1H, s), 8.60 (1H, d, $J = 2.3$ Hz), 8.52 (1H, d, $J = 2.3$ Hz), 8.43 (1H, d, $J = 2.9$ Hz).
Hz), 7.52 (2H, dd, J = 2.0, 6.6 Hz), 7.37 (1H, t, J = 2.0 Hz), 7.15 (dt, 1H, J = 1.1, 8.0 Hz), 7.10 (1H, t, J = 8.0 Hz), 6.90 (2H, dd, J = 2.0, 6.6 Hz), 6.48–6.45 (1H, m); $^{13}$C NMR (100 MHz, d$_2$-DMSO): $\delta$ 162.6, 157.5, 157.0, 147.6, 142.4, 140.6, 130.0, 129.6, 129.5, 129.2, 128.1, 126.4, 118.8, 116.0, 110.6, 110.1, 109.5, 107.0; HRMS (ESI+) Calc. for C$_{20}$H$_{15}$N$_3$O$_3$ [M + Na]$^+$ 368.1006, found 368.1005. IR (neat, cm$^{-1}$): $\tilde{\nu}$ 3393, 3091, 2921, 1639, 1365. HPLC: 99.5% (HPLC Method A), RT: 17.4 min.

6.5 Biology evaluation

All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). Primary antibodies against DYRK1A (#8765), DYRK1B (#2703), DYRK2 (#8143) and p38$\alpha$ (#9218), secondary anti-rabbit (#7074) and anti-mouse (#7076) HRP-linked antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). EGFR (#sc03) and $\beta$-actin (#A5441) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and Sigma Aldrich (St. Louis, MO, USA), respectively. CLK1 antibody (#ab209681) was purchased from Abcam (Cambridge, UK). EGF was purchased from Life Technologies (Carlsbad, CA, USA).

6.5.1 Kinase inhibition assay

Active DYRK1A, DYRK1B, DYRK2 and CLK1 (all Life Technologies) were assayed in Tris buffer (50 mM Tris-HCl, pH 7.5) containing 0.1 mM EGTA, 15 mM DTT, MgAc/ATP cocktail (0.5 mM HEPES pH 7.4; 10 mM Mg(CH$_3$COO)$_2$; 0.1 mM ATP), [$\gamma$-$^{32}$P]-ATP 100–300 cpm/pmol and test compounds diluted in deionised water. As substrate, Woodtide (50 $\mu$M, Genscript) was used in DYRK1A, DYRK1B and DYRK2 activity assays, and RS repeat peptide [KKGRSRSRSRSRSR] (20 $\mu$M, Genscript) was used in CLK1 activity assays. The reaction was initiated with 1 ng/$\mu$L DYRK1A or 0.5 ng/$\mu$L DYRK1B, DYRK2 and CLK1. The reaction mixture was incubated at 30 $^\circ$C for 10 min (DYRK1A) or 40 min (DYRK1B, DYRK2, CLK1). Reaction was stopped by pipetting 10 $\mu$L of the reaction mixture onto P81 paper (Reaction Biology) and washing with 0.75% w/v H$_3$PO$_4$ and acetone. P81 papers were transferred to sample bags containing Optiphase Supermix scintillation cocktail.
(Perkin Elmer) and radioactivity (cpm) was measured with MicroBeta Trilux 2 counter (Perkin Elmer). Compounds that inhibited DYRK1A activity by more than 50% at 10 µM were tested in an eight-point serial dilution at a Log₃ scale. Data were normalised to controls (set as 100% activity) and IC₅₀ values calculated by non-linear regression analysis using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). IC₅₀ values represent mean ± SEM from 3 independent experiments performed in triplicate.

6.5.2 Cell culture

U251 and A172 cell lines were obtained from the European Collection of Cell Cultures (EACC, Salisbury, UK) through Cell Bank Australia in 2014. Cells were cultured in DMEM medium supplemented with 10% FBS and Antibiotic-Antimycotic (both Life Technologies) at 37 °C and 5% CO₂. Primary glioblastoma cell lines (RN1, JK2, WK1, SJH1) were derived from glioblastoma specimens, characterised as described²⁹¹-²⁹² and cultured in KnockOut DMEM/F-12 basal medium supplemented with StemPro NSC SFM supplement, 2 mM GlutaMAX-ICTS, 20 ng/mL EGF, 10 ng/mL FGF-β and Antibiotic-Antimycotic solution (all Life Technologies) as adherent cells on flasks coated with MatriGel Matrix (BD Falcon). The protocols were approved by the Human Ethics Committee of the University of Sydney (HREC2013/131) and the Human Ethics Committee of the Royal Brisbane & Women’s Hospital (RBWH 2004/161). All cell cultures were routinely tested for mycoplasma infection and the cumulative length of culturing did not exceed 10 passages.

