A Fragment-Based Drug Discovery Approach for the Development of Selective Inhibitors of Protein Kinase CK2

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Over the last twenty years, fragment-based drug discovery (FBDD) has emerged as a highly successful way to provide lead compounds for subsequent optimisation into drug candidates. Initial hits usually exhibit lower potency than those identified by more traditional techniques, such as high-throughput screening (HTS), but the optimisation phase of FBDD is highly efficient, thus providing superior lead-like compounds. The recent application of FBDD in a variety of protein kinase campaigns has successfully led to the identification of novel binding sites and highly efficient chemical ligands. This demonstrates the utility of the FBDD strategy against well-established kinase targets, where selectivity is otherwise challenging due to significant conservation of the ATP-binding site.

Protein kinase CK2 is a ubiquitously expressed and constitutively active regulator of cell growth, proliferation and apoptosis. Elevated levels of CK2 protein and activity have historically been involved in human cancer, including lung, cervical and head and neck cancer types, and its overexpression is associated with worse prognosis. A number of CK2 inhibitors are currently available displaying activity against multiple cancers in vitro and in the clinic, however these candidates target the ATP-binding site and thus display poor selectivity in kinase panel assays.

Here, the application of FBDD towards the development of potent and selective inhibitors of the catalytic α-subunit of CK2 is explored. This project exploits a novel, conserved binding site, named the αD pocket, for the generation of allosteric inhibitor molecules. Following characterisation of a previously unreported binding mode and structure-based optimisation of a potent inhibitor series, a fragment linking strategy between the lead αD-site fragment and a low-affinity pseudosubstrate peptide is investigated. This work validates the utility of FBDD towards the discovery of new binding modes and presents a first in class CK2α allosteric inhibitor series. Furthermore, it provides the first X-ray crystal structure of protein kinase CK2 in complex with a ligand binding in the substrate-binding channel.
Declaration

This dissertation is the result of my own work. It includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. It is not substantially the same as any that I have submitted, or is being concurrently submitted, for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution. I further state that no substantial part of my dissertation has already been submitted, or is being concurrently submitted, for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution. The length of this dissertation does not exceed the word limit prescribed by the Physics and Chemistry Degree Committee.

Sophie Mitchell

May 2018

Queens’ College, University of Cambridge
Abstract
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<tr>
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<tr>
<td>Akt</td>
<td>protein kinase B</td>
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<td>aq.</td>
<td>aqueous</td>
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<td>ATP</td>
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<td>benzyl</td>
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<tr>
<td>Bu</td>
<td>butyl</td>
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<td>cat.</td>
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<td>capillary electrophoresis</td>
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<td>CK2</td>
<td>casein kinase 2</td>
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<tr>
<td>CLL</td>
<td>chronic lymphocytic leukaemia</td>
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<tr>
<td>cLogP</td>
<td>calculated logarithm of the octanol/water partition coefficient</td>
</tr>
<tr>
<td>cm⁻¹</td>
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<tr>
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<tr>
<td>d</td>
<td>D-aspartic acid</td>
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<tr>
<td>Da</td>
<td>dalton(s)</td>
</tr>
<tr>
<td>d.b.</td>
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</tr>
<tr>
<td>dba</td>
<td>dibenzylideneacetone</td>
</tr>
<tr>
<td>deprot.</td>
<td>deprotection</td>
</tr>
<tr>
<td>d.i.</td>
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<td>DIC</td>
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</tr>
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<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DME</td>
<td>1,2-dimethoxyethane</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>5,6-di-chloro-1-β-D-ribofuranosylbenzimidazole</td>
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<td>dishevelled</td>
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<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal response concentration</td>
</tr>
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<td>eq.</td>
<td>equivalent(s)</td>
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<tr>
<td>ESI</td>
<td>electron spray ionisation</td>
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<td>FBDD</td>
<td>fragment-based drug discovery</td>
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<td>FDA</td>
<td>US Food and Drug Administration</td>
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<td>fluorenylmethyloxycarbonyl</td>
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<tr>
<td>g</td>
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<tr>
<td>GI&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>h</td>
<td>hour(s)</td>
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<td>hydrogen bond acceptor</td>
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<td>HMBC</td>
<td>heteronuclear multiple bond correlation</td>
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<td>HS1</td>
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<td>heat shock protein 90</td>
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<td>heteronuclear single quantum correlation</td>
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<td>HTC</td>
<td>hepatoma tissue culture</td>
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<td>high-throughput screening</td>
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<tr>
<td>/</td>
<td>iso</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
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<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IP</td>
<td>intellectual property</td>
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<tr>
<td>IQA</td>
<td>5-oxo-5,6-dihydroxyindolo- [1,2-α]quinazolin-7-ylacetic acid</td>
</tr>
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</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<tr>
<td>J</td>
<td>coupling constant</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<td>$K_d$</td>
<td>dissociation constant</td>
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<td>inhibitory constant</td>
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<td>$K_M$</td>
<td>Michaelis constant</td>
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<td>I</td>
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<td>LCMS</td>
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<td>LE</td>
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<td>LEF</td>
<td>lymphoid enhancer-binder factor</td>
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<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>M</td>
<td>molar/methionine</td>
</tr>
<tr>
<td>$m/z$</td>
<td>mass to charge ratio</td>
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<td>Me</td>
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<tr>
<td>mGlu</td>
<td>metabotropic glutamate</td>
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<td>minute(s)</td>
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<td>min.</td>
<td>minimal</td>
</tr>
<tr>
<td>m.p.</td>
<td>melting point</td>
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<td>MS</td>
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<tr>
<td>MST</td>
<td>microscale thermophoresis</td>
</tr>
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<td>MW</td>
<td>molecular weight</td>
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<td>nano</td>
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<td>NS3</td>
<td>non-structural protein 3</td>
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<td>Oxyma</td>
<td>ethyl (hydroxyimino)cyanoacetate</td>
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<td>PAINS</td>
<td>pan-assay interference compounds</td>
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<td>PDB</td>
<td>protein data bank</td>
</tr>
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<td>PDPK</td>
<td>phosphoinositide-dependent protein kinase</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
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<tr>
<td>pH</td>
<td>logarithm of the reciprocal of hydrogen ion concentration</td>
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<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
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<tr>
<td>Pim</td>
<td>Moloney murine leukaemia virus</td>
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<tr>
<td>pin</td>
<td>pinacol</td>
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<td>PIP3</td>
<td>phosphatidylinositol 3,4,5 trisphosphate</td>
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<td>POM</td>
<td>polyoxometalate</td>
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<td>PPI</td>
<td>protein-protein interaction</td>
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<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog deleted on chromosome 10</td>
</tr>
<tr>
<td>Py</td>
<td>pyridine</td>
</tr>
<tr>
<td>R</td>
<td>unspecified substituent</td>
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<td>RBF</td>
<td>round-bottom flask</td>
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<td>R_f</td>
<td>retention factor</td>
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<td>right hand side</td>
</tr>
<tr>
<td>r.t.</td>
<td>room temperature</td>
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<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<tr>
<td>SBDD</td>
<td>structure-based drug design</td>
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<tr>
<td>S-Phos</td>
<td>2-dicyclohexylphosphino-2',6'-dimethoxybiphenyl</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid-phase peptide synthesis</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>T</td>
<td>tertiary</td>
</tr>
<tr>
<td>TAT</td>
<td>trans-activator of transcription</td>
</tr>
<tr>
<td>TBB</td>
<td>4,5,6,7-tetabromobenzotriazole</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell specific transcription factor</td>
</tr>
<tr>
<td>Tf</td>
<td>trifluoromethanesulfonyl (triflyl)</td>
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<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<td>TIPS</td>
<td>triisopropyl silyl</td>
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<tr>
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<td>thin layer chromatography</td>
</tr>
<tr>
<td>t_r</td>
<td>retention time</td>
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<td>v/v</td>
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<tr>
<td>w.t.</td>
<td>weight</td>
</tr>
<tr>
<td>w/w</td>
<td>weight for weight</td>
</tr>
<tr>
<td>zm</td>
<td>Zea mays</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
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Δ heat
ΔG change in Gibbs free energy
μ micro
μw microwave
$\nu_{\text{max}}$ absorption maximum

Standard one and three letter codes are used for all amino acids.
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Chapter One: Introduction

1.1 Fragment-Based Drug Discovery (FBDD)

1.1.1 General Principles

Over the last twenty years, fragment-based drug discovery (FBDD) has emerged as a highly successful way to provide lead compounds for subsequent optimisation into drug candidates.\(^1\) To date, two FBDD-derived oncology drugs have been clinically approved\(^2-4\) and more than thirty other examples are in clinical trials\(^5,6\) for disease states including cancer,\(^7\) Alzheimer’s disease\(^8\) and type 2 diabetes.\(^9\)

FBDD has not only been adopted as a mainstream technique within pharma, but is also well-established within biotech and academic institutions, reliably generating high quality chemical leads against a range of targets.\(^1,6,10,11\)

The core concept of FBDD is the detection of low affinity fragments that bind to a biological target with well-defined interactions.\(^12\) These low molecular weight (MW) hits can then be efficiently elaborated into larger, more potent compounds while maintaining the key binding interactions.\(^12\) High affinity fragments derived from this process can be used to probe the biology of the target or as a lead for drug discovery.\(^13\)

With this in mind, the FBDD process can be described in three main steps: fragment library design, fragment screening, and hit-to-lead generation. The FBDD pipeline is depicted in Figure 1, and further detail on each stage is given below and in the sections that follow.

![Figure 1: General workflow of the FBDD approach. After fragment library screening and hit validation, a ligand is optimised into a drug candidate by iterative cycles of rational design, chemical synthesis and affinity measurement. The relative potency at each stage is shown. \(K_d, K_i, IC_{50}\) are dissociation constant, inhibitory constant, and potency, respectively.](image)

Typically, an FBDD project begins with the design of a suitable fragment library for screening against a pre-selected target. These fragment libraries are relatively small collections (500-3000 compounds)
of diverse, low MW compounds (<300 Da) featuring 10-20 ‘heavy’ (or non-hydrogen) atoms.\(^6\) Owing to the small size of fragments, biological binding affinities are in the mM range, and therefore library screening requires sensitive biophysical methods and high concentration biochemical assays.\(^{13}\) Following initial identification, a hit is validated through use of a secondary screening technique, commonly X-ray crystallography or NMR spectroscopy. These methods provide detailed structural information, thereby enabling characterisation of the fragments’ binding pose.\(^{10,14,15}\) The final step involves iterative cycles of fragment elaboration, structural screening, and bio-affinity measurements to facilitate hit-to-lead generation. The use of X-ray crystallography is particularly prevalent at this stage as it provides visualisation of the protein-ligand complex, thus promoting rational structure-based drug design (SBDD).\(^1,16\) X-ray crystallography reveals ways to boost binding affinity whilst the chemist maintains physicochemical properties within an acceptable range\(^17\) – an underappreciated factor in drug discovery and a well-known source of attrition for small molecule drugs.\(^18\)

In recent years, FBDD has proven itself as a viable alternative to more traditional drug discovery techniques, such as high-throughput screening (HTS) and virtual screening. In contrast to FBDD, HTS uses collections of millions of compounds with MW ~500 Da in search of nM range binding affinities.\(^{19}\) While HTS has led to many successful drug leads, more recently it has yielded fewer hits and more false positives.\(^{20,21}\) This has encouraged a transition to FBDD, which typically yields higher hit rates and has proven itself against more challenging targets for which HTS has failed, e.g. protein-protein interactions (PPIs).\(^{22-24}\)

Furthermore, despite the lower potency of initial hits, FBDD is considered more efficient than HTS in the optimisation phase.\(^{10,14}\) Analysis suggests that FBDD generates smaller, less lipophilic hits and leads than HTS, and strict SBDD ensures every moiety of the compound contributes significantly towards binding. Figure 2 depicts the efficiency of HTS relative to FBDD hits. It emphasises that higher MW compounds, as used in HTS, adopt suboptimal binding modes with the protein target whereas fragments, as used in FBDD, engage more efficiently (Figure 2, a vs. b respectively). If multiple fragment hits are identified, then they can be combined into an optimised lead during hit-to-lead generation (Section 1.1.4).
The following sections consider the key components of an FBDD program in greater depth. The main advantages and limitations of the technique are highlighted and the relevance of FBDD in the development of kinase inhibitors is introduced.

1.1.2 Fragment Library Design

The outcome of an FBDD program is very much dependent on the quality of the initial fragment hits. With this in mind, it is essential that the fragment library is carefully designed, with thorough consideration of multiple factors including fragment size, complexity and properties.

As mentioned in Section 1.1.1, most fragment libraries are small in number, just a few thousand compounds, aimed at providing a reasonable number of hits without overwhelming the screening methods. Additionally, the size of each fragment is limited to 300 Da which, in theory, provides more efficient sampling of chemical space than larger molecules. It has been calculated that the size of chemical space increases approximately 8-fold for every heavy atom in a molecule and therefore, a diverse library of 1000 fragments, with MW 190 Da, covers chemical space in an equivalent manner to $10^{18}$ molecules of MW 440 Da. As a result, a concentrated, fragment-sized screening library should be capable of sufficiently probing the surface of multiple, varied biological targets.

Once the size and MW range of the fragment library has been determined, attention must be given to the individual members of the collection. There are five areas to consider when assessing whether a fragment is appropriate for an FBDD screening library:
1. **Fragment complexity, diversity and shape**

As modelled by Hann, a molecule needs to have sufficient features to make interactions with a binding site, but not so many that binding is prevented.\textsuperscript{29} Therefore, a careful balance between fragment complexity and MW is required. Furthermore, diverse scaffolds with a variety of 2D and 3D shapes should improve molecular recognition against varied and challenging biological targets.\textsuperscript{26}

2. **Physicochemical properties**

In 2003, Congreve \textit{et al.} analysed a diverse range of fragment hits from the Astex archive. They observed that the average physical properties of successful hits fell within different orders of three: MW <300 Da, hydrogen bond donors (HBDs) ≤3, hydrogen bond acceptors (HBAs) ≤3 and CLogP ≤3.\textsuperscript{30} Fifteen years on, the ‘Rule of Three’ concept is still widely used as a guiding criteria when constructing fragment libraries. Properties commensurate with biophysical screening at high concentration, such as high aqueous solubility, are also vital in order to avoid fragment precipitation and aggregation.\textsuperscript{31}

3. **Reactive properties**

Fragments bearing functional groups known to be reactive towards proteins, associated with aggregation in solution and/or the generation of false positives, should be avoided.\textsuperscript{32} These traits mean compounds can register as hits in an assay without having specific binding affinities that can be further enhanced.\textsuperscript{6} Some compounds can be easily recognised, \textit{e.g.} Michael acceptors, alkyl halides and epoxides, while others can be identified by comparison to known pan-assay interference compounds (PAINS).\textsuperscript{33}

4. **Fragment analogues and exit vectors**

During hit optimisation, the availability of fragment analogues, either commercially or through efficient chemical synthesis, is essential.\textsuperscript{26} For the latter, fragments bearing multiple exit vectors, or so-called synthetic ‘handles’, enable growth in 3-dimensions so that new binding interactions can be accessed.

5. **Fragment quality control**

Regular quality control checking must be possible for the fragments so that high concentration screening can be performed reliably.\textsuperscript{34} Quality control includes checking for compound purity, stability, precipitation, aggregation and pH change.\textsuperscript{35}

Practitioners working carefully within these fragment library guidelines have reported multiple cases of high quality protein-hit interactions.\textsuperscript{1} Many of these hits have then been successfully used as starting points in hit-to-lead generation.
### 1.1.3 Biophysical Techniques

Until recent years, conventional biophysical methods, such as differential scanning fluorimetry (DSF) and mass spectrometry (MS), have not been sufficiently sensitive to identify the modest affinity of fragments for a protein target.\(^{36,37}\) As a result, biophysical techniques compatible with the smaller size and reduced complexity of fragments have been developed.\(^{26}\) From 2011 to 2013, a shift in screening method preference was observed (Figure 3, a). This is owed in part to the strides in technology but also to increased accessibility.\(^{38}\) Since 2016, three biophysical techniques have dominated the majority of FBDD efforts: NMR spectroscopy - specifically ligand-observed NMR experiments, X-ray crystallography and surface plasmon resonance (SPR) (Figure 3, b).\(^{39}\)

**Figure 3:** Biophysical techniques used for screening in FBDD programs. a) Poll results adapted from the blog Practical Fragments,\(^{38}\) reporting a change in fragment screening techniques between the years 2011 and 2013. b) Analysis adapted from Swain in 2016,\(^{39}\) in which the screening techniques in 165 published FBDD programs were considered. SPR - surface plasmon resonance; MS - mass spectrometry; ITC - isothermal titration calorimetry; CE - capillary electrophoresis; BLI - biolayer interferometry; MST - micro-scale thermophoresis.

#### 1.1.3.1 Ligand-Observed NMR

Ligand-observed NMR utilises a combination of 1D NMR experiments to track shifts in fragment NMR peaks before and after the addition of a protein.\(^{34}\) From this, it is possible to determine which ligands have undergone a binding event, but no information on the binding site or affinity can be derived. In practice, it is common to use three different 1D experiments - saturation transfer difference (STD),\(^{40}\) water-ligand-observed gradient spectroscopy (Water-LOGSY)\(^ {41}\) and Carr-Purcell-Meiboom-Gill relaxation dispersion (CPMG RD),\(^ {42}\) and to include a competition step to differentiate between specific and non-specific binding.\(^ {13}\) As this is a measurement in free solution, the solubility and stability of the fragments and protein can be evaluated for each measurement.\(^ {13}\) This minimises the likelihood of false positives and negatives due to aggregation or precipitation.
1.1.3.2 X-Ray Crystallography

In the past, X-ray crystallography was generally viewed as a secondary screening technique. Following recent developments in high-throughput crystallography, it has now found common use in primary screening.\textsuperscript{43} Any hits identified through this route are inherently validated for the target, and the binding pose information provides a platform for rapid and efficient SBDD.\textsuperscript{35} Orthogonal assays are required for biological assessment of the fragment hits, as X-ray crystallography reveals the binding mode and not the potency of a fragment.\textsuperscript{35} Nonetheless, X-ray crystallography remains a highly popular technique and is the screening concept on which the successful biotech company - Astex Pharmaceuticals - was founded.\textsuperscript{6}

1.1.3.3 Surface Plasmon Resonance (SPR)

The key feature of SPR is immobilisation of the target protein to a sensor chip. A solution containing the fragment is then injected over the surface and the refractive index of the interface is monitored. Following a fragment binding event, the protein complex increases in mass near the sensor surface, which is detected as a change in refractive index.\textsuperscript{44} If the ligand solubility is much higher than the dissociation constant ($K_d$), then titration can be used to quantify binding.\textsuperscript{6} However, in practice these values are often in the same range. SPR uses smaller quantities of protein than either NMR or X-ray crystallography and is a relatively rapid screening technique. The main disadvantage of SPR is that the protein can be challenging to immobilise, while retaining its binding integrity.\textsuperscript{13}

1.1.3.4 Isothermal Titration Calorimetry (ITC)

ITC measures the thermodynamics of a protein-ligand interaction via observation of the heat exchange during the binding reaction.\textsuperscript{45} Experiments are performed by titration of a ligand into a sample solution containing the protein. Following each addition, the heat released or absorbed is monitored and thermodynamic analysis then enables quantitative characterisation of the binding process.\textsuperscript{46} Data obtained through ITC measurements include the binding constant ($K_a$), enthalpy ($\Delta H$), entropy ($\Delta S$) and Gibbs free energy of binding ($\Delta G$) and the binding stoichiometry.\textsuperscript{45,47,48} In the past, the use of ITC has been limited due to a lack of sufficient sensitivity, however recent technology developments have yielded instruments capable of measuring heat effects arising from reactions involving nanomole amounts of reactants.\textsuperscript{46} As a result, ITC has become a popular primary screening technique in FBDD.\textsuperscript{49,50}
While focus has been given to four of the most widely used biophysical techniques, other sensitive technologies are also successfully used in screening programs. Those gaining more widespread attention include: protein-observed NMR spectroscopy, \textsuperscript{51} thermal shift analysis (TSA), \textsuperscript{52} computational screening, \textsuperscript{53,54} and mass spectrometry (MS). \textsuperscript{55,56} With every method there is a risk of false positives and negatives, and therefore some FBDD programs combine two or more screening techniques and follow up on mutual hits.\textsuperscript{6} While this approach may reduce time wasted on promiscuous fragments, it can also lead to novel and unusual protein-ligand interactions being overlooked, and thus must be used with caution.