6.5.2.1 Cell viability assay

U251, A172, RN1, WK1 (2 x 10³ cells/well) and JK2, SJH1 (4 x 10³ cells/well) were seeded in 96-well plate and treated on the following day with vehicle or test compounds (0.001–50 µM) for 3 days (U251, A172, RN1, JK2) or 5 days (WK1, SJH1). 10 µL of Cell-Titer Blue reagent (Promega, WI, USA) was added to each well and incubated at 37 °C for 4 h. Fluorescence was measured with Tecan M200 PRO+
microplate reader (Tecan, Switzerland) at 585 nm. Data were normalised to controls (set as 100% viability) and EC<sub>50</sub> values calculated by non-linear regression analysis using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). EC<sub>50</sub> values represent mean ± SEM from 3 independent experiments performed in triplicate.

6.5.2.2 Clonogenic survival

RN1 (2 x 10<sup>3</sup> cells/well) and U251 (0.5 x 10<sup>3</sup> cells/well) were seeded onto 6-well plates and treated on the following day with vehicle or test compounds (0.01–5 µM). Cells were grown for 12 days, fixed with 50% methanol and stained with Toluidine Blue (Sigma Aldrich). Colonies were counted using the ImageJ software ( Colony Area function). Data were normalised to controls (set as 100% survival) and EC<sub>50</sub> values calculated by non-linear regression analysis using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). EC<sub>50</sub> values represent mean ± SEM from 3 independent experiments performed in duplicate. One-way ANOVA, followed by Dunnett’s multiple comparison test (GraphPad Prism 6.0) was used to determine statistical significance. P < 0.05 was considered as significant.

6.5.2.3 Migration and invasion assays

U251 (1.7 x 10<sup>3</sup> cells/well), A172 (1.5 x 10<sup>3</sup> cells/well) and RN1 (2.0 x 10<sup>3</sup> cells/well) were seeded onto ImageLock 96-well plates (IncuCyte). For RN1 cells, the plates were pre-coated with MatriGel Matrix (BD Falcon). After 24 h, each well was scratched using the WoundMaker (IncuCyte) and washed with Dulbecco’s PBS (Sigma Aldrich). Migration assays were performed in 1% FBS in DMEM (A172, U251) or 0.2% neural supplement in StemPro NSC SFM (RN1) containing test compounds or DMSO (control). For the invasion assays, 45 µL of MatriGel Matrix and 5 µL of 10x inhibitor (or DMSO) were added to each well and allowed to solidify at 37 °C for 30–60 min. Once the Matrigel Matrix was set, cells were treated with test compounds or DMSO in 1% FBS in DMEM (A172, U251). The plates were imaged over 72 h (IncuCyte) and the relative wound density (RWD, defined as the cumulative increase of wound confluence as a percentage over time) was analysed with IncuCyte.
Zoom software. Velocity (µm/h) was calculated between 0–20 h, where the RWD curves were steep and their slopes constant, following the Wound Healing Data analysis (ibidi GmbH, Version 1.2; http://tinyurl.com/gv6ehvo).

6.5.2.4 Transient DYRK1A knockdown and EGFR degradation assays

U251 (7–9 x10^5 cells/well) were plated on 6-well plates and incubated overnight or until 60–70% confluence. Cells were washed with PBS and treated with transfection medium containing 10 nM siRNA-DYRK1A (5’ – CCGUAAACUUCAUAACAUUtt – 3’) or 10 nM siRNA-Ctr (both Ambion Silencer Select) diluted in RNAiMAX®, Opti-MEM® and serum free DMEM (all Life Technologies), according to manufacturer’s instructions. Cells were incubated in the transfection medium for 12–16 h and then starved overnight in serum free DMEM. For the EGFR degradation assay, starved cells were incubated with cycloheximide (2 µg/mL, 2 h) and treated with EGF (100 ng/mL). Cells were lysed at indicated time points with lysis buffer (1 M Tris, 0.5 M EDTA, 5 M NaCl, 1 M MgCl₂, Triton X-100, 0.5 M NaF, 10 % v/v glycerol, 1 mM PMSF, 1mM Na₃VO₄) supplemented with protease inhibitor cocktail (Roche), the protein concentration in lysates was determined with the BCA Protein Assay Kit (Life Technologies) and samples were analysed by Western blotting.