1.1.4 Hit-to-Lead Generation

Hit-to-lead generation uses structural biology as a starting point for rational fragment elaboration into a drug candidate.\textsuperscript{16} Most of the success stories to date have utilised a robust model of how the fragment binds, and an analysis of all published hit-to-lead examples from 2015 indicates X-ray crystallography is by far the preferred structural technology.\textsuperscript{2} Molecular docking can also be useful in assisting SBDD at this stage.\textsuperscript{35}

During optimisation, ligand efficiency (LE) metrics are commonly used to judge the relative efficacy of a fragment manipulation.\textsuperscript{57} The calculation determines the binding energy of the ligand per atom and can be calculated by converting the $K_d$ into the Gibbs free energy ($\Delta G$) of binding [Eqn 1] at 300K and dividing by the number of ‘heavy’ (i.e. non-hydrogen atoms) [Eqn 2].\textsuperscript{58} Furthermore, the Gibbs free energy ($\Delta G$) can be substituted for IC\textsubscript{50} [Eqn 3].\textsuperscript{57}

$$\Delta G = -RT.\ln(K_d) \quad \text{[Eqn 1]}$$

$$\text{LE} = \frac{\Delta G}{N_{\text{non-hydrogen atoms}}} \quad \text{[Eqn 2]}$$

$$\text{LE} = 1.4(-\log(\text{IC}_{50}))/N_{\text{non-hydrogen atoms}} \quad \text{[Eqn 3]}$$

Ligand efficiency is a way of normalizing the potency and MW of a compound to provide a useful comparison between compounds with a range of MWs and activities. This provides a guide to show whether the atoms added during a cycle of elaboration are making optimal interactions with the protein.\textsuperscript{58} In theory, the use of LE metrics should enable the medicinal chemist to achieve an appropriate balance of physicochemical properties and binding affinity. Thereby, minimising the chance of clinical failure due to lipophilicity-related safety issues, such as lack of selectivity, poor solubility and low metabolic clearance.\textsuperscript{18,59} Analysis by Schultes \textit{et al.} and Hopkins \textit{et al.} into the LE of FBDD hits that were selected for further optimisation in 2010 and 2013 respectively, reported average starting LE of 0.38.\textsuperscript{57,60}
Three synthetic strategies are typically employed in hit-to-lead generation: fragment linking, growing and merging (Figure 4).\textsuperscript{13}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fragment_optimization.png}
\caption{Strategies for fragment optimisation. \textbf{a)} Fragment linking. \textbf{b)} Fragment growing. \textbf{c)} Fragment merging. Adapted from Lamoree et al.,\textsuperscript{13} Copyright (2018), with permission from Portland Press.}
\end{figure}

In the \textbf{linking} strategy, two or more fragments binding in different regions of the pocket are linked together via rigid or flexible linkers (Figure 4, \textbf{a}).\textsuperscript{25} The linked compound should then have a more favourable $\Delta G$ of binding than the sum of the $\Delta G$ values for the individual fragments. This is described by the concept of ‘super-additivity’.\textsuperscript{61,62} However, in practise, fragment linking is often highly challenging as linkers can constrain the molecule too much. This results in suboptimal interactions with the target and a subsequent loss in binding affinity.\textsuperscript{15}

The earliest example of fragment linking comes from Shuker \textit{et al.} at Abbott in 1996.\textsuperscript{51} This was the first publication of an FBDD project in which a weak $\mu$M affinity hit was successfully developed into a potent nM affinity lead. Shuker \textit{et al.}’s work is considered proof of concept for fragments in drug discovery.\textsuperscript{63}
The most advanced example of fragment linking also comes from workers at Abbott, with their selective Bcl-2 inhibitor ABT-199 (5) (Figure 5). Bcl-2 is an apoptosis regulator protein and its aberrant activity results in evasion of cell death and the development of Bcl-2-dependent tumours. Studies towards this target were as follows: fragment screening by protein-observed NMR, followed by structure determination and optimisation, identified fragments 1 and 2 as inhibitors of Bcl-xL, a prosurvival protein related to Bcl-2 (Figure 5, a and b). Combination of fragments 1 and 2, before further elaboration, provided potent inhibitor 3 (Figure 5, a and c). This was further optimised to ABT-737 (4) (Figure 5, a and d) which entered clinical trials as a dual Bcl-xL/Bcl-2 inhibitor. Subsequent modification of ABT-737 (4) provided final drug candidate ABT-199 (5) (Figure 5, a). In 2016, ABT-199 (5) received approval by the US Food and Drug Administration (FDA) for the treatment of chronic lymphocytic leukaemia (CLL) and was released onto the market as venetoclax.
The second strategy, fragment growing, is the most widely applied strategy and involves growing a validated hit in a stepwise fashion, picking up additional target interactions with each iteration (Figure 4, b). Structure-guided design is prevalent in this method and a modular approach to optimisation allows medicinal chemists to address specific questions such as the utility of adding a HBA/HBD or filling a small lipophilic pocket. This also highlights the importance of including synthetic handles in the design of the initial library.

The most prominent example of fragment growing comes from the development of FDA-approved vemurafenib (9) by Bollag et al. (Figure 6). Vemurafenib (9) is a selective inhibitor of the B-Raf V600E mutant kinase, which is a well-documented oncogene and present in almost half of all melanomas.
An initial hit (6) was identified from a biochemical screen against the kinase Pim-1 (Figure 6, a). X-ray crystallography validated the hit and guided the development of elaborated fragment 7 (Figure 6, a and b). Iterative rounds of SBDD grew into the back cavity of the binding pocket, identifying fragment 8 (Figure 6, a). At this point, fragment 8 was co-crystallised with B-Raf V600E and observed to bind the ATP-site in a similar manner to Pim-1 (Figure 6, c). Addition of a 4-chlorophenyl moiety provided clinical candidate vemurafenib (9) (Figure 6, a and d). A loss in potency between fragment 8 and lead vemurafenib (9) was observed (IC$_{50}$ from 13 to 31 nM), but LE and kinase selectivity were improved. In 2011 vemurafenib (9) became the first drug with FBDD origins to be approved by the FDA and is currently used in the treatment of B-Raf driven melanoma.$^3$

Finally, fragment merging sees the best features of several overlapping fragments merged into a single molecule with higher potency (Figure 4, c).$^{12}$ It relies on the overlay of multiple crystal structures and ambitious SBDD, therefore few successful reports of this technique exist. One example, from the Vernalis archive, shows how fragments were combined with a literature compound in the development of PDPK1-selective inhibitor 16 (Figure 7).$^{70}$ With the use of X-ray crystallography and computational docking, compound 12 was developed from fragments 10 and 11, while species 14 was derived from 13. Overlay of the crystal structures for 12, 14 and literature compound 15, suggested fragment combination to provide final product 16. Compound 16 selectively binds PDPK1 with an IC$_{50}$
of 90 nM, but inhibition of the kinase did not have the desired anti-cancer effect and thus the project was terminated.\textsuperscript{70}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{The discovery of protein kinase PDPK1 inhibitor 16 by fragment merging. Initial hit fragments 10, 11 and 13 were elaborated into fragments 12 and 14. These were combined with the core of 15 to provide final inhibitor 16. The circled portions highlight the structural moieties preserved from each fragment during the merging process. n.d.: no data. Adapted from Hubbard,\textsuperscript{70} Copyright (2018), with permission from the International Union of Crystallography.}
\end{figure}

FBDD projects aim to conclude the hit-to-lead phase once a collection of target-selective, lead-like compounds, with substantial potency, have been generated. These chemical tools should have physicochemical properties resembling drug candidates, rendering them suitable for progression into clinical trials.\textsuperscript{31}

\subsection{Advantages and Limitations of FBDD}

As alluded to in the previous sections, the FBDD process has multiple advantages over traditional drug discovery. Firstly, the binding of a low MW fragment reflects a high intrinsic binding enthalpy overcoming an entropic binding penalty.\textsuperscript{71,72} As a result, fragments can be highly efficient ligands and better starting points for physicochemical-controlled elaboration than higher MW hits from HTS. Additionally, the reduced complexity of fragments lowers the chance of a steric group preventing binding. In turn, this leads to an increase in the number of FBDD hits and easier identification of
functional motifs that are essential for target binding.\textsuperscript{13} High hit rate may raise concerns of low specificity \textit{i.e.} a fragment binding to various proteins and/or to a single protein in several ways. But specificity can be introduced during fragment optimisation, and a broad initial approach enables more thorough interrogation of target druggability\textsuperscript{26} – a vital aspect for chemists investigating new and unusual binding sites and mechanisms. On this note, Saalau-Bethell \textit{et al.} at Astex demonstrated the utility of FBDD towards the discovery of a novel allosteric binding site.\textsuperscript{73} This site was positioned at the interface between two domains of the Hepatitis C viral protein NS3. Their work showcased successful fragment screening against a challenging PPI and the value of FBDD in identifying alternative modes of action.

Limitations of the current FBDD methodology have also been identified. Evaluation of several existing fragment libraries by Morley \textit{et al.} reported limited shape diversity to the screening sets, with predominantly (hetero)aromatic-derived chemotypes.\textsuperscript{36} They surmised that this ‘flatness’ might predispose the fragment library success for certain target classes, and limit its tractability against new targets that require pharmacophores with alternative substitution vectors.

Analysis by Johnson \textit{et al.}, into the protein target class featured in FBDD projects from 2015, goes some way to supporting the hypothesis made by Morley (Figure 8).\textsuperscript{1} Johnson \textit{et al.} showed that previously exploited, literature-rich protein kinases and proteases were dominant among the successful targets in 2015. In contrast, emerging classes such as PPIs and membrane-bound proteins (\textit{i.e.} GPCRs) barely featured. The preparation of fragment libraries drew on knowledge from previously successful FBDD studies, and therefore heavily featured core scaffolds known to hit existing targets. These biased libraries ultimately led to exaggerated discoveries on the same, well-documented proteins which, in agreement with Morley, interact well with planar, sp\textsuperscript{2}-rich compounds.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure8.png}
\caption{Protein target class analysis for 27 fragment-to-lead examples with publication year 2015. GPCR – G-protein coupled receptor; PPI – protein-protein interaction. Reprinted with permission from Johnson \textit{et al.}\textsuperscript{1} Copyright (2018) American Chemical Society.}
\end{figure}
A further observation by Johnson et al. was the reliance of FBDD practitioners on X-ray crystallography – 85% of the FBDD projects in 2015 used X-ray structural data for hit validation and optimisation.\textsuperscript{1} Earlier work by Murray et al. in 2010 reported similar findings, from which they hypothesised whether the lack of a protein crystal structure had the potential to render a target off-limits.\textsuperscript{16} With this in mind, a second explanation for the lack of diversity in protein class targets was put forward by Johnson et al. In this analysis the ease of crystallisation of the target protein was considered.\textsuperscript{1} They observed that the challenges of obtaining crystal structures for membrane-bound proteins, or ligands in complex with PPIs, were well documented, whereas kinase and protease targets are often readily-crystallised.\textsuperscript{74–77} This provides an alternative explanation for the distribution reported in Figure 8.

Despite the above limitations – with respect to library diversity and target crystallisation, encouraging FBDD progress has recently been reported for multiple PPIs and one membrane protein.\textsuperscript{78–82} Of greatest note is FDA-approved venetoclax (5) (Section 1.1.4), which acts to disrupt the PPI between Bcl-2 and BIM, instigating apoptosis in the treatment of chronic lymphocytic leukaemia (CLL).\textsuperscript{4} Additionally, Christopher et al. at Heptares published a promising membrane protein example in 2015.\textsuperscript{82} They demonstrated the use of biochemical assays and X-ray crystallography for the development of an advanced, lead-like modulator of the mGlu\textsubscript{5} receptor. mGlu\textsubscript{5} is a member of the metabotropic glutamate receptor family, and modulates synaptic transmission in response to the neurotransmitter glutamate.\textsuperscript{83} Dysfunction in this signalling is linked to numerous disorders including Parkinson’s disease,\textsuperscript{84} neuropathic pain,\textsuperscript{85} anxiety\textsuperscript{86} and depression.\textsuperscript{87}

To summarise, low affinity fragment hits can be found for most targets, including those that fail in HTS.\textsuperscript{88} As X-ray crystallography techniques continue to develop,\textsuperscript{43} and attention is given towards fragment libraries with greater three-dimensionality,\textsuperscript{36} further success against these previously ‘undruggable’ targets is expected.

### 1.1.6 Kinase Inhibitors and FBDD

Protein kinases catalyse the transfer of the terminal phosphate group of adenosine triphosphate (ATP) onto an amino acid residue, commonly serine, threonine or tyrosine, within a polypeptide chain.\textsuperscript{89} Their activity is vital in the mediation of intracellular signalling and as such, they are deemed key regulators of cellular processes.\textsuperscript{90} Aberrant kinase activity has been linked to a large number of disease states including cancer and inflammation as well as metabolic, autoimmune and neurological conditions.\textsuperscript{91–93} As a result, protein kinases are considered one of the most important target classes in modern drug discovery.\textsuperscript{90} Research into the ‘druggable kinome’ (\textit{i.e.} members of the kinome related to human disease and susceptible to modulation by drugs) has identified more than 150 potential
kinase targets to date. As of March 2017, a total of 32 kinase inhibitors had been approved by the FDA and more than 1000 clinical studies were ongoing.

Despite the aforementioned value of exploiting protein kinases in disease therapy, a number of key challenges exist. All kinases utilise ATP as a cofactor and as a result, the ATP-binding site has been thoroughly investigated and forms the basis for the majority of kinase inhibitor projects. However, drugs with ATP-site competitive modalities are rife with well-documented difficulties:

1. **Competition with ATP**
   
   Inhibitors must outcompete mM levels of intracellular ATP in order to bind the kinase target.

2. **Protein kinase selectivity**
   
   There are over 500 human protein kinases, all of which bind ATP in a similar manner, thereby presenting an obvious selectivity challenge when designing inhibitors of just one kinase.

3. **Physicochemical properties**
   
   The ATP-binding site is relatively flat and lined on two sides with hydrophobic residues. Therefore, unless care is taken, inhibitors that are lipophilic and sp²-rich are produced – characteristics with poor prognosis in clinical trials due to lack of selectivity, poor solubility and low metabolic clearance.

4. **Intellectual property (IP)**
   
   The last 20 years has seen an intense interest in kinases by the pharma industry, with the majority of lead-candidates mimicking the adenine group of ATP. As a result, the IP around this template is highly congested.

   However, despite these difficulties, it is important to note that some ATP-site competitive kinase inhibitors are remarkably selective for individual kinases when in vitro. The high observed potency of these inhibitors can be best explained by the Cheng-Prusoff equation [Eqn 1].

   This states that a drug IC₅₀ value (concentration of drug at which 50% of the kinase activity is inhibited) depends on the intrinsic affinity of the inhibitor (the dissociation constant, Kᵅ) as well as the competition from intracellular ATP (governed by the [ATP], usually 1-5 mM, and the Kₘ,ATP).

   \[
   IC₅₀ = \frac{Kᵅ}{1 + \frac{[ATP]}{Kₘ,ATP}}
   \]  

   [Eqn 1]

   Thus, the Cheng-Prusoff equation establishes that the affinity of the kinase target for ATP (expressed as Kₘ,ATP) is critical in determining the potency of an ATP-site competitive inhibitor. This means that inhibitors with similar intrinsic affinities (Kᵅ values) against multiple kinases in vivo will more potently
inhibit kinases with higher $K_m, \text{ATP}$ values when in cells.\textsuperscript{101} Therefore, superior selectivity \textit{in vitro} can sometimes be observed for drugs targeting kinases that display weaker competition with ATP.

Returning to the difficulties outlined previously, the application of FBDD in the search for kinase inhibitors has become of great interest. Screening diverse fragment libraries provides the potential for novel scaffold and mechanism discovery. This works in new IP space and offers the opportunity of developing inhibitors that bind to alternative positions on the protein. Careful hit elaboration allows control over physicochemical properties\textsuperscript{95} and the wealth of available kinase structural data accelerates the optimisation step, affording clinically acceptable lead compounds in a timely manner.\textsuperscript{99} To highlight the uptake of FBDD by kinase practitioners, Johnson \textit{et al.} analysed all fragment-to-lead projects from 2015. They found that protein kinases constituted the largest target class (Figure 8).\textsuperscript{1} Furthermore, in 2016 over one third of FBDD candidates in clinical trials were kinase inhibitors.\textsuperscript{5} Finally, as summarised in Section 1.1.4, vemurafenib (9) was the first FDA-approved drug with origins in FBDD and is a potent and selective kinase inhibitor.\textsuperscript{2}

However, even with the dawn of FBDD, development of kinase inhibitors displaying modalities distinct from the ATP-binding site remains a significant challenge.\textsuperscript{95} To date, very few examples of allosteric kinase inhibitors exist in the literature, but in 2011 two interesting fragment-based approaches were reported (Figure 9 and Figure 10).\textsuperscript{104,105}

The first, from Pollack \textit{et al.}, used SPR to screen a diverse fragment library against protein kinase p38\textalpha.\textsuperscript{104} This led to the identification of a novel lipid binding pocket (Figure 9). X-ray crystallography of hit fragment 17 in complex with p38\textalpha verified the binding position and subsequent SBDD improved binding affinity tenfold. Overlay of ligand-protein X-ray crystal structures with the Apo and active forms of p38\textalpha suggested an allosteric mechanism of action for the inhibitor series. Their work continues towards the synthesis of more potent analogues.
The second example comes from Betzi et al. who utilised X-ray crystallographic screening to identify an allosteric site on protein kinase CDK2 (Figure 10). When hit fragment 18 binds in this pocket, the C-helix undergoes a conformational change and the ATP-binding site is reduced in size. As a result, the crucial interaction between CDK2 and cyclin A is disrupted and CDK2 kinase activity is lost. Their work on elaborating initial hit 18 is ongoing.
In conclusion, the successful approval of vemurafenib (9), and the emergence of allosteric kinase inhibitors, validates the power of FBDD in the identification of efficient fragments and new binding pockets. Continued work in this area should facilitate the development of novel kinase modulators with properties superior to traditional ATP-site competitive drugs.

1.2 Protein Kinase CK2
Protein kinase CK2 is a ubiquitous serine/threonine kinase that is highly conserved in eukaryotic cells.106 Since its discovery in 1954,107 over 600 substrates modulated by CK2 phosphorylation have been identified.108 CK2 interacts with diverse and essential cellular pathways including cell growth,106 proliferation,109 survival,110 morphology111 and transformation.112 Furthermore, its role in signalling pathway activation,113 angiogenesis,114,115 embryonic development116 and circadian rhythm117 is well documented. Extensive research into CK2 signalling has established a definitive link between increased expression/activity and disease states such as cancer,112 cardiac hypertrophy,118 multiple sclerosis119 and inflammation.120 Additionally, in contrast to the majority of eukaryotic kinases, CK2 is constitutively active, i.e. active in the absence of phosphorylation and/or specific stimuli.106

The breadth of CK2 impact on cellular machinery has given it the status of a ‘master regulator’ within the cell,121 and the last twenty years have seen increased interest in the development of chemical modulators for this kinase.122 The following section reviews the structure of CK2, before its role in cancer is examined and its targeting in cancer therapy is considered. An overview of published CK2 inhibitors is given and the therapeutic potential of a novel binding site, discovered by FBDD, is introduced.

1.2.1 CK2 Structure
The CK2 holoenzyme is a tetramer comprising two catalytic alpha subunits (α and α’) supported on a regulatory beta (β) subunit dimer (Figure 11).123 The most stable form of the protein is in its tetrameric complex. However, studies show the widespread presence, and full kinase activity, of CK2α and α’ subunit monomers within cells.124,125 Crystal structures of the holoenzyme126 and individual subunits127,128 have been published and over 70 structures of CK2 in complex with its inhibitors have been deposited in the protein data bank (PDB).108 Structural analysis of these crystals revealed CK2 conserves most of the major structural motifs reported for all kinases (i.e. the P-loop, substrate binding site, catalytic loop and activation loop). Furthermore, comparison of all known CK2 primary sequences suggests CK2α and α’ are well conserved within eukaryotes.123 The CK2β subunit primary
sequence is similarly well conserved in higher eukaryotes, but two homologues have been identified in yeast and three in *Arabidopsis thaliana*.\textsuperscript{123,129–131}

![Diagram of human CK2 holoenzyme complex](image)

*Figure 11: Overview of human CK2 holoenzyme complex (PDB: 1JWH). The tetramer consists of two CK2α catalytic subunits (green and yellow) and a CK2β regulatory dimer (red and blue). A molecule of AMPPNP is shown in the ATP-binding site of one CK2α subunit and two Zn\textsuperscript{2+} ions are shown in the CK2β dimerization region. Reprinted from Niefind et al.,\textsuperscript{132} Copyright (2018), with permission from Elsevier.*

1.2.1.1 The Catalytic α and α’ Subunits

The catalytic subunits are responsible for binding ATP and the substrate, and catalysing the phosphorylation event.\textsuperscript{128} In mammals, the CK2 catalytic subunit is composed of two genes, CK2α (CSNK2A1) and CK2α’ (CSNK2A2), which share 75% sequence similarity.\textsuperscript{123,133} Of the two, the CK2α protein has broader and higher-level tissue expression, but specific roles for the two isoforms have not yet been established.\textsuperscript{134–136}

Drawing from all known crystallographic information, the architecture of the CK2 α-subunits can be described by two main topological determinants (Figure 12). The first is the N-terminal domain, also known as the ‘N-lobe’, and includes β strands 1-5 in an antiparallel arrangement and the αC helix. The second is the C-terminal domain, also known as the ‘C-lobe’, and is dominated by an α-helical fold (helices αD-N) featuring two small double-stranded β-sheets (β6-9).\textsuperscript{128,137} The two lobes are connected by a short loop, called the ‘hinge region’ (Glu114-Asn118), which is the basis for the ATP-binding site.\textsuperscript{128,137} Overall, this is a typical kinase fold structure.\textsuperscript{138}
Figure 12: Overview of Zea mays catalytic subunit, CK2α, with AMPPNP and Mg$^{2+}$ ions occupying the ATP-binding site (PDB: 1LP4). The key structural features of the subunit are labelled and the short loop between β5 and αD represents the hinge region. Reprinted from Niefind et al., Copyright (2018), with permission from Elsevier.

The catalytic subunits are both related to the CMGC group kinase subfamily, named after the initials of some members including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPks), glycogen synthase kinases (GSKs) and CDK-like kinases. This group has a diversity of functions in cell cycle control, growth and stress response, cell signalling, splicing and metabolism. However, the α and α′ subunits display some sequence characteristics unique to CK2. Most notable are three differences:

1. **High concentration of basic residues around the substrate-binding channel**

In contrast to the rest of the CMGC kinase class, CK2 is characterised by an acidophilic substrate recognition site. In particular, helix αC (Lys74-Arg80) and residues Lys49 and His160 are acidic in all other kinases within the CMGC family. In CK2, these residues are important for the recognition of substrates at the n+3, n+2 and n-2 positions respectively (Figure 13, b). Additionally, the activation segment of CK2 (Figure 13, a) hosts an unusual basic triplet (Arg191, Arg195 and Lys198), responsible for the recognition of the substrate at position n+1 (Figure 13, b). In line with these observations, CK2 substrates are characterised by multiple acidic residues surrounding the phosphoacceptor amino acid.
Figure 13: Representation of CK2α with an enlargement of the substrate binding region. 

**a)** Human CK2α (green) with typical kinase subdomains highlighted and ANP (blue) occupying the ATP-binding site (PDB: 1JWH).

**b)** CK2α substrate binding channel (green) with substrate mimic 19 (yellow) modelled. The most important protein-peptide interactions are highlighted. Adapted from Cozza et al., Copyright (2018), with permission from John Wiley and Sons.

In 1994, Marin et al. developed a CK2-selective dodecapeptide substrate mimic (sequence: RRRADDSDDDDD, 19). Point-mutation studies in both the protein and peptide corroborated the sequence binding position as the substrate channel (modelled in Figure 13, b). However, to date, CK2 has not successfully been crystallised with the peptide, or a genuine substrate, so this has not been unambiguously verified. Peptide 19 demonstrates the ability of CK2 to recognise and bind highly acidic substrates (considered in greater depth in Results and Discussion, Section 2.3).

2. Absence of a regulatory phosphorylation site in the substrate recognition domain

Both catalytic subunits lack the common phosphorylation mechanism by which other members of the class downregulate the substrate-binding site activity. Absence of this reputed phosphorylation site, either a threonine or tyrosine in the substrate recognition domain, contributes to the observed constitutive activity of CK2.

3. Absence of a regulatory phosphorylation site in the activation loop

Neither catalytic subunit presents any serine or threonine residues in the activation loop (Asp175-Tyr188) and no phosphorylation event near this region has ever been reported. In contrast, all other
CMGC and eukaryotic serine/threonine kinases require phosphorylation of this loop to promote a transition from the inactive to active state. This transition promotes maximal enzyme activity. In the case of CK2, which is always in its active state, it has been shown that the unusual position of the N-terminal domain is instead essential. This blocks the activation loop in an open, and consequently active, conformation.

### 1.2.1.2 The Regulatory β Subunits

Within the CK2 tetrameric complex, the regulatory subunit dimer, (CK2β)₂, acts as a central scaffold, alters CK2 substrate specificity and modulates kinase activity. CK2β is encoded by the gene CSNK2B and is ubiquitous in the eukaryotic kingdom. It ranges in size from 26 - 46 kDa and bares no sequence similarity with any other known proteins except the stellate protein from *Drosophila melanogaster*.

The primary sequence of CK2β presents an interesting distribution of acidic and basic residues located at the N- and C-terminals respectively (Figure 14, a). X-ray crystallographic data and biochemical experiments have clarified the structures and functions of these regions. The N-terminal domain has an α-helical fold and is involved in the negative regulation of CK2 activity. The C-terminal domain contains β sheets 1-5, two α-helices and a zinc-binding site. It is responsible for the positive regulation of CK2, β-β dimerization, association with the α-subunit and preservation against unfolding and proteolysis.

The Zn²⁺-binding motif (residues 105-161, C-terminal) provides a highly efficient dimerization interface for the CK2β subunits (Figure 14, b). First, multiple hydrophobic interactions are formed between the zinc-finger motifs of two CK2β monomers. This pulls the two subunits into close contact. Then, the CK2β tail of each monomer, consisting a small two-stranded antiparallel β-sheet (β4/β5), crosses the dimer interface to nestle against the second CK2β unit. This sets the stage for two CK2α subunits to associate independently with the dimer. When this happens, the β4/β5 segment inserts into a shallow hydrophobic groove formed by β-sheets 1-5 in the N-lobe of CK2α. This PPI is 832 Å² and referred to as the CK2α/CK2β-subunit interface.
1.2.1.3 The Tetrameric Holoenzyme $\alpha_2(\alpha')\beta_2$

As mentioned previously, in Section 1.2.1, protein kinase CK2 is usually isolated as a tetrameric structure, $\alpha_2(\alpha')\beta_2$ (Figure 11). The tetramer displays greater stability than individual CK2$\alpha$
monomers,\textsuperscript{154} and the activity of isolated CK2α subunits is generally lower with respect to the holoenzyme.\textsuperscript{155} Some studies suggest that a transition from an isolated tetramer to an inactive multimeric form of CK2 is possible \textit{via} electrostatic contacts. These hypothesised multimeric structures include holoenzyme dimers,\textsuperscript{156} holoenzyme linear tetramers\textsuperscript{157} and holoenzyme circular tetramers.\textsuperscript{158}

1.2.2 CK2 as a Cancer Driver

An association between CK2 and neoplasia has been known for a long time.\textsuperscript{159–161} In particular, a cause-effect link between abnormally elevated CK2 expression/activity and the enhancement of the tumour phenotype has been established.\textsuperscript{162} The following sections summarise the impact of raised cellular CK2 level on cancer (Figure 15) and introduce the concept of CK2 addiction in cancer cells.

1.2.2.1 Elevated CK2 Level

A striking observation is that CK2 activity is invariably more elevated in tumours than in corresponding normal tissues and cells.\textsuperscript{160,163} Over the years, multiple studies have shown that CK2 genes, especially CK2α and α’, are overexpressed in many common cancer types. These include lung,\textsuperscript{164} head and neck,\textsuperscript{165} mammary gland,\textsuperscript{166} prostate\textsuperscript{167} and kidney cancers.\textsuperscript{167} Furthermore, CK2 level can be used as both a diagnostic and prognostic marker.\textsuperscript{162,168} To demonstrate, Kaplan-Meier Plotter analysis by Ortega \textit{et al.} found strong correlation between CK2 expression level and lower patient survival rate for lung, breast and ovarian cancer types.\textsuperscript{169} Similarly, analysis into head and neck tumours by Gapany \textit{et al.} gave the same unfavourable prognosis.\textsuperscript{170}

When considering the role of CK2 in healthy cells, it is hardly surprising that raised activity should have such a devastating effect. The ability of CK2 to promote cell proliferation,\textsuperscript{109} growth\textsuperscript{106} and survival,\textsuperscript{110} modulate diverse signalling pathways,\textsuperscript{171} enhance cellular transformation\textsuperscript{115} and support neovascularisation\textsuperscript{172} all provide selective advantage to a tumour, and are characteristic hallmarks of cancer.\textsuperscript{173} The oncogenic nature of CK2 is further supported by experimental studies in mice,\textsuperscript{174} whereby site-specific overexpression of CK2 leads to cancer development \textit{via} enhanced pro-survival and anti-apoptotic signalling.\textsuperscript{175,176} CK2α-subunit “knock-in” experiments also result in promoted neoplastic transformation.\textsuperscript{177,178}

Finally, elevated CK2 level is known to contribute toward the multi-drug resistant phenotype\textsuperscript{179} – one of the major problems in the pharmacological treatment of tumours. In CK2, this counteracts the
efficacy of anti-tumour agents, most notably imatinib\textsuperscript{180,181} and melphalan.\textsuperscript{182} Their effectiveness is consequently restored by CK2 inhibition.

\begin{figure}[h]
\centering
\includegraphics[width=0.6\textwidth]{figure15.png}
\caption{Summary of the effects of abnormally high CK2 level.}
\end{figure}

1.2.2.2 Addiction to CK2

The term ‘addiction’ was originally coined by Weinstein.\textsuperscript{183} It was used to highlight the fact that tumour cells, enriched in a particular oncogene, may critically rely on a sustained level of this gene to a greater extent than cancer cells not overexpressing the gene. As a result, the malignant phenotype can be reverted toward normal, or apoptotic, by just lowering the level of the oncogene the tumour is addicted to. Consequently, the proteins associated with these oncogenes represent potential drug targets.\textsuperscript{184}

Over recent years, it has been observed that not all addictive proteins are activated by mutations to their genes. This phenomenon is now referred to as ‘non-oncogene addiction’.\textsuperscript{184,185} It applies to situations where a protein which is not subjected to obvious mutation becomes overexpressed to a degree of causing an undue reliance on itself. Protein kinase CK2 is a pertinent example of this class.\textsuperscript{115} There are no gain-of-function CK2 mutants known, yet its elevated expression level is associated with a host of cancer types, and the majority of these display a reliance on it for survival.

CK2 is expressed in all cells but its level significantly differs, with higher expression levels observed in cancer cells. This suggests specific roles of CK2 in malignancies and a stricter reliance on its functionality for survival. This concept is supported by the observation that primary tumour cells treated with CK2 inhibitors are more susceptible to cell death than healthy precursor cells treated in
the same manner. This has been found for multiple myeloma, acute myeloid leukaemia and T-acute lymphoblastic leukaemia cells.

In summary, CK2 may not be a senso stricto ‘tumour promoter’ but whenever its activity is abnormally increased, a favourable cellular environment for cancer onset results. Extensive data provides evidence for elevated CK2 as a biomarker for cancer, and addiction studies reveal CK2 as necessary for cancer cell survival. Therefore, the development of potent and selective cell-permeable CK2 inhibitors, devoid of undesirable side effects, represent a valuable tool for the treatment of a wide range of oncological diseases.

1.2.3 CK2 Signalling and Cancer

The ability of CK2 to promote tumours may be largely due to its ability to regulate a broad range of signal transduction pathways. These include the NF-κB, Wnt, PI3K/Akt, Hedgehog and JAK/STAT pathways. Modulation of these signalling cascades results in cells displaying distinct liabilities, such as growth advantage, enhanced survival and dynamic adaptation to stress. Ultimately, these alterations can lead to tumorigenesis and therefore the signalling mechanisms represent avenues by which CK2 can induce cancer.

Illustrated below are three pathways in which CK2 is a multi-site regulator (Figure 16). Under healthy conditions, CK2 modulates transcriptional activity and enables regular development. When CK2 activity is elevated, the transcriptional pathways are abnormally reactivated and lead to oncogenesis.
a) **NF-κB signalling**

NF-κB normally binds to its inhibitor IκB and is sequestered in the cytosol. Activation of kinase IKK, by stress or a cytokine signal, promotes proteolytic degradation of IκB. This releases free NF-κB which then translocates into the nucleus and functions as a transcription factor for anti-apoptotic and pro-proliferative genes (Figure 16, a).\(^{115}\)

CK2 acts at different levels of this process. Firstly, it can phosphorylate IKK thereby promoting IκB degradation and enabling NF-κB signalling.\(^{192}\) Secondly, it can directly phosphorylate IκB, providing an alternative pathway for promoting IκB proteolysis.\(^{193,194}\) Finally NF-κB itself can be phosphorylated and activated by CK2.\(^{195,196}\)

Aberrant activation of NF-κB has been documented in several cancers including mammary gland, prostate and head and neck cancer.\(^{197,198}\) Knockdown of individual CK2 subunits also results in a decrease in NF-κB gene expression.\(^{199}\)

b) **β-Catenin/Wnt signalling**

The Wnt signalling cascade regulates cell proliferation (Figure 16, b).\(^{115}\) When in the presence of Wnt, the stabilising protein dishevelled (Dvl) inhibits the ‘destruction complex’ associated with kinase GSK3. As a result, β-catenin is not targeted to the proteasome and its cellular level remains high. It then acts as a co-factor for transcription factors of the TCF/LEF family and promotes survival.
CK2 can affect this pathway at multiple levels: phosphorylation of Dvl, β-catenin and the β-catenin/TCF/LEF complex are all possible. This promotes their stabilisation and activates survival gene transcription.\textsuperscript{200,201}

Song et al. showed that β-catenin is upregulated in mice overexpressing CK2α in mammary glands.\textsuperscript{200}

c) PI3K/Akt signalling

PI3K activation leads to the phosphorylation and activation of the oncogene Akt, which has downstream anti-apoptotic effects (Figure 16, c).\textsuperscript{202} The pathway is antagonised by PTEN, a phosphatase which dephosphorylates PIP3 and maintains PI3K/Akt signalling down, under resting conditions.\textsuperscript{203}

In this pathway, CK2 is again a multi-site upregulator. It acts indirectly by phosphorylating PTEN and inhibiting its phosphatase ability, thus resulting in unregulated Akt-dependent signalling.\textsuperscript{204} It also acts directly, by phosphorylating Akt and inducing a hyper-activated state.\textsuperscript{205} In addition, phosphorylation of the chaperone protein Hsp90 is possible by CK2. This results in a stabilised Akt-Hsp90 association that protects Akt from dephosphorylation.\textsuperscript{206}

Aside from the role of CK2 as a cell survival agonist, it also acts as an antagonist in the apoptotic program.\textsuperscript{115} Its action is most notable in the disruption of caspase-mediated cleavage. Caspases are a family of protease enzymes that play an essential role in programmed cell death.\textsuperscript{207} Their activation leads to controlled degradation of cellular components, and caspase deficiencies or mutations are a cause of tumour development. Analysis of the caspase cleavage consensus sequence shows significant similarity with the CK2 substrate consensus sequence.\textsuperscript{208} This has led to the common observation that sequence phosphorylation by CK2 hampers subsequent cleavage by caspases. This results in inhibition of apoptosis and promotes tumourogenesis. This has been recognised for Bid,\textsuperscript{209} Max,\textsuperscript{210} hematopoietic lineage cell-specific protein 1 (HS1),\textsuperscript{211} presenilin,\textsuperscript{212} connexin 45.6\textsuperscript{213} and PTEN proteins.\textsuperscript{214}

In summary, it is clear that CK2 plays a global role as a pro-survival and anti-apoptotic agent, which potently contributes to its oncogenic activity.

1.2.4 CK2 as an Anticancer Target

In developing novel avenues for effective cancer therapy, the ultimate goal is to eradicate all tumour cells in the host and achieve a complete cure of the disease.\textsuperscript{121} Thus, it is important to consider targeting a protein that is uniquely indispensable for cell survival, otherwise tumour cells will escape
cell death by recruiting an alternative pathway.\textsuperscript{161,215} The molecular targeting of ‘non-critical’ species might limit their therapeutic utility and hinder productive treatment of the disease.\textsuperscript{216}

CK2 plays a key role in many cellular processes that have been demonstrated as essential for cell survival. The knock-out of CK2\(\alpha\) is lethal in mice\textsuperscript{217} and \textit{Saccharomyces cerevisiae},\textsuperscript{218} while the deletion of CK2\(\beta\) alleles results in abnormal development in the early stages of mouse growth.\textsuperscript{219} Additionally, downregulation of CK2 impacts not only cell growth and proliferation, but also induces widespread apoptosis and inhibition of angiogenesis, all to the benefit of cancer cell elimination.\textsuperscript{114,220–223} In line with this, gene silencing targeted against CK2 provides the second most effective induction of apoptosis across the whole kinome when tested in pancreatic tumour cell lines.\textsuperscript{224} Finally, as far as the literature reports, there appear to be no redundant pathways to compensate for CK2 downregulation.\textsuperscript{217–219} Ultimately these findings validate CK2 as an attractive and suitable target for cancer therapy.

It is important to mention here that the ubiquitous and essential nature of CK2 functionality raises issues of host toxicity. This therefore casts doubt on its ‘druggability’. However, studies show that normal cells exhibit relative resistance to apoptosis induction in response to CK2 inhibitors, compared to cancer cells. This observation suggests that malignancies may be more sensitive to CK2 modulators than healthy cells.\textsuperscript{220,222} The concept of cancer cell addiction to CK2 was discussed more thoroughly in Section 1.2.2.2, Addiction to CK2.

In conclusion, dysregulated CK2 activity has been identified as a key player in the development and maintenance of the cancer phenotype. Elevated CK2 level reflects the pathological status of a tumour and serves as a biomarker for cancer studies. Furthermore, it is an unfavourable prognostic marker in the clinic. Subunit knock-out studies and gene silencing have shown CK2 as an essential-for-survival cellular component, and downregulation of CK2 activity in cancer cell lines has a marked effect against the malignant state. These results validate CK2 as an anticancer target and suggest that development of potent and selective CK2 inhibitors could provide a valuable toolkit for the treatment of multiple cancer types.

1.2.5 CK2 Inhibitors to Date

The recent emergence of CK2 as a potential anticancer target has led to the development of a number of cell-permeable CK2 inhibitors.\textsuperscript{225} These inhibitors have been shown to have activity against multiple cancer types \textit{in vitro}\textsuperscript{171} and one candidate, silmitasertib (20, formerly CX-4945), gained FDA-approved orphan drug status in January 2017.\textsuperscript{226} Silmitasertib 20 is used for the treatment of advanced
cholangiocarcinoma and is currently undergoing phase I/II clinical trials against lung, cervical and head and neck malignancies (ClinicalTrials.gov Identifier: NCT02128282).\textsuperscript{227}

The majority of published CK2 inhibitors have relied on a rational structure-based approach in their design. This has been possible thanks to a wealth of multidisciplinary work bridging chemical synthesis, biological assays and X-ray crystallography.\textsuperscript{228} Furthermore, an analysis of the oncomine database revealed that the dominant CK2α subunit is overexpressed in 5 out of the 6 most important cancer types in the United States, thereby channelling attention toward this particular portion of the protein rather than the α’ or β subunits.\textsuperscript{169}

The following sections review the most notable CK2 inhibitors and their binding sites. ATP-competitive inhibitors are considered first, followed by a discussion regarding the need for alternative binding sites. A selected summary of successful exosite-binding inhibitors is then given.

\textbf{1.2.5.1 ATP-Competitive Inhibitors}

More than 40 X-ray crystal structures of CK2α in complex with different ATP-competitive inhibitors have been deposited in the PDB.\textsuperscript{229} This has made it possible to efficiently exploit molecular docking for the proposal, analysis and corroboration of hypotheses about CK2 binding.\textsuperscript{230,231} As a result, the majority of published CK2 inhibitors have been rationally designed to compete at the ATP-site on CK2α.\textsuperscript{123}

Structural analysis of the catalytic site of CK2 has revealed a handful of unique properties specific to CK2 over other kinases. One particular feature is that hydrogen bonding with the hinge region is not essential for binding (Figure 17, a). As such, the common pharmacophore for CK2 activity is instead composed of an important hydrophobic/aromatic area in the centre of the pocket (Figure 17, b).\textsuperscript{234} This motif is essential and is present in all sub-micromolar inhibitors (Figure 17, c). It potentially accounts for the specific inhibition of CK2 by relatively small molecules.\textsuperscript{232}
Figure 17: ATP-competitive inhibitors of CK2. a) Zea mays CK2α hinge region (black) overlaid with other eukaryotic protein kinases to demonstrate relative proximity of the protein backbone to ATP (grey). H-bonding between ATP and Glu127 (red) in CAPK is highlighted (dashed lines) and equivalent residue Asp120 (grey) in CK2α is shown in its distal position. CAPK – Cyclic-AMP Protein Kinase; CDK2 – Cyclin-Dependent Kinase 2; IRK – Insulin Receptor Kinase. Reprinted from Niefind et al., 132 Copyright (2018), with permission from Elsevier. b) Superposition of ATP-competitive CK2 inhibitors silmitasertib 20 (green) (PDB: 3PE1), IQA 21 (blue) (PDB: 1OM1) and TBB 22 (magenta) (PDB: 1J91) binding in the ATP-site without making polar interactions to the hinge region Glu114, Val116 or Asp120 (white). The aromatic cores of the three inhibitors overlap, highlighting the main CK2 pharmacophore responsible for the binding potency. c) Chemical structures of three prominent ATP-competitive CK2 inhibitors with corresponding IC_{50} values.123

Clinical candidate silmitasertib 20, mentioned in Section 1.2.5, is an ATP-site competitive inhibitor and the only drug currently in human trials against CK2 (Figure 17, c). The inhibitor is well tolerated despite the ubiquitous role of CK2 in cellular pathways, and reports describe it as ‘highly selective’.233,234 However, silmitasertib 20 inhibits at least 12 other kinases with nanomolar IC_{50} values, and is more effective against Clk2 than CK2α.235,236 This results in clear cellular effects that are not linked to the inhibition of CK2, such as widespread CK2α-independent alteration in alternative splicing for numerous genes, and suppression of the phosphorylation of serine/arginine-rich proteins in mammalian cells.236 In fact, multiple ATP-competitive inhibitors described as being ‘selective’ against
CK2 are also the most potent known inhibitors of other kinases. This is especially true within the DYRK, HIPK and Clk protein families.\textsuperscript{236,237}

The aforementioned observations highlight a common issue with ATP-site competitive inhibitors: a lack of selectivity owed to conservation of the ATP-binding site across all kinases. Furthermore, compounds that do disclose specificity for CK2, instead exhibit other physiological effects in cells. These off-target effects require further investigation, and reflect the fact that ATP-binding sites are not unique to just protein kinases.\textsuperscript{113} Off-target activity of this type limits the use of ATP-competitive drugs and impedes their development in cancer therapy. As a result, there is an increased interest in the development of alternative inhibitors that utilise more selective binding positions outside of the active site.