6.5.4.2.1 EGFR degradation assay upon drug treatment

U251 (3 x 10^5 cells/well) were seeded onto 6-well plates and starved in serum free DMEM overnight. Starved cells were incubated with test compounds (2.5 µM) or DMSO (control), treated with cycloheximide (30 µg/mL; 1 h) and EGF (100 ng/mL) for indicated time points. Cells were lysed with lysis buffer (as above), protein concentration was determined with the BCA Protein Assay Kit (Life Technologies) and samples were analysed by Western blotting.
6.5.4.2.2 Cellular thermal shift assay

U251 (8 x 10^6 cells/plate) were seeded onto 100 mm Petri dishes and incubated overnight. Cells were treated with test compound (10 µM) or DMSO for 1 h, harvested, washed with PBS and resuspended in PBS containing protease inhibitor cocktail (Roche). Cell suspensions were divided into 100 µL aliquots in 0.2 mL PCR tubes. Each tube was heated at indicated temperatures (40–70 °C) for 3 min using the Veriti thermal cycler (Life Technologies) followed by 3 min cooling at 25 °C, and then snap-frozen using liquid nitrogen.

For the ITDRF assay, U251 (8 x 10^6 cells/plate) were seeded onto 100 mm Petri dishes and incubated overnight. Cells were treated with test compound (0–20 µM) or DMSO for 1 h, harvested, washed with PBS and re-suspended in PBS containing protease inhibitor cocktail (Roche). Cell suspensions were divided into 100 µL aliquots in 0.2 mL PCR tubes and heated at 54 °C for 3 min using the Veriti Thermal Cycler (Life Technologies) followed by 3 min cooling at 25 °C, and then snap-frozen using liquid nitrogen. The heat-treated cells were lysed by 3 freeze-thaw cycles using liquid nitrogen and 37 °C water bath. Cell lysates were centrifuged at 15,000 rpm for 20 min at 4 °C. Clear supernatants were removed, subjected protein quantification using the BCA Protein Assay Kit (Life Technologies, following manufacturer’s instruction) and analysed by Western blotting.

6.5.3 Western blotting

Cell lysates (20–60 µg) or CETSA samples (30 µg) were resolved on 8% or 4–12% SDS-PAGE gels and transferred onto PVDF membranes (both Life Technologies). Membranes were blocked with 5% (w/v) skim milk or 15% (w/v) BSA in TBST and incubated with primary antibody overnight at 4 °C. After washing with TBST, membranes were incubated with their respective secondary antibody for 1 h at room temperature. Detection was performed with Immobilon Western HRP Substrate
Luminol-Peroxidase reagent (MerckMillipore) and the ChemiDoc MP System (BioRad).
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84. Lochhead, P. A.; Sibbet, G.; Morrice, N.; Cleghon, V. Cell, 2005, 121 (6), 925-936.


Chapter 7


Appendix - Selected spectra

4,4’-(ethane-1,2-diylbis(azanediyl))diphenol (100a)

$^1$H NMR (500 MHz, d-DMSO), $^{13}$C NMR (125 MHz, d-DMSO), HPLC (Method A)
**N-(4-(3-(4-hydroxyphenyl)-1H-pyrrolo[2,3-b]pyridin-5-yl)phenyl)acetamide (120a)**

1H NMR (300 MHz, d-DMSO), 13C NMR (75 MHz, d-DMSO), HPLC (Method A)
Sample Name: zq1035
Date Acquired: 7/10/2015 7:59:29 PM

Injection Volume: 10.00 ul
Date Acquired: 7/10/2015 7:59:29 PM

Run Time: 51.0 minutes

Peak Information

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Note: The peak information table includes retention times (RT), peak areas, and percentage areas.
Appendix

\(N,3\text{-bis}(4\text{-hydroxyphenyl})-1\text{-H-pyrrolo}[2,3-b]pyridine-5\text{-carboxamide}\) (126a)

\(^1\text{H NMR}\) (500 MHz, \(d\text{-DMSO}\)), \(^{13}\text{C NMR}\) (125 MHz, \(d\text{-DMSO}\)), \(\text{HPLC}\) (Method A)
\[ N,5\text{-bis}(4\text{-hydroxyphenyl})-1H\text{-pyrrolo}[2,3-b]\text{pyridine-3-carboxamide (130a)} \]

\(^1\text{H NMR (400 MHz, }d\text{-DMSO), }^{13}\text{C NMR (100 MHz, }d\text{-DMSO), HPLC (Method A)}\]