1.2.5.2 Alternative Site Inhibitors

The complex and multi-domain architecture of protein kinase CK2 lends itself to the discovery of alternative binding sites for novel small molecule modulators (Figure 18).\textsuperscript{238} The development of inhibitors targeting different surfaces of the kinase could avoid the shortcomings of conventional ATP-competitive inhibitors. These include poor selectivity, \textit{in vitro} competition with a high ATP concentration and reliance on highly conserved residues in the ATP-binding site, that are susceptible to mutation.\textsuperscript{239,240}
One strategy is to identify and exploit a negative allosteric mode of action. Small molecules that bind allosteric pockets exert control over a protein’s function by perturbing the conformation of essential enzymatic residues. This can then lead to protein activity inhibition.\(^\text{242–244}\) These sites represent valuable targets for drug design, but the discovery of new allosteric pockets is challenging and often occurs serendipitously by analysis of protein-ligand structural complexes.\(^\text{245,246}\) To date, only Raaf \textit{et al.} have fully characterised an allosteric site on CK2\(\alpha\), located at the CK2\(\alpha/\)CK2\(\beta\) interface (Section 1.2.5.2.1).\(^\text{247}\) However, ongoing work by Prudent \textit{et al.} suggests the existence of a second allosteric site (Section 1.2.5.2.2).\(^\text{238}\)

Work towards other alternative inhibitors has also been published, and this includes targeting CK2 substrates,\(^\text{248,249}\) targeting the CK2\(\beta\) subunit\(^\text{250}\) and the development of CK2\(\alpha\) bisubstrate inhibitors (Section 1.2.5.2.3).\(^\text{251,252}\)

\subsection*{1.2.5.2.1 \textit{CK2}\(\alpha/\)CK2\(\beta\) Subunit Interface Inhibitors}

The crystal structure of DRB \textit{23}, an old and relatively selective CK2 inhibitor,\(^\text{253}\) in complex with human CK2\(\alpha\) revealed a new binding position.\(^\text{247}\) It showed that DRB \textit{23} is able to bind not only the canonical ATP pocket, but also a secondary site at the interface with CK2\(\beta\) (Figure 19, a). This hydrophobic pocket
is lined by β-sheets 1-5 in the N-terminal domain of CK2α. The discovery of this site presented a new ‘druggable’ position on CK2, with potential for inducing selective disruption of CK2α/CK2β subunit assembly. It was hypothesised that the development of potent small molecules binding this site may result in inhibition of CK2α activity.

Figure 19: Inhibitors binding the CK2α/CK2β subunit interface. a) X-ray crystal structure of DRB 23 (green) in complex with human CK2α (grey) (PDB: 3H30), showing hydrophobic pocket (β1-5) in the N-lobe of CK2α at the CK2α/CK2β interface. b) Chemical structures of four prominent CK2α/CK2β interface-binding inhibitors with IC₅₀ values. n.d.: no data.

Structure-based design at the CK2α/CK2β interface, by Laudet et al., led to the development of Pc peptide 24 (Figure 19, b). This truncated CK2β mimic was particularly effective at antagonising the assembly of the holoenzyme, and inhibiting phosphorylation of CK2β-dependent substrates. This work, in combination with the DRB 23 crystal structure, validated the subunit interface as a targetable exosite amenable to pharmacological modulation.

Rational design of low molecular weight antagonists, also by Laudet et al., resulted in a HTS of a collection of podophyllotoxine indolo-analogues. This led to the identification of the first chemical ligands capable of binding the CK2α/CK2β interface and inducing full CK2α inhibition. Optimisation produced their most potent compound, W16 25, and kinetic analysis showed non-competitive inhibition of CK2α without interfering with the ATP-cleft (Figure 19, b). No X-ray crystal structure has been reported for W16 25 in complex with CK2α, but inhibition is alleviated by the addition of CK2β or Pc peptide 24. This suggests that W16 25 binds in, or near, the hydrophobic interface pocket. The
proximity of the ATP-site to the CK2α/CK2β pocket makes a functional connection between them likely, therefore supporting an allosteric mode of action.

More recent work by Bestgen et al. led to the development of a cell permeable version of Pc peptide 24, called TAT-Pc13 (26) (Figure 19, b). This was shown to promote CK2 holoenzyme disruption within living cells, and rapidly induce caspase-independent cell death.256 Continued work by this group aims to utilise TAT-Pc13 26 towards the identification of CK2β-dependent substrates and selective ways to target them.

1.2.5.2.2 Polyoxometalates (POMs) Target an Alternative Exosite

Polyoxometalates (POMs) are a unique class of inorganic compounds that are potent inhibitors of CK2.257 They are complexes of early-transition metal ions and oxo ligands discovered by Prudent et al. through HTS of highly diverse chemical libraries. The best inhibitor reported is K4[P2Mo18O62] 27 which has an IC50 of 1.4 nM and displays high specificity for CK2 against a panel of 29 kinases.257 Steady state kinetic analysis and site-directed mutagenesis showed that POMs do not target CK2α in the ATP- or substrate-binding sites or at the CK2α/CK2β interface. Biophysical experiments are underway to further decipher this unexpected inhibition mode, and Figure 18 indicates a proposed POM binding site. These non-classical kinase inhibitors may provide an exploitable allosteric mechanism, allowing the future development of potent CK2α drugs with enhanced selectivity relative to ATP-mimetic inhibitors.238

1.2.5.2.3 Bisubstrate Inhibitors

An emerging strategy to increase the selectivity of ATP-site competitive kinase inhibitors is based on the design of so-called ‘bisubstrate’ (or bifunctional) inhibitors.251 These species consist of two combined moieties, one targeted to the ATP-binding site, and the other shaped in such a way that it mimics the phosphoacceptor substrate, and therefore competes for the substrate-binding site. At variance with ATP-competitive inhibitors, which often bind with high affinity but low selectivity, and with pseudosubstrate inhibitors, which are in principle quite selective but generally bind very weakly, bisubstrate inhibitors are expected to be both very potent and selective. This is owed to the cooperative effects of their dual mode of binding. In the last decade, several successful examples of this strategy have been reported for a variety of kinases258,259 and now attention has turned to CK2. Unfortunately, a common feature of all reported bisubstrate inhibitor-protein complexes is the failure to determine the whole structure of the ligand by X-ray crystallography.260–262 Attempts often lead to
the detection of the ATP-competitive moiety and no defined electron density for the pseudosubstrate portion.

One successful CK2 bisubstrate inhibitor comes from Cozza et al., where a purely ATP-competitive CK2 inhibitor – K137 28 – was derivatized at its 3-amino position with a peptidic fragment composed of four glutamic acid residues (Figure 20, a). The bifunctional inhibitor, K137-E4 29, gave an IC₅₀ value of 25 nM against the CK2 holoenzyme, which is several fold better than original ATP-site fragment 28 (IC₅₀ = 130 nM). Furthermore, K137-E4 29 effectively demonstrated the synergistic effect of anchoring via inhibitor K137 28, as the isolated glutamic acid tetramer was originally devoid of efficacy. K137-E4 29 was more selective than parent inhibitor K137 28, failing to inhibit any kinase other than CK2 in a panel of 140 kinases. In contrast, 35 kinases were inhibited more potently than CK2 by K137 28. Residue mutation studies and molecular docking experiments confirmed the double mode of binding for K137-E4 29 (Figure 20, b).
Figure 20: CK2 bisubstrate inhibitor K137-E4 29. a) Chemical structures of K137 28 and K137-E4 29 with IC$_{50}$ values against CK2 holoenzyme.$^{251}$ b) In silico interaction between K137-E4 29 (yellow) and CK2α (PDB: 3Q04). Residues involved in the interaction are highlighted in grey. The IC$_{50}$ values of corresponding Ala mutants are indicated for comparison with that of wild-type CK2 (black box outlines). K74-77A means K74A, K75A, K76A and K77A. Reprinted from Cozza et al.,$^{251}$ Copyright (2018), with permission from Portland Press.

A second example of a bisubstrate inhibitor has been published by Rahnel et al.$^{252}$ Their biligand inhibitor, ARC-772 31, was constructed by conjugating ATB 30 with a carboxylate-rich peptoid via an optimised hydrophobic linker (Figure 21). ARC-772 31 was found to bind CK2 with a $K_d$ of 0.3 nM and showed remarkable CK2 inhibitory selectivity in a panel of 140 kinases – highly superior to ATB 30 alone.$^{263}$ Ester prodrug, ARC-775 32, was efficiently taken up by HeLa cancer cells and observed to activate apoptosis marker caspase-3 with an EC$_{50}$ of 0.3 μM – a 20-fold lower extracellular
concentration than clinical candidate silmitasertib 20 (EC$_{50}$ of 6.5 μM). No discussion of the binding mode for ARC-772 31 is reported at this time.

![Chemical structures](image)

**Figure 21**: Chemical structures of ATP-site competitive inhibitor ATB 30, bisubstrate inhibitor ARC-772 31 and prodrug ARC-775 32. Upon penetration of the cell plasma membrane, the acetoxymethyl ester moieties on ARC-775 32 are cleaved by esterases to release active drug ARC-772 31.

Results from bisubstrate inhibitors K137-E4 29 and ARC-772 31, showcase the potential of this strategy in drug development against CK2. They both demonstrate how moderately potent, and quite promiscuous, ATP-competitive inhibitors can be converted into more effective, and very selective, CK2 antagonists by elaboration with pseudosubstrate moieties.

In conclusion, the design, testing and synthesis of compounds that interact differentially with the surface areas of CK2α, outside of its catalytic cleft, has already led to the identification of new potent inhibitors. Furthermore, recent crystal structure analysis across multiple kinases, including CK2, has revealed how dynamic their structures are. This has suggested that exploitable, and as of yet undiscovered, allosteric networks exist throughout each protein. HTS has proved fruitful in initial attempts to identify these binding sites and mechanisms on CK2α, but the application of FBDD could be a more efficient means of further interrogation. The use of low molecular weight fragments in initial screening, partnered with sensitive X-ray crystallography or NMR spectroscopy, could identify promising small molecules for drug development. Detection of any new inhibitor binding pocket must
be urgently followed up with full structural and biophysical characterisation, in order to understand the mechanism of action and promote SBDD towards improved drug candidates.

1.2.6 Discovery of the αD Pocket on CK2α

A recent FBDD program by the Spring/Hyvönen group collaboration at the University of Cambridge led to the discovery of a novel binding pocket for small molecules on CK2α, named the αD pocket. A high concentration crystallographic screen of a commercial fragment library was performed to probe the surface of CK2α. The original aim was to screen for new allosteric inhibitors at the CK2α/CK2β interface. Instead, a hit (33) was identified that induced the opening of a previously unseen cryptic pocket close to the ATP-binding site (Figure 22, b). The newly identified αD pocket is located behind the αD helix (Asp120-Thr127) and is largely hydrophobic in character. This binding site is either closed or partially open in all other Apo structures of CK2α, with either Phe121 (PDB: 3FWQ), Tyr125 (PDB: 5CVH) or Leu124 (PDB: 5CVG) occupying the pocket (Figure 22, c). Upon binding fragment hit 33, the residue occupying the αD pocket is displaced, and Met225 rotates to open the bottom of the pocket. The αD helix is released from the C-lobe and adopts an open helical arrangement.

Figure 22: Discovery of the αD pocket on CK2α. a) Chemical structure of hit fragment 33. b) X-ray crystal structure of hit 33 (orange) in complex with CK2α (grey) (PDB: 5CLP). The position of αD-site fragment 33 relative to other CK2α features is shown and the αD helix is highlighted in yellow. c) Cross section of the αD pocket with hit 33 (orange) bound. The size of the αD pocket in the closed CK2α structure (PDB: 3FWQ) is represented by the black dashed line and the αD pocket in the partially open CK2α structure (PDB: 3WAR) is represented by the red dashed line.

Following hit validation, further exploration into the druggability of this site was undertaken by the Spring and Hyvönen groups. Elaboration of fragment 33 using SBDD led to high affinity αD-
selective binding fragment 34, but no inhibition of CK2α kinase activity was observed (Figure 23, a). A fragment-linking strategy was then proposed, whereby benzylamine fragment 34 would be linked to a low affinity ATP-site competitive inhibitor via an optimised flexible linker. ATP-competitive fragment 36 was identified in a high concentration crystallographic screen of a commercial fragment library, and deemed suitable for linking to αD fragment 34. The linker approach was rationalised on the grounds that the αD-pocket appears to be a unique feature of CK2α compared to other human kinases. Thus it could be exploited as an anchor site to deliver a weak ATP-site inhibitor selectively onto CK2α over other kinases (Figure 23, b and c).

Efficient execution of the linking strategy, using iterative linker growth and computational modelling, led to chemical probe CAM4066 35 (Figure 23). Linked species 35 demonstrated the highest CK2 selectivity of any reported inhibitor to date when tested against a panel of 52 kinases. A $K_d$ of 320 nM was also recorded, which is a 1000-fold improvement on starting fragment 34. An IC$_{50}$ of 370 nM was reported for CAM4066 35, a vast improvement on original fragments 34 and 36, as neither showed efficacy up to 500 μM. Poor cell permeability led to the development of pro-CAM4066 37, a prodrug version of CAM4066 35, for use in cellular assays (Figure 23, b). When pro-CAM4066 37 was tested against HCT116, Jurkat and A549 cancer cell lines, GI$_{50}$ values of 9, 6 and 20 μM respectively were reported. This profile compares well to the corresponding values for clinical candidate silmitasertib (20) (5, 5 and 17μM GI$_{50}$ respectively).
Figure 23: Development of optimised, linked inhibitor CAM4066 35. a) Chemical scheme demonstrating hit-to-lead generation of CAM4066 35. Hit fragment 33 was elaborated into αD-site selective binder 34 which was linked to ATP-competitive inhibitor 36 via an optimised linker to produce potent, CK2-selective inhibitor CAM4066 35. $K_d$ values are reported where possible. n.d.: no data. b) Representation of CAM4066 35 binding to CK2α and structure of pro-CAM4066 37. Zwitterionic elements are coloured in green, amide bonds in blue and the difference between CAM4066 35 and prodrug pro-CAM4066 37 is highlighted by the purple box. The interaction between CAM4066 35 and highly conserved Lys68 is shown as a red dashed line. The flexible linker is circled in orange and the αD pocket and ATP-binding site are reported as black curves. Reprinted with permission from Iegre et al. Published by the Royal Society of Chemistry. c) X-ray crystal structure of CAM4066 35 (green) in complex with CK2α (grey) (PDB:5CU4).
CAM4066 35 provides valuable proof of concept, validating both the use of FBDD on CK2α and the development of anchored inhibitors binding in the novel, poorly conserved αD-pocket. CAM4066 35 is a new generation of selective CK2α inhibitor and lays the groundwork for further elaboration into higher affinity candidates with improved pharmacokinetics. Furthermore, full characterisation of the novel αD exosite now enables easier investigation into the utility of this binding pocket for allosteric kinase inhibition. More broadly, the strategy of using cryptic pockets outside the active site could be applied to other protein classes where selectivity is otherwise hard to achieve.

1.3 Project Aims

The project discussed in this thesis built on the αD pocket work previously reported by the Spring/Hyvönen groups (Section 1.2.6). The aim was to further investigate the druggability of the new αD-site and explore its utility as a binding pocket for small molecule inhibitors with negative allosteric modalities. It was believed that the development of inhibitors binding the poorly conserved αD pocket would provide far superior CK2α selectivity than any published inhibitor to date.

The specific research objectives were two-fold: first, develop αD-site selective fragments with higher binding affinities than previously reported, and secondly, achieve CK2α kinase inhibition by binding solely within the αD pocket. This would avoid growth into the ATP-binding site and the involvement of potentially promiscuous ATP-competitive warheads (i.e. CAM4066 35, Figure 24, a).

As a starting point, the biaryl benzylamine scaffold of known αD-site binding fragment 34 would be exploited (Figure 24). It was envisioned that SBDD around the lower aryl ring (species 38 - 40) would probe the flexibility of the αD pocket and lead efficiently to improved binding fragments.
Figure 24: Illustration of the project aims. In the centre is shown αD-site binding fragment 34, the starting point for SBDD in this project. Following the left arrow shows the X-ray crystal structure of CAM4066 35 (green) (PDB: 5CU4) binding simultaneously in the αD pocket and ATP site. This work was previously reported by Brear et al.265 Following the right arrow shows the X-ray crystal structure of fragment 34 (blue) (PDB: 5CHS) in the αD pocket and scaffolds 38 - 40 investigated in this project. R, R’ and R” represent substitution positions on fragment 34 for investigation. $K_d$ values are given265 and the numbering system for the lower phenyl ring is shown in blue for later reference.

X-ray crystal structure analysis of fragment 34, and the analogues leading to its development, revealed the extent of the αD loop flexibility, and thus the variability in volume of the αD pocket (Figure 25). In growing from initial hit 33 to lead fragment 34, Brear et al. had also observed large rearrangements in the αD loop of up to 23.8 Å, for fragment 42.266 This movement was significantly more fluid than in previous descriptions268 and suggested to us that it might be possible to accommodate fragments larger than biaryl 34 within the αD pocket. Based on this, we hypothesised that αD-site fragment growth to a critical size, and with optimal ligand-protein interactions, might cause sufficient conformational disruption of CK2α so as to inhibit kinase activity. The impact of this work would be a first in class CK2α allosteric inhibitor series with superior selectivity, instilled by exploiting the unique
αD binding site. This would validate the continued use of FBDD in kinase inhibitor programs and could lead to the development of novel anticancer therapies.

Figure 25: Investigating the flexibility of the αD loop. a) Chemical structures of key fragments in hit-to-lead generation from hit 33 to lead 34. b) Superposition of X-ray crystal structures showing the relative position of the αD loop when fragments 33 (green) (PDB: 5CLP), 41 (magenta) (PDB: 5CVF), 42 (purple) (PDB: 5CS6) and 34 (blue) (PDB: 5CSH) are bound to CK2α. Key residues Phe121 and Tyr125 are shown to highlight the flexibility of the αD loop upon binding a fragment. The largest rearrangement was observed for fragment 42 which moved 23.8 Å from its original position in the closed Apo structure (PDB: 3FWQ). Reprinted with permission from De Fusco et al. Copyright (2018) Elsevier.

Investigation into our hypothesis began by substituting positions around the lower aromatic ring of biaryl 34 to provide species of the form 38 - 40 (Figure 24). This portion of the fragment had not been optimised in previous publications so presented an unexplored starting point for fragment elaboration. Furthermore, it would effectively interrogate the flexibility of the bottom of the pocket. Three positions of substitution were considered (38 - 40) and the workload divided between three members of the Spring group. The focus in this thesis was substitution at the 3-position, highlighted by R’ on scaffold 39 in Figure 24.
A suitable project approach was envisaged as follows:

1. Design a target-oriented fragment library utilising commercially available building blocks to ensure rapid and efficient later synthesis.

2. Screen the library in silico using the protein-bound X-ray crystal structure of lead fragment 34 and Schrödinger’s Maestro package.

3. Perform chemical synthesis for library members with most promising docking results.

4. Submit synthesised compounds for X-ray crystallography by our Hyvönen group collaborator, Dr. Paul Brear, from the University of Cambridge Biochemistry Department.

5. Determine binding affinity (by ITC) and inhibitory activity (by CK2α kinase assay) for fragments displaying αD-site selectivity. Also to be performed by Dr. Paul Brear.

6. Analyse combined structural and biochemical results to verify the binding position, compare with computational modelling predictions, identify false positives/negatives and establish fragments superior to current ‘top leads’ (i.e. benzylamine 34).

7. Perform iterative cycles of structure-based optimisation, biochemical evaluation and combined analysis (steps 1-6) until lead-like compounds have been obtained.

In summary, we were searching for improved αD pocket binding fragments that could inhibit CK2α kinase activity. Final candidates needed to exhibit good ligand efficiency (LE>0.38) and present physicochemical properties commensurate with the ‘Rule of Three’. This aim followed the typical SBDD sequence used in hit-to-lead generation during FBDD programs, as outlined in Section 1.1.4. It also represented the first complete application of fragment-based drug discovery to protein kinase CK2.
Chapter Two: Results and Discussion

2.1 Target-Oriented Fragment Library Design, Synthesis and Testing

2.1.1 Fragment Library Design

The fragment screening library required by this project had a different aim to the typical broad, commercial fragment libraries purchased for the start of FBDD programs. In this case, Brear et al. had already reported the identification of a hit fragment (33) and its subsequent development into an αD-site specific lead (34). Therefore, this project began at the SBDD stage of FBDD (introduced in Section 1.1.4) and utilised the X-ray crystal structure of current lead 34 for the rational design of a target-oriented fragment library.

Design of the structure-guided library began with analysis of the key protein-ligand binding elements for lead compound 34. In order to best exploit the biaryl core, it was essential that the interactions between 34 and the αD pocket were understood and maintained. Analysis of the X-ray crystal structure of 34 in complex with CK2α (PDB:3CSH) determined that the benzylamine moiety plays a crucial role in positioning the fragment within the αD pocket (Figure 26, a). The amine, protonated under crystallography conditions, makes HBD interactions to Pro159 and Val162 and participates in a water bridge with Asn118, Thr119 and two water molecules. It was also recognised that the αD pocket is lined by hydrophobic residues and the space is filled well by the biaryl core of 34 (Figure 26, b). Finally, the lower phenyl ring stacks against Met225 which locks the position of the ring within the lower part of the pocket (Figure 26, c). Furthermore, the ortho-substituted chlorine may force the biaryl into a pre-formed conformation that aids binding in the αD pocket. Based on this assumption, the chlorine substituent would be maintained.
With these factors in mind, the lower portion of the αD pocket was investigated to determine whether substitution of the bottom phenyl ring at the 3-position (43) would provide fragments likely to bind (Figure 27, a). Visual inspection of biaryl 34 bound to CK2α showed a vacant space in the pocket adjacent to the 3-position (Figure 27, b). This cavity is lined by hydrophobic residues (Met137, Ile140, Leu218, Leu222 and Met225) and appeared suitable to accommodate a substituent (Figure 27, c). Furthermore, given the reported flexibility of the αD helix, and observed scope for the αD pocket to deepen in the presence of larger fragments, substitution at the 3-position was deemed likely to be tolerated. Overall, available structural data for Brear et al.’s lead fragment 34 provided a strong precedent for the development of analogues of the form 43.
Following the above analysis, design of the target-oriented fragment library began. A diverse range of 3-position substituents were desired, but rapid and efficient chemical synthesis of each compound was also vital. As a result, only fragments derived from commercially available building blocks, bearing features suitable for Suzuki-Miyaura cross coupling, were considered. This provided an analogue library of 75 fragments that was screened \textit{in silico} against CK2α using Glide from the Schrödinger package, set to default parameters.\textsuperscript{269–272} Subsequent protein-ligand visualisation and image rendering was performed using Pymol from the Schrödinger package.\textsuperscript{273}

The predicted binding modes for the top docking results were visually inspected and species maintaining the benzylamine H-bonding pattern, upper ring orientation and Met225 stacking interaction were prioritised. Within this refined collection, fragments predicted to efficiently occupy the 3-position hydrophobic cavity were further analysed. Fragments with polar groups, such as dibenzylamine 44, were predicted to make HBD interactions with the backbone of Met221 (Figure 28, a). Fragments with non-polar groups, such as methyl 45 and vinyl 46, were predicted to occupy the cavity without steric clash (Figure 28, b and c respectively). Overlay of the predicted structures in Figure 28 with a modelled structure of lead 34 and the reported structure of lead 34 (Figure 27, b) indicated precise alignment of the biaryl cores in all cases. This validated the use of computational modelling software to predict binding modes of close analogues.
The target-oriented fragment library was finally refined to 11 fragments which would efficiently interrogate the predicted interactions reported in Figure 28. This collection was considered accessible from readily available starting materials and would provide a variety of polar and non-polar substitution groups for X-ray crystallography and biochemical assessment.

2.1.2 Fragment Library Synthesis

Following fragment library design, an efficient and modular approach for subsequent synthesis was desired. Ideally this would involve robust experimental conditions with broad substrate scope so that the fragment analogues could be made in as few synthetic steps as possible. Taking inspiration from Brear et al., a retrosynthesis of benzylamine scaffold 43 was envisaged as follows (Scheme 1): first, the benzylamine moiety of 43 could be prepared by reduction of nitrile 47. Biaryl scaffold 47 could come from Suzuki-Miyaura cross coupling between triflate species 48 and commercially available boronic acid/ester 49. Triflate 48 could be prepared from commercially available alcohol 50.
Scheme 1: Retrosynthetic analysis for benzylamine scaffold 43. \( R \) represents the 3-position substituent, \( BR' \) represents either a boronic acid or ester and \( X \) represents the counter-ion of the benzylamine salt.

In the forward direction, experimental protocols from well-precedented literature (in particular those relating to lead 34) were considered in order to provide the following conditions for testing (Scheme 2): application of a standard triflation protocol\(^{265,274}\) to alcohol 50 would provide cross coupling partner 48, which would then be combined with boronic acid/ester 49 under typical Suzuki-Miyaura cross coupling conditions.\(^ {265,274}\) This would give benzonitrile 47 which, upon reduction of the nitrile moiety using conditions developed by Brear et al.,\(^ {265,266}\) would provide the crude free amine of final product 43. Purification of 43 by manual flash column chromatography or semi-preparative HPLC would be decided following \(^1\text{H} \) NMR, LCMS and TLC analysis of the crude reaction mixture. In the case of manual flash column chromatography, the free amine would then be converted to a benzylamine chloride salt.\(^ {265,266}\) Semi-preparative HPLC would directly provide the trifluoroacetate salt. Salt formation would provide stability during storage and ease of later handling.

Scheme 2: Proposed forward synthesis for benzylamine scaffold 43.\(^ {265,266,274}\) \( R \) represents the 3-position substituent, \( BR' \) represents either a boronic acid or ester and \( X \) represents the counter-ion of the benzylamine salt.

Library fragment synthesis began as suggested in Scheme 2 with the triflation of commercially available alcohol 50 (Scheme 3).\(^ {274}\) The reaction provided Suzuki-Miyaura cross coupling partner 48 in high yield, and these conditions are referred to as ‘General Method A’ hereafter (Section 4.2.1, Experimental General Methods). The proposed Suzuki-Miyaura protocol was attempted using boronic pinacol ester 51b but failed to provide desired benzonitrile 51a.\(^ {265,274}\) Instead, \(^1\text{H} \) NMR and LCMS
analysis of the crude reaction mixture indicated that the majority of triflate 48 had been hydrolysed to starting alcohol 50. Traces of two other unidentified products were also observed, neither of which had $^1$H NMR peaks or LCMS mass consistent with benzonitrile 51a.

Scheme 3: Attempted synthesis of first fragment 51a using proposed literature conditions. The triflation conditions provided 48 from 50 in high yield and were repeated on later substrates under 'General Method A'.

Suzuki-Miyaura cross coupling conditions failed to give benzonitrile 51a.

Following this, three alternative sets of Suzuki-Miyaura cross coupling conditions were attempted (Table 1). Entry 1 repeated the protocol from the proposed synthesis but with heating under reflux instead of microwave irradiation. Analysis of the crude reaction mixture gave the same results as Scheme 3, with majority triflate hydrolysis to alcohol 50. The coupling partners used in Entry 2 had the boron and pseudohalide moieties swapped such that benzonitrile building block 52 bore a boronic acid and phenolic partner 51c had an iodine. However, these conditions resulted in a complex mixture of unidentified products when the crude reaction mixture was analysed by $^1$H NMR and LCMS. Entry 2 was not investigated further. Returning to boronic pinacol ester 51b and switching the pseudohalide for bromide 53, in Entry 3, provided desired benzonitrile 51a in good yield. Subsequent optimisation of reagent equivalents led to the conditions in Entry 4, where 1.1 eq. of bromide 53 and 1.0 eq. of boronic pinacol ester 51b gave product 51a in the highest yield of 93%. These optimal Suzuki-Miyaura cross coupling conditions, described by Entry 4, were used for the synthesis of all subsequent library fragments and are referred to as ‘General Method B’ (Section 4.2.2, Experimental General Methods).
Table 1: Screening of Suzuki-Miyaura cross coupling conditions for the synthesis of benzonitrile 51a. Entry 4 describes the conditions referred to as General Method B hereafter.

With benzonitrile fragment 51a in hand, reduction of the nitrile moiety was performed as proposed in Scheme 2. Subsequent purification by semi-preparative HPLC provided final benzylamine fragment 51 in moderate yield (Table 2). This result ended the synthesis of the first benzylamine compound for the target-oriented fragment library. Following this success, the optimised Suzuki-Miyaura conditions (General Method B) and nitrile reduction protocol (General Method C) were applied in the synthesis of a further eight biphenyl, benzylamine fragments, as summarised in Table 2.

Suzuki-Miyaura cross coupling reactions between bromide 53 and a range of boronic acids and esters (Table 2) provided benzonitrile products 44a – 46a and 54a – 58a. The products were isolated in excellent yields of over 80% in all cases except one, nitrile derivative 44a, which gave a good 75% yield. Subsequent reduction of the nitrile and purification by semi-preparative HPLC yielded the final library fragments as trifluoroacetate benzylamine salts 44 – 46 and 54 – 58. Yields for this step ranged from poor, 6% for methylene alcohol 54 and 19% for alkyne 57, to good, 71% for ethyl 55 and 77% for fluoro 56. Methylene alcohol 54 was observed to co-elute with an unidentified side-product which led to poor recovery.
**Table 2:** Synthesis of target-oriented fragment library with biphenyl, benzylamine scaffolds. Isolated yield of the final fragment and benzonitrile precursor is provided, along with the identity of the boron species used in the Suzuki-Miyaura cross coupling.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R’</th>
<th>Compound</th>
<th>R</th>
<th>Yield (%)</th>
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<tr>
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<td>-CHCH₂</td>
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<td>78</td>
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</tbody>
</table>

Two additional fragments, with an adjusted scaffold core, were also synthesised for the fragment library (Scheme 4). These fragments had thiophene moieties in place of the lower phenyl ring and looked promising in computational modelling. The sulfur lone pair on the lower ring was predicted to fill space within the bottom of the αD pocket in both cases. Synthesis of isomers 59 and 60 followed the same route as the biphenyl fragments, except final purification was performed by manual flash column chromatography and the benzylamine chloride salt was formed.
Benzonitrile precursors 59a and 60a (Scheme 4) were prepared in comparable yields to the biphenyl fragments in Table 2. Reduction of the nitrile to provide benzylamines 59 and 60 proceeded without complication and in yields slightly higher than the average reported in Table 2. This may be owed to the relatively poor recovery rate often observed during semi-preparative HPLC purification.

In conclusion, a robust and modular synthetic approach for efficiently accessing biaryl benzylamine fragments was developed. The route was applied successfully in the synthesis of 11 target-oriented library fragments (Table 2 and Scheme 4), hosting a range of hydrophobic and polar moieties on the lower phenyl ring. Compounds were isolated in high yields following the Suzuki-Miyaura cross coupling step (General Method B) and good yields following nitrile reduction (General Method C) and salt formation.

2.1.3 Fragment Library Screening and Biochemical Assessment

The target-oriented fragment library, prepared in Table 2 and Scheme 4, Section 2.1.2, was submitted to our University of Cambridge Biochemistry collaborator, Dr. Paul Brear, for structural analysis and biochemical assessment. He co-crystallised the fragments with a K74A mutant of CK2α before sending them for X-ray crystallography at the Diamond Light Source Synchrotron, Didcot. Samples exhibiting selective binding in the αD pocket, relative to the ATP- and CK2α/CK2β interface-sites, were then carried forward into biochemical testing via ITC to determine the binding constant ($K_d$). A CK2α kinase assay was performed, using the ADP-Glo™ kinase assay kit (Promega), to determine whether the fragments could inhibit activity of the enzyme. For fragments capable of inhibition, a measurement of the % inhibition of kinase activity at a sample concentration of 100 μM was recorded.

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*For this project, all raw X-ray crystal structure data was collected and refined by Dr. Paul Brear, Department of Biochemistry, University of Cambridge. He also performed all biochemical assays for the collection of $K_d$, % inhibition and IC$_{50}$ data. The analysis of this data, presented in this thesis, is my own work.*
The results from X-ray crystallography and biochemical testing are presented in Table 3. Four fragments (methyl 45, alcohol 51, methylene alcohol 54 and thiophene 60) were not observed to bind to CK2α. They presented no electron density at any binding site on subunit α and were not considered further. The remaining seven fragments did bind to CK2α and site-selectivity for the αD pocket was reported. Three of these fragments, derivatives ethyl 55, fluoro 56 and 2-thiophene 59, occupied the αD pocket with the same binding mode as lead fragment 34, i.e. with the benzylamine moiety projecting towards the ATP-site and the lower phenyl ring stacked with Met225 (Figure 29, a). The binding positions also matched those predicted by the computational modelling, whereby the ethyl (55) and fluoro (56) groups and sulfur lone pair (59) fill the hydrophobic cavity at the 3-position (Figure 29, b, c and d respectively). Fluoro derivative 56 was observed to fill the αD pocket in two binding poses (Figure 29, c). This is potentially due to the small size of the fluoro substituent and flexibility of the pocket to accommodate a 180° rotation in the biaryl positioning.

![Figure 29: X-ray crystal structures of benzylamine fragments binding within the αD pocket (grey) and adopting the same binding mode as lead fragment 34. a) Lead fragment 34 (blue). b) Ethyl derivative 55 (magenta). c) Fluoro derivative 56 (yellow) with two binding modes, both optimally utilising the αD pocket volume. d) 2-Thiophene derivative 59 (purple) with sulfur lone pair projecting into the vacant space. Polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.]

These three fragments were tested for binding affinities (K_d) but all measured over 750 μM (Table 3). This was well above the K_d reported by Brear et al. for their original lead 34, of 270 μM. The fragments were also tested in a CK2α kinase assay but none were reported to inhibit activity. Based on this, these fragments were deemed too weak to use in future SBDD and were not considered further.
Table 3: X-ray crystallography and biochemical testing results for target-oriented fragment library.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ar</th>
<th>αD-site selective? [a]</th>
<th>αD-site binding mode [b]</th>
<th>$K_i$ (μM) [b,c]</th>
<th>% inhib. @ 100 μM ± SEM [b,d]</th>
</tr>
</thead>
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<td>n.a.</td>
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<tr>
<td>45</td>
<td>Me</td>
<td>n.d.</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>46</td>
<td></td>
<td>Y</td>
<td>Novel</td>
<td>&gt;750</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>51</td>
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<td>n.d.</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>54</td>
<td>OH</td>
<td>n.d.</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>55</td>
<td>Me</td>
<td>Y</td>
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<td>&gt;750</td>
<td>n.a.</td>
</tr>
<tr>
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<td>&gt;750</td>
<td>n.a.</td>
</tr>
<tr>
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<td></td>
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<td>28 ± 0.4</td>
</tr>
<tr>
<td>58</td>
<td>CF₃</td>
<td>Y</td>
<td>Novel</td>
<td>750</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>59</td>
<td>S</td>
<td>Y</td>
<td>Consistent with literature</td>
<td>&gt;750</td>
<td>n.a.</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>n.d.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

[a] Determined from X-ray crystal structure data. [b] Measured by ITC. [c] Results are of one experiment. [d] Measured by CK2α ADP-glo™ kinase assay. [e] Tests carried out in triplicate and reported as the mean. Y: yes. n.d.: no density observed on CK2α. αD-site binding mode: novel or consistent with Brear et al. reported binding mode for lead 34. n.a.: not active at concentration tested.
The remaining four fragments, dibenzylamine 44, vinyl 46, alkyne 57 and trifluoro 58, were observed to bind in the αD pocket with binding modes that were previously unreported and unpredicted by computational modelling. The first of these modes was adopted by dibenzylamine derivative 44 (Table 3). Fragment 44 had originally been predicted to bind in the same manner as lead fragment 34 and interact with Met221 via a HBD (Figure 30, a). Instead, the 3-position benzylamine group displaced the original benzylamine moiety from its binding position (Figure 30, b). This forced the biaryl core to rotate 90° within the αD pocket. The rearrangement forced a new pocket opening and the benzylamine group on the chlorophenyl ring projected through it. This positioned the benzylamine moiety away from the protein and into solvent, where no polar interactions were reported. Overlay of lead fragment 34 with dibenzylamine species 44 highlights the fragment rotation within the αD pocket (Figure 30, c).

When tested by ITC, the binding affinity for dibenzylamine derivative 44 was above 750 μM and it failed in the kinase inhibition assay (Table 3). The interesting binding mode presented an opportunity for new SBDD, but the poor quality of the biochemical data halted further interest in this fragment scaffold.
The final three fragments, derivatives vinyl 46, alkyne 57 and trifluoro 58, also adopted a new αD-site binding mode (Figure 31). Relative to original lead 34 (Figure 31, a), these fragments were rotated clockwise by 90° and the benzylamine moiety induced an alternative pocket opening into the substrate-binding channel (Figure 31). In all cases, the amine group made HBD interactions with the sidechain of Glu230 and water molecules at the opening of the pocket. For vinyl 46, an acetate molecule sat in the pocket opening and acted as a HBA for the benzylamine (Figure 31, b). The new binding mode is discussed further in Section 2.1.4, Analysis of the New Binding Mode.

Figure 31: X-ray crystal structures comparing lead fragment 34 with the new binding mode towards the substrate-binding channel, indicated by orange arrow. a) Lead fragment 34 (blue). b) Vinyl derivative 46 (pink) with acetate (purple). c) Alkyne derivative 57 (magenta). d) Trifluoro derivative 58 (yellow). Pro159 and Val162 (white) have been highlighted in each image as reference to the binding position of lead fragment 34. Polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.

Biochemical assessment of fragments 46, 57 and 58 returned binding affinities of >750, >750 and 750 μM respectively (Table 3). Pleasingly, in the kinase assay all three fragments showed inhibition of CK2α activity. Trifluoro derivative 58 performed the best, giving 32% activity inhibition, but the statistical
error of 5% overlaps with alkyne derivative 57. To the best of our knowledge, this was the first observation of CK2α inhibition by small molecules binding exclusively within the αD pocket. Furthermore, the novel binding mode adopted by these fragments had not been predicted by computational modelling and provided a new exit vector from the αD pocket.

In conclusion, a target-oriented fragment library consisting 11 members was screened against CK2α by X-ray crystallography. Seven fragments were observed to bind selectively within the αD pocket, displaying both predicted and unpredicted binding modes. These fragments were subjected to ITC for binding affinity determination which showed very poor binding for four fragments (44, 55, 56 and 59) and weak binding for three fragments (46, 57 and 58). Final assessment of the library fragments by CK2α kinase inhibition assay revealed three derivatives capable of inhibiting protein activity: 46, 57 and 58. Interestingly, these successful candidates all bind in the αD pocket with an unexpected and novel binding mode (Figure 31).

The discovery of a new binding mode within the αD pocket highlights one of the major benefits of screening by X-ray crystallography rather than in silico or by biochemical assay. If structural biology had not featured so heavily in this project, then the novel binding pose may have gone undetected and the inhibitor series reported in Section 2.2 would not have been developed.

From this point forward, the new binding orientation adopted by vinyl 46, alkyne 57 and trifluoro 58 derivatives became the focus of this project. To better understand the alternative binding mode, analysis of overlaid X-ray crystal structures was first performed. Based on this knowledge, development of an optimised inhibitor fragment series was then conducted followed by an investigation into the utility of the substrate-binding channel exit vector. The upcoming sections work through these aims in order.

2.1.4  Analysis of the New Binding Mode

As introduced in Section 2.1.3, fragments bearing vinyl (46), alkyne (57) or trifluoro (58) groups at the 3-position of the lower phenyl ring were observed to bind via a new mode within the αD pocket. Overlay of the X-ray crystal structures for these three fragments showed that the biaryl cores aligned precisely and that the 3-position substituent occupied a hydrophobic pocket (Figure 32, a). Superimposing lead fragment 34 onto this structure revealed the relative positioning of the old and new binding modes (Figure 32, b). The 3-position substituents sit where the lower phenyl ring of 34 sat, and the stacking interaction with Met225 is lost. By visual inspection of the overlaid X-ray crystal structures, all fragments reach the same depth within the αD pocket.
As the binding mode for all three derivatives (vinyl 46, alkyne 57 and trifluoro 58) was demonstrated to be consistent (Figure 32), the fragment providing the best resolution X-ray crystal structure and most promising biochemical data was used as the lead compound for further analysis. This was trifluoro benzylamine 58, and the rest of this section focuses on comparing the structural data of 58 with that of Brear et al.’s lead fragment 34.

Comparison of the αD pocket cavity for benzylamine 34 and trifluoro derivative 58 revealed the crucial rearrangement of three protein residues: Phe121, Tyr125 and Glu230 (Figure 33). The rest of the residues lining the pocket overlay well, with minimal movement observed.

1. **Movement of Phe121**

When fragment 34 (blue) is bound in the αD pocket, Phe121 lines the wall of the cavity in a position adjacent to the chlorophenyl ring (Figure 33, a). Upon binding trifluoro species 58 (yellow), Phe121 is displaced by the chlorophenyl ring and forced to rotate 90° anticlockwise. This movement creates an opening between the αD pocket and the substrate-binding channel, through which the benzylamine moiety of 58 protrudes. The sidechain of Phe121 displaces the two water molecules used by
benzylamine 34 for H-bonding and also blocks the ATP-site pocket opening, thus closing this exit vector.

Figure 33: X-ray crystal structure comparison of original lead 34 (blue) and trifluoro fragment 58 (yellow). a) View of the αD pocket (grey) highlighting the relative movements of Phe121 and Tyr125 upon the binding of 58. Water molecules from original lead 34 are shown and αD helix is labelled. b) View of the αD pocket (grey) highlighting the relative movement of Glu230 and entry of three water molecules. H-bonding network is shown by green dashed lines.

2. Movement of Tyr125

Tyr125 originally formed part of the pocket wall, however when trifluoro derivative 58 binds, this residue is forced to rotate down and away from the protein (Figure 33, a). This aids in opening the new gateway into the substrate-binding channel and widens the pocket opening to allow the benzylamine group to come through.

3. Movement of Glu230

Finally, Glu230 is pushed away from the protein core when trifluoro derivative 58 occupies the αD pocket (Figure 33, b). This movement helps widen the opening into the substrate-binding channel and allows for three water molecules to enter the pocket mouth. The three waters form a H-bonding network with benzylamine 58 and Glu230 which helps hold the fragment in the pocket.

Re-analysing the computational modelling results for trifluoro species 58 did not provide a clear reason for the new binding mode (Figure 34, a). The prediction software indicated sufficient space within the αD pocket for the accommodation of a trifluoro group, implying that an argument on the basis of sterics was not sufficient. Additionally, the biaryl ring conformation of fragment 58 predicted by the computational software (Figure 34, a) was not dissimilar to the conformation adopted in the X-
ray crystal structure (Figure 34, b). This implied that there was not a significant energy penalty arising from the biaryl ring conformation that would explain preference for one binding mode over the other.

Alternatively, hydrophobic interactions between the trifluoro of 58 and the residues lining the true binding mode (Leu128, Ile133, Tyr136, Met137, Ile140 and Met221) (Figure 34, b), may be more favourable than those in the predicted binding mode (Met137, Ile140, Leu218, Met221 and Met225) (Figure 34, a).

Figure 34: Comparison of trifluoro derivative 58 binding mode from computational modelling and X-ray crystallography. a) Computational modelling result for 58 (magenta) showing predicted binding mode consistent with lead fragment 34. b) X-ray crystallography result for 58 (yellow) showing accommodation of the trifluoro group in an alternative hydrophobic pocket. Residues lining the hydrophobic pockets are shown in white.

With new hit species trifluoro 58 in hand, and binding mode analysis complete, the investigation was continued via traditional hit-to-lead generation (Section 1.1.4). Starting with the X-ray crystal structure of hit 58, an SBDD approach was initiated with the aim of developing more potent CK2α inhibitors (Section 2.2). Given sufficient data, the mode of action for this inhibitor series would then be considered (Section 2.2.5).

2.2 Structure-Based Optimisation of the New Binding Mode

2.2.1 Optimisation Strategy for Hit-to-Lead Generation

X-ray crystal structure analysis of trifluoro hit 58 showed three regions of the fragment that could be optimised: ring A, ring B and the head group (Figure 35). It was envisaged that:
1. The addition of a polar substituent to ring A may be able to replicate the HBD interaction that original lead 34 made with Pro159 and Val162, whilst maintaining the overall binding pose of 58.

2. Alternative substitution pattern on ring B may more efficiently fill space within the middle portion of the αD pocket.

3. Changing the identity of the head group protruding into the substrate-binding channel may alter the position of the fragment within the αD pocket, potentially exploiting the depth of the pocket.

A systematic optimisation at each position was performed, with the best fragment from each round being carried into the next. At each stage, fragments accessible from commercially available starting materials were screened in silico, before chemical synthesis of ligands with the best docking results was performed. If a fragment with biochemical data superior to that of the current lead was identified, then SBDD moved forward to the next optimisation site. In moving forward, the core scaffold of the improved fragment was maintained. This method efficiently provided the potent αD-selective inhibitor series reported herein (Sections 2.2.2 – 2.2.4).

2.2.2 SBDD on Ring A

An X-ray crystal structure overlay of hit 58 and original lead 34 suggested that the introduction of a HBD group at the 6-position of ring A might mimic the benzylamine of 34 (Figure 36, b). It was hypothesised that this polar group would be capable of replicating the HBD interactions with Pro159 and Val162 that were lost in discovery of the new binding mode. An alcohol substituent was added to fragment 58 at the 6-position (61) (Figure 36, a) and computational modelling was performed. The results looked promising, with the alcohol predicted to contact Pro159 and an αD pocket water (Figure 36, c). The predicted positions of the biaryl core and amine head group were consistent with hit 58.
Computational modelling was followed by the synthesis of two molecules: HBD fragment 61 and analogue 62, lacking the trifluoro group (Figure 36, a). Analogue 62 had been modelled in silico and was predicted to bind with the same interactions as 61 (Figure 36, c). We were interested to determine the importance of the trifluoro group towards promoting the observed binding mode and thus comparison of 61 and analogue 62 was deemed relevant.

Synthesis of HBD fragment 61 was performed under the general conditions developed in Section 2.1.2 (Scheme 5). Previous synthesis of alcohol fragments 51 and 54 (Table 2, Section 2.1.2) had shown that an alcohol protecting group was not necessary, so commercially available boronic acid 61c was converted to a pinacol ester and the benzyl group removed. This gave Suzuki-Miyaura cross coupling partner 61b in excellent yield over two steps. Deprotection of 61c benzyl group was also attempted without initial boronic acid conversion to a pinacol ester, but recovery of the product boron species was very poor. With boronic ester 61b in hand, General Method B was applied to 61b and bromide 53 to provide benzonitrile 61a in good yield. Finally, reducing conditions C and purification by semi-preparative HPLC gave desired product 61 in moderate yield.
Scheme 5: Synthesis of optimised ring A fragment 61 from commercially available 61c. Following boronic ester formation and benzyl alcohol deprotection to give 61b, General Methods B and C were applied to provide final product 61. Isolated yield of 61b is reported over two steps.

Synthesis of alcohol analogue 62 was performed under the same conditions as fragment 61, but instead starting from commercially available boronic acid 62b, which did not need protecting group removal (Scheme 6). General Method B provided Suzuki-Miyaura product 62a in good yield and reduction of the nitrile by General Method C, followed by manual flash column chromatography and hydrochloride salt formation, gave final product 62 cleanly.

Scheme 6: Synthesis of fragment analogue 62 from commercially available boronic acid 62b by General Methods B and C. Isolated yield of 62 is reported over two steps.

Optimised ring A fragments 61 and 62 were submitted for X-ray crystallography and biochemical assessment. Trifluoro fragment 61 was observed to bind the αD pocket selectively and as predicted by computational modelling (Figure 37, a). The 6-position alcohol formed a HBD interaction with Pro159 and a HBA interaction with an αD pocket water molecule. Interactions between the
benzylamine moiety and three substrate-binding channel water molecules were maintained but the contact with Glu230 was lost.

Figure 37: X-ray crystallography results for optimised fragments 61 and 62 in complex with CK2α (grey). a) Optimised trifluoro species 61 (magenta) adopts the binding mode predicted by modelling. b) Analogue 62 (pink) reverts back to the original binding pose of lead 34. Analogue 62 was observed to bind the αD pocket with two chlorophenyl ring orientations: chlorine pointing towards front or back of pocket. Polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.

Fragment 62, on the other hand, did not bind CK2α as predicted and instead adopted the original fragment binding mode reported by Brear et al.265 (Figure 37, b). HBD interactions between the benzylamine and Pro159, Val162 and a water molecule were identified. The chlorophenyl ring was accommodated in the αD pocket via two binding modes, with the chlorine projecting either to the back of the pocket or to the front of the pocket.

Based on the X-ray crystallography data in Figure 37, it was decided that the 3-position trifluoro moiety of 58 and 61 was essential in promoting the new binding mode. Therefore, all future fragment analogues would bear this functional group. As alcohol 62 was not consistent with the binding mode under investigation, it was not considered further.

Benzylamine fragment 61, bearing optimised ring A, was taken forward for biochemical assessment (Table 4). When tested in the CK2α kinase inhibition assay, optimised benzylamine 61 performed better than previous lead 58, reporting an improved 44% activity inhibition compared to an original 32%, although the statistical errors overlap by 1%. An IC₅₀ value of 100 μM was also recorded for optimised 61. The improved activity of ortho-alcohol substituted 61, relative to fragment 58, could partly be owed to the pre-formed conformation of the biaryl moiety. The chlorine and alcohol ortho-
substituted groups restrict free rotation of the two aryl rings and therefore reduce the entropic binding penalty associated with fragment 61 binding in the αD pocket.

Table 4: Biochemical testing results for optimised ring A fragment 61 in comparison to hit fragment 58.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>$K_d$ (μM)$^{[a,b]}$</th>
<th>% inhib. @ 100 μM ± SEM$^{[c,d]}$</th>
<th>IC$_{50}$ ± SEM (μM)$^{[c,e]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>-H</td>
<td>750</td>
<td>32 ± 5</td>
<td>n.d.</td>
</tr>
<tr>
<td>61</td>
<td>-OH</td>
<td>n.d.</td>
<td>44 ± 8</td>
<td>100 ± 18</td>
</tr>
</tbody>
</table>

[a] Measured by iTC. [b] Results are of one experiment. [c] Measured by CK2α ADP-glo™ kinase assay. [d] Tests carried out in triplicate and reported as the mean. [e] Tests carried out in duplicate and reported as the mean. n.d.: no data.

In conclusion, two ring A-optimised fragments (61 and 62) were designed, modelled and synthesised. Following X-ray crystallography, fragment 61 was observed to maintain the binding mode of hit 58, but analogue 62 adopted Brear et al.’s published binding mode.$^{265}$ The alcohol group added to fragment 61 successfully replicated the H-bonding network previously reported for the benzylamine moiety of Brear’s lead fragment 34.$^{266}$ Biochemical assessment of optimised species 61 provided superior inhibition data relative to hit fragment 58, and thus 61 became the new lead compound for future SBDD.

2.2.3 SBDD on the Amine Head Group

The benzylamine functional group at the head of biaryl scaffold 61 was considered next for optimisation. It was observed that the existing polar amine substituent was protonated and solvent exposed when bound in the αD pocket (Figure 37, a). Therefore, it was hypothesised that the energy penalty from fragment desolvation may not be balanced by the interactions made to the protein following binding. Based on this theory, we wanted to test alternative functional groups with reduced H-bonding ability.

An in silico screening library was constructed, guided by commercially available Suzuki-Miyaura cross coupling partners bearing a head group in the 1-position, a chlorine in the 3-position and a bromine in the 4-position. Results from computational modelling predicted that three species with alternative head groups would overlay well with the biaryl core of current lead 61: nitrile 61a, aldehyde 63 and methylene alcohol 64 (Figure 38). They were not predicted to make any interactions with Glu230 or
the three substrate-binding channel waters, but were expected to maintain the HBD/HBA interactions on optimised ring A.

![Figure 38: Computational modelling results for optimisation of the amine head group on lead fragment 61. a) Nitrile 61a (blue). b) Aldehyde 63 (orange). c) Methylene alcohol 64 (green). All three molecules were predicted to maintain the ring A interaction with Pro159 (white) and the αD pocket water, shown by dashed yellow lines. None of the molecules were predicted to interact with Glu230 (white) or the waters at the pocket opening.]

Moving forward, synthesis of nitrile 61a had already been performed en route to benzyamine 61 (Scheme 7, a). Aldehyde 63 was synthesised by Suzuki-Miyaura cross coupling between boronic ester 61b and appropriate bromide 63a (Scheme 7, b). This provided desired fragment 63 in good yield and subsequent aldehyde reduction under literature conditions delivered methylene alcohol 64 without complication.

![Scheme 7: Synthesis of optimised head group fragments 61a, 63 and 64. a) Chemical structure of nitrile 61a previously synthesised in Scheme 5. The numbering pattern used for ring B is shown in green. b) Synthesis of aldehyde 63 by General Method B and alcohol 64 by NaBH₄ reduction.]

The three fragments were submitted for structural analysis by X-ray crystallography, which revealed binding modes consistent with the computational modelling (Figure 39). Overlay of the biaryl core for nitrile 61a with lead benzylamine 61 showed similar positioning of the fragments within the αD pocket,
and the interactions between ring A, Pro159 and the αD pocket water were maintained (Figure 39, a). Repeating this overlay for aldehyde 63 and alcohol 64 gave the same results as for nitrile 61a.

Figure 39: X-ray crystal structures following head group optimisation. a) Structure overlay of current lead 61 (magenta) and nitrile 61a (blue) showing good overlap of the biaryl cores and maintenance of the optimised ring A interactions. b) Aldehyde 63 (orange). c) Alcohol 64 (green). Polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.

In further analysis, the nitrile group of 61a was observed to make HBA interactions with two water molecules at the pocket mouth, and one of these waters had not been observed in prior structures (Figure 39, a). Aldehyde 63 and alcohol 64 both formed one HBA interaction with a substrate-binding channel water (Figure 39, b and c respectively). No interaction with Glu230 was observed for any fragment, which was probably due to repulsion between the carboxylate sidechain and head group lone pairs.

Biochemical assessment of the three fragments was conducted and the results compared to lead benzylamine 61 (Table 5). Exchanging the benzylamine for a nitrile (61a) gave superior kinase activity inhibition of 80% compared to 44%, and an improved IC50 of 51 relative to 100 μM. Aldehyde 63 inhibited kinase activity to a poorer extent (34%) than lead 61 and was not investigated further. Alcohol 64 was a weaker binding fragment than nitrile 61a (Kd of 68 compared to 22 μM) and was also not investigated further. Overall, benzonitrile 61a provided the best improvement on lead benzylamine 61 and was tested against HCT116 cancer cell lines to provide a GI50 of 69 μM. The HCT116 cancer cell line was chosen in order to be continuous with previous publications on clinical candidate silmitasertib 20 and Brear et al.’s pro-CAM4066 37. However, the cell line does not explicitly overexpress CK2α and no western blots were performed for this project, therefore effects

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b All GI50 data reported in this thesis was collected by Dr. Maxim Rossman, Department of Biochemistry, University of Cambridge. The analysis of this data, presented herein, is my own work.
may be due to off-target activity of the compounds. Further investigation, which is beyond the scope of this project, would be required to confirm activity against CK2α in cells.

Table 5: Biochemical testing results for optimised head group fragments 61a, 63 and 64 in comparison to lead fragment 61.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>$K_i$ (µM)[a][b]</th>
<th>% inhib. @ 100 µM ± SEM[c][d]</th>
<th>IC$_{50}$ ± SEM (µM)[e][f]</th>
<th>GI$_{50}$ ± SEM (µM)[f]</th>
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</thead>
<tbody>
<tr>
<td>61</td>
<td>-CH$_2$NH$_3^+$</td>
<td>n.d.</td>
<td>44 ± 8</td>
<td>100 ± 18</td>
<td>n.d.</td>
</tr>
<tr>
<td>61a</td>
<td>-CN</td>
<td>22</td>
<td>80 ± 8</td>
<td>51 ± 10</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>63</td>
<td>-CHO</td>
<td>n.d.</td>
<td>34 ± 2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>64</td>
<td>-CH$_2$OH</td>
<td>68</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

[a] Measured by ITC. [b] Results are of one experiment. [c] Measured by CK2α ADP-glo$^\text{TM}$ kinase assay. [d] Tests carried out in triplicate and reported as the mean. [e] Tests carried out in duplicate and reported as the mean. [f] Inhibition of proliferation tested in HCT116 cell lines. n.d.: no data

In conclusion, optimisation of the amine head group was investigated and provided new lead benzonitrile 61a. X-ray crystallography revealed an alternative H-bonding network at the αD pocket mouth for nitrile 61a compared to benzyamine 61. A new interaction to a water molecule was observed and no contact to Glu230 was reported. In biochemical testing, CK2α inhibition data for nitrile 61a was superior to starting fragment 61.

2.2.4 SBDD on Ring B

The third step in our optimisation strategy was to investigate the substitution pattern of ring B (Figure 40). Inspection of the X-ray crystal structure for benzonitrile lead 61a showed an opportunity to fill more space in the hydrophobic cavities around the 3-position chlorine and vacant 5-position. A polar interaction with the sidechain of Ser224 was also hypothesised if a HBD substituent were added at the 2-position.
Figure 40: Optimisation strategy for ring B. a) Chemical structure of 61a highlighting three positions of interest for substitution: 2-, 3- and 5-positions of ring B. The numbering scheme for ring B is shown in green. b) X-ray crystal structure of 61a (blue) in complex with CK2α (grey) to show space around ring B (red circles) and Ser224 (white).

To test this hypothesis, a selection of commercially available benzonitrile building blocks with a 4-position bromine were identified. These were narrowed down to species bearing relevant 2-, 3- or 5-position substitution. The biaryl fragments accessible from these building blocks were then modelled against CK2α in silico. Modelling results predicted reasonable overlay of the biaryl cores with lead 61a for eight fragments. The optimised polar interactions for ring A were maintained in all cases, and water contacts with the nitrile were predicted for the majority of fragments. Hydrophobic ring substituents, such as 3-methyl species 65, were predicted to occupy the chlorine binding pocket (Figure 41, a). Polar substituents, such as 2-amino derivative 66, were expected to contact Ser224 and a water molecule in the αD pocket opening (Figure 41, b). Disubstituted fragments such as 3,5-dichloro biaryl 67 were predicted to occupy the original chlorine binding pocket and also the vacant space in the 5-position (Figure 41, c).
Synthesis of six out of the eight benzonitrile fragments was performed by General Method B (Table 6). Suzuki-Miyaura cross coupling between bromides hosting a 3-position substituent and boronic ester 61b provided 3-methyl 65, 3-trifluoro 68 and 3-amino 69 derivatives in moderate yields. Under the same conditions, 2-position substituted benzonitrile products 2-amino 66, 2-alcohol 70 and 2-methylsulfonyl 71 were also isolated and in slightly better yields. Co-elution of the bromide starting material and biaryl product during manual flash column chromatography was observed for 3-trifluoro 68, 3-amino 69 and 2-methylsulfonyl 71 derivatives thus lowering the isolated yields.
Table 6: Synthesis of a benzonitrile fragment library to investigate alternative substituents at the 2- and 3-positions of ring B.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R’</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>-H</td>
<td>-Me</td>
<td>43</td>
</tr>
<tr>
<td>66</td>
<td>-NH₂</td>
<td>-H</td>
<td>56</td>
</tr>
<tr>
<td>68</td>
<td>-H</td>
<td>-CF₃</td>
<td>35</td>
</tr>
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<td>69</td>
<td>-H</td>
<td>-NH₂</td>
<td>39</td>
</tr>
<tr>
<td>70</td>
<td>-OH</td>
<td>-H</td>
<td>58</td>
</tr>
<tr>
<td>71</td>
<td>-SO₂Me</td>
<td>-H</td>
<td>40</td>
</tr>
</tbody>
</table>

The final two fragments of interest in the structure-based optimisation of ring B were 3,5-dichloro and 3,5-dimethyl substituted benzonitriles 67 and 72. Unfortunately the bromide cross coupling partner (67a) for 3,5-dichloro fragment 67 was not readily available. Instead, application of Sandmeyer conditions to commercially available amine 67b yielded desired bromide 67a in moderate yield, following a difficult work-up (Scheme 8, a). Commercially available dichloro alcohol 67d was also converted quantitatively to triflate species 67c following General Method A (Scheme 8, b). This provided an alternative Suzuki-Miyaura cross coupling partner for later investigation.

Scheme 8: Synthesis of Suzuki-Miyaura cross coupling partners for the later synthesis of 3,5-dichloro benzonitrile fragment 67. a) Sandmeyer conditions to provide bromide 67a. b) Application of General Method A to provide triflate 67c.

With bromide 67a and triflate 67c in hand, studies towards the synthesis of 3,5-dichloro biaryl 67 began (Table 7). Application of General Method B, optimised for unhindered Suzuki-Miyaura cross
coupling, failed to provide desired benzonitrile 67 (Entry 1). Instead, by-product 73 was isolated in substantial yield and the structure identified as 4-ethoxybenzoic acid. Literature reading revealed that molecules bearing a trifluoro group para to a strong electron donating group are susceptible to hydrolysis when in base and at elevated temperatures.\textsuperscript{281,282} Therefore, it was hypothesised that by-product 73 could have arisen from the decomposition of boronic ester 61b under Suzuki-Miyaura cross coupling conditions and in the presence of the EtOH/H\textsubscript{2}O solvent system. Furthermore, steric hindrance around the bromide of cross coupling partner 67a could reduce the rate of oxidative addition to palladium-(0).\textsuperscript{283–285} This would expose boronic ester 61b to the palladium catalyst for an extended period of time and promote reductive elimination with a hydride, leading to by-product 73. Interestingly, trifluoride hydrolysis was observed only twice more during the project (Section 2.3.3), despite the use of base and elevated temperature for multiple reactions.

Table 7: Suzuki-Miyaura cross coupling conditions tested in the synthesis of 3,5-dichlorobenzonitrile 67.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67a (1.0 eq.), 61b (1.2 eq.), General Method B</td>
<td>0% 54% N</td>
</tr>
<tr>
<td>2</td>
<td>67c (1.0 eq.), 61b (1.2 eq.), General Method B</td>
<td>0% 0% Y</td>
</tr>
<tr>
<td>3</td>
<td>67c (1.0 eq.), 61b (1.2 eq.), General Method B, reflux, 4 h</td>
<td>0% 20% Y</td>
</tr>
</tbody>
</table>

[a] Isolated yield of by-product 73, fully characterised to provide data consistent with CAS: 619-86-3.  
[b] Presence determined by \textsuperscript{1}H NMR and LCMS analysis of the crude reaction mixture. Y: yes. N: no.

Following rationalisation of by-product 73, attention turned to alternative Suzuki-Miyaura cross coupling partner, triflate 67c. Literature suggested that the rate of oxidative addition of hindered aryl triflates to palladium-(0) is comparable to that of aryl bromides, and in some cases faster.\textsuperscript{286} To test this, triflate 73 was subjected to General Method B (Entry 2). Analysis of the crude reaction mixture by \textsuperscript{1}H NMR and LCMS did not show the presence of by-product 73. However, instead it revealed hydrolysis of triflate 67c back to starting alcohol 67d – an observation reported for earlier triflate 48 as well (Table 1, Section 2.1.2). Entry 2 was repeated with heating under reflux instead of microwave irradiation to provide Entry 3. This time, both by-product 73 and hydrolysed species 67d were observed. For all three entries, no trace species matching the predicted \textsuperscript{1}H NMR peaks or LCMS mass
for desired product 67 were observed. General Method B was therefore determined unsuitable for the synthesis of 3,5-dichloro species 67.

In parallel to this work, investigation into the synthesis of 3,5-dimethyl benzonitrile 72 was undertaken (Table 8). Application of General Method B (Entry 1) to commercially available bromide 72a and boronic ester 61b gave a complex mixture of unidentified products, as determined by 1H NMR and LCMS analysis of the crude reaction mixture. Repeating these conditions with heating under reflux (Entry 2) resulted in the sole isolation of by-product 73 in moderate yield. Alternative Suzuki-Miyaura conditions adapted from Negoro et al.287 were attempted in Entry 3 and pleasingly provided desired 3,5-dimethyl product 72 in good yield.

Table 8: Suzuki-Miyaura cross coupling conditions tested in the synthesis of benzonitrile 72 and the development of General Method D, described by the conditions in Entry 3.287

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72a (1.0 eq.), 61b (1.2 eq.), General Method B</td>
<td>n/a[a] n/a[a]</td>
</tr>
<tr>
<td>2</td>
<td>72a (1.0 eq.), 61b (1.2 eq.), General Method B, reflux, 4 h</td>
<td>0% 60%</td>
</tr>
<tr>
<td>3</td>
<td>72a (1.0 eq.), 61b (1.2 eq.), Pd(OAc)₂ (cat.), S-Phos, K₂PO₄, Toluene, H₂O₂, 60 °C, 18 h</td>
<td>64% 0%</td>
</tr>
</tbody>
</table>

[a] Isolated yield of desired product 72. [b] Isolated yield of by-product 73, fully characterised to provide data consistent with CAS: 619-86-3. [c] Complex mixture of unidentified products as determined by 1H NMR and LCMS analysis of the crude reaction mixture.

Following successful Suzuki-Miyaura cross coupling to provide 3,5-dimethyl fragment 72 (Table 8, Entry 3), the conditions were repeated using 3,5-dichloro bromide 67a (Scheme 9). Gratifyingly, desired product 67 bearing chlorine substituents at the 3- and 5-positions of ring B was isolated without trace of by-product 73. A lower yield of 35% compared to 64% (for 3,5-dimethyl species 72) was isolated due to remaining bromide 67a despite 18 hours heating under reflux. These Suzuki-Miyaura cross-coupling conditions, suitable for hindered bromide cross-coupling partners, form ‘General Method D’ (Section 4.2.4, Experimental General Methods).
The eight fragments 65 – 72 hosting varying substitution patterns on ring B were submitted to Dr. Paul Brear for analysis by X-ray crystallography and biochemical assay. Inspection of the X-ray crystallography results found all eight fragments bound selectively in the αD pocket of CK2α and adopted binding modes consistent with lead benzonitrile 61a. A closer look at the binding pose of ring A revealed all fragments interacted with Pro159 via a HBD interaction and the αD pocket water via a HBA interaction. Additionally, all fragments except 3-trifluoro species 68 were reported to engage in an additional, new HBD interaction directly with Val162 (Figure 42, b).

It was observed that the position of ring B within the αD pocket was variable and depended on the ring substituent. Considering the 3-position substituents first, 3-methyl species 65 occupied the pocket with almost precise overlay of lead 61a (Figure 42, a) and maintained the same HBA interactions between the nitrile and two substrate-binding channel water molecules (Figure 42, b). 3-Trifluoro derivative 68, on the other hand, sat further forward in the αD pocket relative to lead 61a (Figure 42, a). As a consequence, one HBA interaction with a water at the pocket mouth was lost (Figure 42, c). The electron density for ring B of 3-amino fragment 69 was particularly poor and could not be resolved accurately. As a result, this species was not considered further.
Incorporation of a polar group at the 2-position of ring B successfully resulted in a H-bonding interaction with Ser224 (Figure 43). In further detail, ring B of 2-amino species 66 sat lower down in the αD pocket relative to lead 61a (Figure 43, a). As a result both HBA interactions between the nitrile and substrate-binding channel water molecules were lost (Figure 43, b). Ring B of 2-alcohol derivative 70 was forced to sit higher in the αD pocket than lead 61a (Figure 43, a). The HBA interactions at the pocket opening were maintained (Figure 43, c). Last, 2-methylsulfonyl fragment 71 overlaid exactly with 2-alcohol species 70 and contacted two substrate-binding channel waters, one via the nitrile moiety and one via the methylsulfonyl group (Figure 43, d).
Finally, the X-ray crystal structures for disubstituted species 67 and 72 were analysed. Relative to current lead 61a, ring B of both 3,5-dichloro 67 and 3,5-dimethyl 72 derivatives overlaid well (Figure 44, a). The 3-position chlorine of lead 61a almost perfectly eclipsed the 3-position chlorine and methyl of 67 and 72 respectively. The 5-position substituent was observed to occupy a previously vacant hydrophobic cavity towards the front of the αD pocket, as computationally predicted (Figure 44, b and c). In both cases, the nitrile moiety maintained contact with the two water molecules in the mouth of the αD pocket.
Following structural interrogation, the ring B substituted fragments (excluding 3-amino derivative 69, due to poor structural data, discussed above) were tested for inhibition in a CK2α kinase assay (Table 9). Pleasingly, all fragments 65 – 68 and 70 – 72 showed inhibition of CK2α enzyme activity and the percentage inhibition was used as a relative measure to rank the compounds.

In comparison to current lead fragment 61a, two species performed better: 3,5-dichloro 67 and 2-amino 66 with 95% and 91% kinase activity inhibition respectively (Table 9). The statistical errors for 3,5-dichloro 67 and 2-amino 66 were reported as 6% and 1% and therefore the data points for these two ligands overlap. As a result, 3,5-dichloro 67 and 2-amino 66 were considered to have comparable activity against CK2α. 2-Methylsulfonyl derivative 71 reported inhibition of activity by 79%, which was comparable to the 80% reported for lead 61a. There was then a distinct drop in inhibitory activity to 65% for fifth best fragment 3-methyl species 65 and another drop to 52% for sixth best candidate 3-trifluoro derivative 68. The poorest performers of the fragment library – derivatives 2-alcohol 70 and 3,5-dimethyl 72 – presented substantially weaker CK2α inhibition.

The IC₅₀ of fragment 3,5-dichloro 67 was determined as 9 µM which was a significant improvement on current lead 61a, of 51 µM (Table 9). 3,5-Dichloro species 67 was then submitted for a cell-based assay. When tested against HCT116 cancer cell lines a GI₅₀ of 16 µM was recorded. This was also a significant improvement on current lead 61a (GI₅₀ of 69 µM), and was comparable to Brear et al.’s best lead, pro-CAM4066 37, which recorded a GI₅₀ of 9 µM.²⁶⁵ It is possible that addition of the second ortho-substituted chlorine on biaryl 67, relative to fragment 61a, locks the biaryl moiety such that the conformation required for binding in the αD pocket is pre-formed. This would minimise the energy
penalty due to loss of entropy upon binding and lead to a higher affinity fragment, as observed in Table 9.

Table 9: Biochemical testing results for substituted ring B fragment library in comparison to lead fragment 61a. Compounds are reported according to relative rank order.

<table>
<thead>
<tr>
<th>Relative rank</th>
<th>Compound</th>
<th>Ring B</th>
<th>% inhib. @ 100 μM ± SEM[a][b]</th>
<th>IC_{50} ± SEM (μM)[c][d]</th>
<th>GI_{50} ± SEM (μM)[e][f]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>![Cl][CN][F3C]</td>
<td>95 ± 6</td>
<td>9 ± 5</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>![NH2][CN][F3C]</td>
<td>91 ± 1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>61a</td>
<td>![Cl][CN][F3C]</td>
<td>80 ± 8</td>
<td>51 ± 10</td>
<td>69 ± 69</td>
</tr>
<tr>
<td>4</td>
<td>71</td>
<td>![SO2Me][CN][F3C]</td>
<td>79 ± 0.02</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>![Me][CN][F3C]</td>
<td>65 ± 0.01</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>68</td>
<td>![F3C][CN][F3C]</td>
<td>52 ± 0.03</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>![Me][OH][F3C]</td>
<td>34 ± 4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>![Me][CN][F3C]</td>
<td>22 ± 2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

[a] Measured by CK2α ADP-glo™ kinase assay. [b] Tests carried out in triplicate and reported as the mean. [c] Tests carried out in duplicate and reported as the mean. [d] Inhibition of proliferation tested in HCT116 cell lines. n.d.: no data

In conclusion, a benzonitrile fragment library was synthesised in order to optimise the substituent on ring B. Three positions around the ring were investigated: the 2, 3, and 5-positions using hydrophobic and polar moieties. The approach towards 3,5-disubstituted fragments also led to the development of Suzuki-Miyaura cross coupling conditions for hindered substrates (General Method D). X-ray crystallography and biochemical assessment of the fragment library revealed two fragments superior to lead 61a: 2-amino 66 and 3,5-dichloro 67. Due to potential promiscuous reactivity of the amine
moiety of 2-amino derivative 66, with respect to both future synthetic chemistry and protein binding, it was decided that 3,5-dichloro species 67 was the most suitable final lead compound from this series.

Overall, an optimised inhibitor fragment, 3,5-dichloro 67, was developed by rational SBDD from initial benzylamine hit 58 (Table 10). Iterative rounds of fragment elaboration led to the introduction of a polar group to ring A (61), a nitrile group at the head of ring B (61a) and a second chlorine to ring B (67). Hit compound 58 provided a poor 32% inhibition of kinase activity, which was improved to 95% for 3,5-dichloro lead 67. The IC$_{50}$ value was also greatly improved from 51 to 9 μM following the addition of a second chlorine to benzonitrile 61a. Furthermore, a promising GI$_{50}$ of 16 μM was reported for final lead 67 when tested against HCT116 cancer cell lines. This activity level is comparable to Brear et al.’s best lead, pro-CAM4066 37, which recorded 9 μM.265

Table 10: Hit-to-lead generation of 3,5-dichloro benzonitrile 67 from benzylamine 58.

<table>
<thead>
<tr>
<th></th>
<th>Hit fragment</th>
<th>Lead fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (μM)</td>
<td>750</td>
<td>n.d.</td>
</tr>
<tr>
<td>% inhib. @ 100 μM</td>
<td>32</td>
<td>44</td>
</tr>
<tr>
<td>IC$_{50}$ (μM)</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td>GI$_{50}$ (μM)</td>
<td>n.d.</td>
<td>69</td>
</tr>
</tbody>
</table>

n.d.: no data

2.2.5  Negative Allosteric Mode of Action

The final lead compound, 3,5-dichloro benzonitrile 67, was observed to inhibit CK2α kinase activity by 95% when tested at a 100 μM assay concentration. Furthermore, studies in HCT116 cancer cell lines reported growth inhibition with a GI$_{50}$ value of 16 μM. Inspection of the X-ray crystal structure for fragment 67 revealed selective binding within the αD pocket and no ligand electron density at the ATP- or CK2β-binding sites. These observations implied a non-competitive mode of inhibition and thus a negative allosteric mode of action for the benzonitrile inhibitor series was considered.

Herein, three different potential mechanisms for the observed allosteric effects are proposed. These arguments are based on analysis of overlaid X-ray crystal structures and literature precedent.
Confirmation of these theories would require extended protein-ligand studies that are beyond the scope of this project, but a comparison to Brear et al.’s fragment 34 is provided as a starting point for further investigation.

2.2.5.1 Hypothesis One: Disruption of the ATP-Binding Site

In the active form of CK2α, ATP can enter its binding site and form interactions with the hinge region (Gln114-Asn118). A phosphorylation event can then occur to leave ADP bound in the active site. Subsequent release of this molecule enables catalytic turnover of the kinase. When in the active form, the αD helix (Asp120-Thr127) adopts an open conformation, where it is released from the C-spine of the protein (Figure 45, a).

By comparing CK2α in the open conformation (PDB: 5CVH) with the X-ray crystal structure of lead 67, it was possible to gain insight regarding the effect of fragment 67 on the ATP-binding site. Overlay of the structures revealed that upon binding fragment 67, the αD helix is pushed away from the protein core to a greater extent than in the open conformation (Figure 45, a). This movement causes a slight rearrangement of the hinge region, with effect most notable on residue Asn118.

To understand the importance of this observation, the open conformation was analysed further (Figure 45, b). Phe121, Tyr125 and Met225 occupy the αD pocket and Asn118 forms polar contacts with Thr119, Ile164 and a water molecule. The Asn118 H-bonding network holds the residue away from the mouth of the active site so that ATP can enter and ADP can leave.

When lead fragment 67 is bound to CK2α, Phe121, Tyr125 and Met225 rotate out of the αD pocket so that a cavity forms for ligand binding (Figure 45, c). The movement of Phe121 has a pronounced knock-on effect at the hinge region. The upward rotation of the phenyl sidechain displaces Asn118 and the water molecule from their original positions. Asn118 is forced to rotate towards the mouth of the ATP-binding site and HBD interactions with Thr119 and Ile164 are lost. A new water molecule occupies the space adjacent to Phe121 and adopts polar interactions with Thr119 and Ile164. In its new position, Asn118 creates an electrostatic network involving ADP and three water molecules. The interactions bridge the mouth of the ATP-binding site and potentially inhibit catalytic turnover by blocking the release of ADP and entry of ATP. Additionally, the H-bonding contacts made to ADP might stabilise the molecule within the active site and hinder its release.
Figure 45: X-ray crystal structures showing disruption of the ATP-binding site following lead 67 binding. A molecule of ADP (magenta) is shown in the ATP-binding site. a) Overlay of CK2α open conformation (green) (PDB: 3CVH) and CK2α following lead 67 binding (brown) with relative positioning of αD helix and Asn118 highlighted. b) CK2α open conformation (PDB: 3CVH) with key polar contacts (yellow) and residues (green) highlighted. c) Fragment 67 bound to CK2α with key residues highlighted (brown). New water molecule (blue) adjacent to Phe121 and electrostatic network waters (orange) are shown. Polar interactions are included as dashed lines.

In summary, movement of residue Asn118 to its position across the mouth of the ATP-binding site was hypothesised as a mechanism for allosteric inhibition. In order to confirm this theory, protein residue mutation studies involving Asn118 and Phe121 would be necessary.264,288–290
2.2.5.2 Hypothesis Two: Disruption of the Substrate-Binding Site

Molecular dynamics simulations predict that the substrate-binding channel terminates at the \( \alpha D \) helix. By comparing CK2\( \alpha \) in the active conformation (PDB: 5CVH) with the X-ray crystal structure of lead 67, it was possible to gain insight regarding the effect of fragment 67 on the substrate-binding site. Overlay of the structures revealed that upon binding fragment 67, the \( \alpha D \) helix is pushed away from the protein core to a greater extent than in the open conformation (Figure 46, a). This movement may disrupt the terminal region of the substrate-binding channel such that substrate recognition, and thus enzyme activity, is lost.

Furthermore, upon binding fragment 67, Tyr125 is forced to rotate away from the protein core (Figure 46, a). Tyr125 now sits within the original substrate-binding channel and potentially acts as a steric block towards substrate binding (Figure 46, b). The nitrile moiety of lead fragment 67 also protrudes from the \( \alpha D \) pocket and sits within the substrate-binding channel, contributing to the steric blockade.

In conclusion, it was hypothesised that exaggerated movement of the \( \alpha D \) helix might disrupt the terminal end of the substrate-binding channel such that substrates are no longer recognised. The observed rotation of Tyr125 and protrusion of benzonitrile 67 into the binding site helps further support this theory. A study into the ability of CK2\( \alpha \) to recognise different substrates would be necessary in order to confirm this mechanism of action. This could be possible by monitoring the substrate binding channel through protein-observed NMR when in the presence/absence of different...
substrates and fragment 67. Protein mutation studies involving Tyr125 would also determine the role of this residue in the inhibition mechanism.264,288–290

2.2.5.3 Hypothesis Three: Transition Between the Open and Closed Forms of CK2α

During the catalytic cycle, the αD helix of CK2 adopts two common positions, referred to as open and closed. As mentioned above, in Section 2.2.5.1, the open conformation is the active form of the protein and is adopted in the presence of ATP/ADP. In this form, the αD helix is released from the protein core. The closed form is adopted following the release of ADP from the active site and has the αD helix pulled in tight to the C-spine. It is believed that the transition from the open to closed conformation aids catalytic turnover.132

To better illustrate this, the closed form (PDB: 3FWQ) was overlaid with the open form of CK2α arising from fragment 67 binding (Figure 47, a). When closed, residues Phe121 and Tyr125 occupy the αD pocket and the αD helix is seen buried in the core of the protein. Upon binding of fragment 67 the αD helix pulls away from the protein and takes the open conformation. While fragment 67 occupies the αD pocket, the αD helix is blocked from transitioning back to the closed conformation. As this transition is reported to aid catalytic turnover,132 inhibition of movement was considered a mechanism for allosteric inhibition.

Furthermore, it was observed that when lead inhibitor 67 is bound, Tyr125 occupies a new position parallel with Arg228 (Figure 47, b). It was proposed that these two residues may form a π-stacking interaction that rigidifies the αD helix in the open conformation. This could contribute towards hindering transition to the closed form.
Figure 47: X-ray crystal structures demonstrating inhibition of the transition between open and closed forms of CK2α following lead 67 binding. a) Overlay of CK2α closed conformation (blue) (PDB: 3FWQ) and CK2α following lead 67 binding (brown) with relative positioning of αD helix, Phe121 and Tyr125 highlighted. b) Fragment 67 (brown) in complex with CK2α (grey). π-stacking between Tyr125 and Arg228 hypothesised by the parallel alignment of residues (brown).

In summary, it was hypothesised that binding lead fragment 67 inhibits transition of the αD helix between the open and closed conformations. Not only does the fragment physically block movement of the αD helix, but a potential π-stacking interaction between Tyr125 and Arg228 could also stabilise the protein in the open form. In order to confirm this theory, molecular dynamics simulations and NMR studies of labelled CK2α would be required.\textsuperscript{264,289} In these studies, the movement of the αD helix could be tracked and compared to the rate of ATP turnover. This would determine whether transition between the open and closed forms of CK2α is essential for catalysis.

2.2.5.4 Experimental Evidence

Following development of the three hypotheses presented above, X-ray crystal structure comparison with Brear et al.’s αD-site selective fragment 34 was proposed. Biaryl 34 was reported to bind the αD pocket with a $K_d$ of 270 μM but was inactive in the kinase assay.\textsuperscript{265} Therefore overlaying the X-ray crystal structure for fragment 34 in complex with CK2α (PDB: 5CSH) with the open form of CK2α (PDB: 5CVH), the closed form of CK2α (PDB: 3FWQ) and lead fragment 67 could validate/invalidate the three suggested mechanisms for allosteric inhibition.

By comparing CK2α in the open conformation (PDB: 5CVH) with the X-ray crystal structure of Brear et al.’s fragment 34 (PDB: 5CSH), it was possible to gain insight regarding the effect of biaryl 34 on the
ATP-binding site. Overlay of the X-ray crystal structures revealed that upon binding fragment 34, the αD helix is not pushed away from the protein core to any greater extent than in the open conformation (Figure 48). As a result, there is no rearrangement of residue Asn118 and thus no disruption to the ATP-binding site. This suggests that the ATP-binding site mechanism, outlined in Section 2.2.5.1, could be responsible for the allosteric inhibition observed for lead fragment 67.

![Figure 48: X-ray crystal structure overlay of CK2α open conformation (green) (PDB: 3CVH) and CK2α following fragment 34 binding (blue) (PDB: 5CSH) with relative positioning of αD helix and Asn118 highlighted. A molecule of ADP (magenta) is shown in the ATP-binding site.](image)

Following this, a similar analysis was performed with respect to the substrate-binding channel. The X-ray crystal structures for Brear et al.’s fragment 34 (PDB: 5CSH) and lead fragment 67 were overlaid with the protein surface of CK2α in the open confirmation (PDB: 5CVH) (Figure 49). This revealed that Tyr125 rotates into the substrate-binding channel following αD pocket binding of both fragments 34 and 67. Upon closer inspection, Tyr125 blocks the terminal position of the substrate binding channel to a greater degree following the binding of fragment 67 than 34, which could potentially explain the kinase activity inhibition observed for fragment 67 over 34. Overall, this X-ray crystal structure overlay is not sufficient to fully validate allosteric inhibition by disruption of the substrate-binding channel, as presented in Section 2.2.5.2.
Finally, analysis of the closed form of CK2α (PDB: 3FWQ) relative to the protein structure following binding of Brear et al.’s biaryl 34 was performed (PDB: 5CSH) (Figure 50). The X-ray crystal structure of lead fragment 67 was also overlaid to enable clearer comparison of the αD helix positions. From this it was clear that binding fragment 34 displaced the αD helix to a similar position as for lead fragment 67. Furthermore, whilst fragment 34 occupies the αD pocket the transition between the open and closed forms of CK2α is inhibited. Brear et al.’s fragment 34 is not reported to inhibit CK2α kinase activity therefore this analysis discounts the allosteric inhibition mechanism put forward in Section 2.2.5.3.
To conclude, three mechanisms by which optimised benzonitrile inhibitor 67 could induce negative allosteric control over CK2α have been presented. The third hypothesis, based on inhibition of the transition between the open and closed forms of CK2α, was then discounted following X-ray crystal structure comparison to Brear et al.’s inactive fragment 34.

To the best of our knowledge, fragment 67 represents a first-in-class allosteric inhibitor, binding selectively in the αD pocket. The arguments put forward in this thesis require further investigation but could form a basis for the future development of selective allosteric inhibitors. A recent publication by Jiang et al. goes some way towards supporting the allosteric mode of action hypothesised above.239 Jiang et al. utilised their Allosite algorithm to computationally identify potential allosteric sites on CK2α. One of their results suggested a site composed of residues Phe121, Leu128 and Met225, which corresponds well with the αD pocket. No further work on this site has been published by them yet.

2.3 αD Pocket to Substrate-Binding Channel Linking Investigation

2.3.1 Background

To date, the substrate-binding channel of CK2 has not been investigated using small molecules, and limited success has been reported with peptides.251,292 One promising lead comes from Marin et al. as introduced in Section 1.2.1.1. Marin designed a pseudosubstrate (19) comprised of 12 amino acids and showed it to be selective for CK2, reporting a Michaelis constant, $K_M$, of 19 μM.146 Follow-up work by Sarno et al. used protein and peptide mutation studies to confirm that dodecapeptide 19 binds in the substrate channel.142,143 This work highlighted CK2α residues that were vital for substrate recognition and mapped them to the relevant positions on dodecapeptide 19 (Figure 51, a). It was observed that His160 recognises the $n$-2 position, Arg191, Arg195 and Lys198 recognise the $n$+1 position, Lys49 recognises the $n$+2 position and Lys74-Lys77 recognise the $n$+3 position. Further work by Enkvist et al.262 and Cozza et al.251 corroborated these findings and also showed that substrate mutations from Asp to Ala at the $n$+1 and $n$+3 positions were the most detrimental to activity. As a result, these two positions are deemed the most important within the consensus sequence of CK2 substrates.293

Since the publication of Marin’s pseudosubstrate (19) in 1994, no X-ray crystal structure of dodecapeptide 19, or any other peptide, genuine substrate or bisubstrate inhibitor, in complex with CK2α has been successfully resolved. Therefore, the exact binding pose adopted by these species has not been unambiguously confirmed. However, computational modelling by Cozza et al. utilised the mutation study data to propose a binding mode for dodecapeptide 19 (Figure 51, b).123 Specific attention was given to the positioning of Ser and the $n$+1, $n$+2 and $n$+3 determinants. The N-terminal
portion was not modelled in detail as the arginyl tail (RRR) exists solely for the purpose of Marin’s phosphocellulose assay, and the neutral Ala residue at n-3 is known to be unnecessary for substrate recognition.

2.3.2 Project Aim and Preliminary Modelling

The work reported in this chapter aimed to develop a chemical probe for the substrate-binding channel. It was envisaged that this could be achieved by linking a high-affinity fragment, anchored in the αD pocket, to a CK2-specific peptide (Figure 52, a). We hoped to achieve an X-ray crystal structure
of this species in complex with CK2α to unambiguously verify the binding position of the peptide within the substrate channel. This could also confirm which protein residues are key for recognition of the CK2 consensus sequence. If successful, this project would provide the first fully resolved X-ray crystal structure of CK2 with a peptide bound in the substrate channel – a PPI acknowledged as ‘highly challenging’.\(^{122}\)

![Diagram](image)

**Figure 52:** Figure to illustrate the project aim. *a*) αD pocket fragment 67 (brown) could be linked to a CK2-selective peptide (green) via a short, optimised linker (yellow). *b*) The optimised biaryl core of 67 would be maintained but nitrile head group would be more synthetically useful as a carboxylate (74).

To start, the αD-site fragment would bear optimised biaryl scaffold 67, as determined in the SBDD program from Section 2.2 (Figure 52, *b*). The nitrile group on ring B of lead fragment 67 would be exchanged for a carboxylate group (74) to provide a more useful synthetic handle for the linking strategy. For the peptide, CK2-specific pseudosubstrate 19, developed by Marin et al., would be truncated to include only the ideal CK2 consensus sequence. Based on the literature reported in Section 2.3.1, this sequence should cover the n-2 to n+3 determinants, and thus hexamer DDSDDD 75 was identified as the target.\(^{295}\) Shortening the sequence to this crucial hexamer should also allow manageable molecular dynamics simulations and efficient synthesis.

To check the viability of the strategy, proposed hexamer 75 was modelled onto CK2α (PDB: 2PVR) by Dr. Paul Brear using molecular dynamics (Figure 53). These results were compared to the literature reported protein-determinant interactions (Section 2.3.1) to ensure a reasonable binding position had been predicted.
Analysis of the molecular dynamics simulation showed that hexamer 75 was positioned as anticipated within the substrate-binding channel. The N-terminal tail of the hexamer projected towards His160, however the n-2 Asp did not make a HBA interaction with it. Instead the n-2 position was predicted to interact with Arg195. The Ser for phosphorylation was positioned adjacent to a phosphate, which was modelled to represent the terminal position of the ATP tail. The backbone of Ser contacted Lys49. The Asp at the n+1 position formed a HBA interaction with Lys198 as desired, but was not close enough to contact Arg191 or Arg195. At the n+2 position, the Asp residue was not predicted to interact with Lys49, but instead formed HBA contacts with Lys74 and Lys77. The final n+3 C-terminal Asp was not predicted to make any polar contacts. Overall, the majority of the key recognition residues were predicted to interact in some way with the hexamer, and this was deemed sufficient to proceed.

Next, modelled hexamer 75 was overlaid with the X-ray crystal structure of optimised lead fragment 67 (Figure 54). The relative position of the peptide and CK2α residues were compared to see whether the conformation of the substrate-binding channel was altered following αD-fragment binding.
Pleasingly, results for the protein of fragment 67 were comparable to the Apo protein (Figure 54 vs. Figure 53). His160 was within range of the N-terminal tail and the n-2 Asp interacted with Arg195. The Asp at n-1 was now predicted to contact Lys49 and n+1 maintained the interaction with Lys198. Both interactions between the n+2 Asp and Lys74 and Lys77 were lost; however, this was due to the CK2α Lys74-Lys76 Ala mutant used for crystallography. Rearrangement of the protein secondary structure in this region resulted in a HBA contact between the Asp at n+3 and Lys77, coincidentally matching that reported in the literature. It thus appeared that the binding of lead fragment 67 in the αD pocket did not have a significant effect on the shape of the substrate-binding channel. Therefore, it was considered reasonable to hypothesise that CK2α could bind an αD-site fragment simultaneously to peptide hexamer 75. Furthermore, the relative positioning of fragment 67 and modelled hexamer 75 implied linkage of the biaryl and N-terminal Asp via a short, optimised linker could be possible (Figure 54). As a result of the molecular dynamics reported above, the allosteric inhibition mechanism relating to disruption of the substrate-binding channel was discounted (outlined in Section 2.2.5.2). Figure 54 implies that
pseudosubstrate hexamer 75 can still bind CK2α in the presence of inhibitor fragment 67 and thus contradicts this inhibition theory.

The work presented in the next sections report the studies towards this project aim. It starts with modification of optimised αD fragment 67 to introduce a carboxylate head group, then development of a suitable linker and finally the fragment-peptide linking step.

2.3.3 αD-Site Fragment Optimisation

In order to prepare αD-site fragment 67 for linker development, the nitrile head group needed to be exchanged for a functional group with greater synthetic tractability. It was envisaged that a carboxylic acid moiety would be most appropriate, as an amine or alcohol linker could be reliably coupled to the fragment under literature conditions.

Monochloride species 61a and lead 3,5-dichloro 67 had been observed to give overlapping binding modes within the αD pocket (Figure 44, Section 2.2.4). With this in mind, synthetic accessibility of the two fragments was considered. Following Suzuki-Miyaura cross coupling, the former (61a) was synthesised in consistently higher yield (77%) than lead 67 (35%). Furthermore, bromide cross coupling partner 53, for the preparation of monochloride 61a, was commercially available, whereas equivalent bromide 67a, for the preparation of 3,5-dichloro 67, required chemical synthesis. It was thus decided that methodology development for the carboxylic acid head group was to be performed on cheaper and more readily accessible fragment 61a.

Initial studies attempted to hydrolyse nitrile 61a directly to carboxylic acid 76 following literature conditions (Scheme 10, a). However, desired product 76 was not observed and instead by-product 77, with a hydrolysed trifluoro group, was isolated in considerable yield. Literature reading revealed that molecules bearing a trifluoro group para to a strong electron donating group are susceptible to hydrolysis when in base and at elevated temperatures. This phenomenon was observed only twice more during the project (Table 7 and Scheme 12), despite the use of base and elevated temperature for multiple reactions.
Scheme 10: Initial studies towards the synthesis of carboxylic acid 76. a) Direct hydrolysis of nitrile 61a. b) Suzuki-Miyaura cross coupling using benzoic acid 76a and General Method B.

Next, Suzuki-Miyaura cross coupling conditions from General Method B were applied to commercially available bromide 76a and optimised boronic ester 61b (Scheme 10, b). The presence of desired product 76 was detected by LCMS of the crude reaction mixture, but the species could not be isolated cleanly from acetic acid when purified by acid-doped flash column chromatography.

Following initial difficulties in obtaining hydrolysed fragment 76, an alternative synthetic approach was sought. A retrosynthesis of desired lithium carboxylate 78 was envisaged as follows (Scheme 11): the carboxylate product 78 could be accessed from carboxylic acid 76, which in turn could have come from hydrolysis of ethyl ester 76c. The biaryl scaffold of 76c could be provided by Suzuki-Miyaura cross coupling between ethyl ester bromide 76b and boronic ester 61b. Coupling partner 76b was not readily available but could be produced in one step from commercially available benzoic acid 76a.

Scheme 11: Retrosynthetic analysis for lithium carboxylate species 78 from commercially available benzoic acid 76a.

In the forward direction, the formation of ethyl ester 76b from benzoic acid 76a proceeded without complication and in high yield (Scheme 12). Suzuki-Miyaura conditions from General Method B then
provided biaryl 76c in excellent yield. Two sets of literature ester hydrolysis conditions were then attempted.297 Hydrolysis using LiOH·H₂O and minimal MeOH solvent failed to provide desired product 78 and instead gave quantitative yield of unexpected product 79. A similar hydrolysis of the trifluoro group was reported in Scheme 10 and it is believe that methyl ester 79 formed under the same mechanism.281,282 Alternative ester hydrolysis by NaOH in dilute THF/MeOH, followed by lithium salt formation, gave desired product 78 in quantitative yield. Conversion from a carboxylic acid to lithium carboxylate salt simplified compound purification, provided ease of handling and offered stability during storage.

Scheme 12: Synthesis of lithium carboxylate species 78 from commercially available benzoic acid 76a and isolation of unexpected product 79.297

With a synthetic route to monochloride carboxylate 78 established, it was envisaged that the same conditions could now be applied to access 3,5-dichloro carboxylate 74. General Method B would be substituted for General Method D, suitable for hindered Suzuki-Miyaura cross couplings.

Unfortunately, 3,5-dichloro benzoic acid 74h was not readily available as a starting material (Scheme 13, a). As a result, 3,5-dichloro ethyl ester 74a was prepared from 3,5-dichloro amine 74c instead (Scheme 13, b). This was performed in two steps: first, benzoic acid 74c was converted to ethyl ester 74b in quantitative yield, then Sandmeyer conditions gave bromide 74a in moderate yield. Application of General Method D to fragments 74a and 61b failed to provided desired biaryl ester 74d. Analysis of the crude reaction mixture revealed 1H NMR peaks and an LCMS mass consistent with traces of product 74d, but majority bromide 74a was observed.
Scheme 13: a) Chemical structure of unavailable starting material 74h. b) Alternative synthesis of bromide 74a by esterification and Sandmeyer reaction. Followed by attempted Suzuki-Miyaura cross coupling towards biaryl 74d.

Following this, six alternative Suzuki-Miyaura cross coupling protocols were attempted using bromide 74a and boronic ester 61b (Table 11). $^1$H NMR and LCMS analysis of the crude reaction for Entry 1 showed a complex mixture of unidentified products. No traces of desired product 74d were detected and no bromide starting material 74a could be recovered. Entries 2-5 tested different palladium catalysts, bases and solvents but unfortunately only starting bromide 74a could be recovered from each reaction. Entry 6 decomposed with heating.
To continue the investigation towards biaryl $74d$, two alternative Suzuki-Miyaura cross coupling partners were prepared (Scheme 14). This was rationalised on the basis that oxidative addition of an aryl halide to palladium(0) is the first step in the Suzuki-Miyaura catalytic cycle, and that the relative reactivity of the halide governs the rate of this step.$^{283,284,303}$ It is well established that the propensity of aryl halides towards oxidative addition increases in the order of ArCl $<$ ArOTf $\sim$ ArBr $<$ ArI and thus iodo and triflate coupling partners $74e$ and $74g$ were considered.$^{286,304}$ Iodo species $74e$ was synthesised in good yield by a Sandmeyer reaction from amine $74b$ (Scheme 14, a)$^{305}$ General Method A then provided triflate fragment $74g$ from commercially available alcohol $74f$, also in good yield (Scheme 14, b).

---

**Table 11: Screening of Suzuki-Miyaura cross coupling conditions for the synthesis of 3,5-dichloro ester $74d$.**$^{287,298-302}$

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>$74d$</th>
<th>$74a^{[a]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pd(PPh$_3)_4$ (cat.), K$_2$PO$_4$, 1,4-dioxane, 90 °C, 16 h</td>
<td>n/a$^{[b]}$</td>
<td>n/a$^{[b]}$</td>
</tr>
<tr>
<td>2</td>
<td>Pd(PPh$_3)_4$ (cat.), Na$_2$CO$_3$, DME, EtOH, H$_2$O, 80 °C, 16 h</td>
<td>0%</td>
<td>55%</td>
</tr>
<tr>
<td>3</td>
<td>PdCl$_2$(PPh$_3)_2$ (cat.), K$_3$PO$_4$, LiCl, 1,4-dioxane, 100 °C, 24 h</td>
<td>0%</td>
<td>61%</td>
</tr>
<tr>
<td>4</td>
<td>Pd(dppf)Cl$_2$•CH$_2$Cl$_2$ (cat.), KOAc, DMF, 90 °C, 16 h</td>
<td>0%</td>
<td>60%</td>
</tr>
<tr>
<td>5</td>
<td>Pd$_2$(dba)$_3$ (cat.), Na$_2$CO$_3$, S-Phos, Toluene, H$_2$O, 100 °C, 24 h</td>
<td>0%</td>
<td>85%</td>
</tr>
<tr>
<td>6</td>
<td>Pd$_2$(dba)$_3$ (cat.), Cs$_2$CO$_3$, PH(Bu)$_3$•BF$_4$, 1,4-dioxane, 90 °C, 18 h</td>
<td>n/a$^{[c]}$</td>
<td>n/a$^{[d]}$</td>
</tr>
</tbody>
</table>

[a] Isolated yield of recovered bromide starting material $74a$. [b] Complex mixture of unidentified products. [c] Reaction mixture decomposition.
Scheme 14: Synthesis of alternative Suzuki-Miyaura cross coupling partners. a) Iodo species 74e. b) Triflate species 74g.

The new cross coupling partners iodo 74e and triflate 74g were tested in a variety of Suzuki-Miyaura cross coupling protocols (Table 12). Entry 1 used iodo species 74e and General Method D, but failed to provide any trace of biaryl 74d when examined by 1H NMR and LCMS. This was also the case for iodo species 74e and the conditions in Entry 2. The final attempt with iodo partner 74e is described by Entry 3 and only recovery of unreacted starting material was possible. Moving on to triflate cross coupling partner 74g, the crude reaction mixture for Entry 4 showed majority alcohol 74f, arising from the hydrolysis of triflate 74g. Pleasingly, entries 5 and 6 both gave desired biaryl product 74d but in low yields of 15 and 22% respectively. During purification of biaryl 74d by flash column chromatography, it was observed that hydrolysed triflate-alcohol 74f co-eluted with desired product 74d, thus reducing the yield. Alternative elution systems were tested but no improvement was observed. At this point, Entry 6 was prioritised over Entry 5 as a superior yield of biaryl 74d had been isolated.
Following the successful synthesis of 3,5-dichloro biaryl 74d, a short optimisation study of the Suzuki-Miyaura cross coupling conditions in Entry 6, Table 12 was performed (Table 13). Entry 1 reports the original protocol used in the discovery of these conditions. Initial optimisation increased the palladium catalyst equivalents from 0.05 to 0.20 (Entry 2). Inspection of the crude reaction mixture by TLC and LCMS showed triflate 74g remaining. The reaction time was then increased from 3 to 24 hours to give Entry 3. Pleasingly this pushed the reaction towards completion and minimal co-elution of alcohol 74f was observed. A substantially improved isolated yield of 76% was obtained under these conditions. Entry 4 repeated Entry 3 except with a lower temperature of 80 instead of 110 °C, hoping to reduce the hydrolysis of triflate 74g. Minimal co-elution of alcohol 74f and biaryl 74d was observed for this Entry, however reaction progress had been reduced and thus a lower yield of 54% was isolated. Returning to the conditions outlined in Entry 3 and lowering the equivalents of boronic ester 61b from 2.5 to 1.2 provided the final test conditions in Entry 5. This was deemed relevant because boronic ester 61b had to be synthesised over two steps from a limited availability starting material (Scheme 5, Section 2.2.2). Unfortunately, this modification saw a significant impact on the yield of biaryl 74d: a drop from 76 to 45%, and was not investigated further.
Table 13: Optimisation of Suzuki-Miyaura cross coupling conditions for the synthesis of 3,5-dichloro ester 74d.

<table>
<thead>
<tr>
<th>Entry</th>
<th>61b eq.</th>
<th>Pd eq.</th>
<th>Temp., °C</th>
<th>Time, h</th>
<th>Result[a][b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
<td>0.05</td>
<td>110</td>
<td>3</td>
<td>22%</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>0.20</td>
<td>110</td>
<td>3</td>
<td>74g remaining[c]</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>0.20</td>
<td>110</td>
<td>24</td>
<td>76%</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>0.20</td>
<td>80</td>
<td>24</td>
<td>54%</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>0.20</td>
<td>110</td>
<td>24</td>
<td>45%</td>
</tr>
</tbody>
</table>

[a] Isolated yield of desired product 74d. [b] Alcohol 74f observed and co-eluted in early fractions of 74d during flash column chromatography. [c] Determined by TLC and LCMS analysis of the crude reaction mixture.

With the optimised conditions from Entry 3 (Table 13) in hand, synthesis of biaryl 74d was repeated on a 2.5 mmol scale – ten times the scale performed during optimisation (Scheme 15). Pleasingly, a high yield of 81% for biaryl 74d was achieved and co-elution with alcohol 74f was negligible.

Scheme 15: Optimised Suzuki-Miyaura cross coupling conditions for the synthesis of 3,5-dichloro ester 74d.

With 3,5-dichloro ester 74d in hand, hydrolysis of the ester moiety and subsequent lithium salt formation was performed (Scheme 16). Synthetic conditions developed for monochloride carboxylate 78 (Scheme 12) were used. This provided the desired 3,5-dichloro lithium carboxylate salt 74 in quantitative yield.
Scheme 16: Synthesis of final product 3,5-dichloro lithium carboxylate salt 74 from ester 74d.

Following the successful synthesis of αD-site fragments 74 and 78, X-ray crystallography was performed to verify the fragment binding modes (Figure 55). Comparison of the biaryl cores for 3,5-dichloro and monochloro carboxylates 74 and 78 with lead fragment benzonitrile 67 showed good structural overlay (Figure 55, a). Ring A, bearing the trifluoro and alcohol groups, remained deep within the αD pocket while ring B filled the middle portion of the pocket and protruded into the substrate-binding channel. It was observed that the carboxylate moieties of 74 and 78 sat higher in the αD pocket relative to the nitrile of 67. In addition, residue Glu230 was pushed further away from the αD pocket opening (Figure 55, a). It was surmised that electrostatic repulsion between the carboxylate head group and Glu230 side chain was responsible for this rearrangement.

Figure 55: X-ray crystallography results for lithium carboxylate salts in complex with CK2α (grey). a) Overlay of lead 67 (brown), 3,5-dichloro derivative 74 (orange) and monochloro derivative 78 (blue) to show relative positioning within the αD pocket. Residue Glu230 has been highlighted to show change in position. b) 3,5-Dichloro derivative 74 (orange). c) Monochloro derivative 78 (blue). Polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.

3,5-Dichloro carboxylate 74 was observed to maintain interactions between the ring A alcohol, Pro159 and Val162, but contact to the αD pocket water molecule was lost (Figure 55, b). HBA interactions between the head group carboxylate and two water molecules in the substrate-binding channel were...
also detected. Analysis of the X-ray crystal structure for monochloro carboxylate 78 revealed the same interactions as for dichloro analogue 74 (Figure 55, c).

Next, hexamer peptide 75 was superimposed onto the X-ray crystal structure for 3,5-dichloro carboxylate 74 (Figure 56). Hexamer 75 was observed to align well with the substrate-binding channel and thus protein residues relevant to substrate recognition were inspected. The relative positions of the residues matched what was expected from earlier studies (Section 2.3.2) but only one interaction was within range for detection: a HBA interaction between the \( n+1 \) Asp and Lys198.

![Figure 56: Overlay of modelled hexamer 75 (green) and 3,5-dichloro carboxylate 74 (orange) protein crystal structure (grey) with ADP (yellow), phosphate (orange) and Mg\(^{2+}\) (purple) in the ATP-site. CK2α residues involved with substrate recognition are highlighted (white) and predicted polar contacts are shown as dashed yellow lines. This protein is a K74-K76 alanine mutant.](image)

This analysis was repeated for monochloro carboxylate 78 (Figure 57). Superimposing hexamer 75 showed good alignment with the substrate-binding channel, and residues responsible for substrate recognition were positioned within appropriate distance. Two HBA interactions were observed involving the Asp at \( n+1 \), Lys198 and Arg191, which match those reported in the literature.
In conclusion, two lithium carboxylate salt analogues were synthesised: 3,5-dichloro carboxylate 74 and monochloro carboxylate 78. The carboxylate moiety was deemed a suitable head group for use in later linker development. X-ray crystallography showed carboxylate fragments 74 and 78 bound selectively in the αD pocket, and the residues lining the substrate-binding channel remained consistent for hexamer 75 binding. Following these promising results, the project progressed into linker development.

Before moving forward, a note on the synthetic accessibility of 3,5-dichloro carboxylate 74 must be mentioned. At the time of linker development, studies towards the Suzuki-Miyaura protocol for 3,5-dichloro ester 74d were ongoing. Therefore 3,5-dichloro carboxylate 74 was not available for use at this stage. Instead, monochloro carboxylate 78 was used in the linker optimisation phase and then substituted for 3,5-dichloro carboxylate 74 at the point of final fragment linking.
2.3.4 Linker Optimisation

2.3.4.1 Optimisation Strategy and Previous Work

Fragment linking is considered the more challenging route of fragment optimisation. Introduction of a linker that does not interfere with the binding mode of the original fragments is difficult and was explained in greater detail in Section 1.1.4, Hit-to-Lead Generation.

In order to maintain the optimised αD fragment binding pose, we envisaged developing a linker via iterative growth of carboxylate fragment 78 towards the substrate-binding channel. Each cycle would involve rational structure-based design of multiple linkers and screening in silico. The most promising candidates would then be synthesised and submitted for structural determination by X-ray crystallography. The best result from each set would serve as the starting point for SBDD in the next round, and thus development of an efficient linker should be possible.

Before proceeding, prior work by Part III masters student, Antanas Radzevičius, was reviewed. This work derivatized aldehyde 63 by reductive amination using commercially available primary amines 80 (Table 14). His aim was to investigate the opening of the αD pocket and utility of this exit vector for binding small molecules in the substrate-binding channel. Radzevičius designed and screened a library of secondary amines bearing scaffold 81 and then synthesised the top hits and submitted them to Dr. Paul Brear for X-ray crystallography (Table 14).
Table 14: Reductive amination fragment library 82–91 synthesised by Antanas Rodzevičius and X-ray crystallography results.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>X-ray cryst. results</th>
<th>Compound</th>
<th>R</th>
<th>X-ray cryst. results</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>-Me</td>
<td>Resolved</td>
<td>87</td>
<td>OH</td>
<td>Unresolved</td>
</tr>
<tr>
<td>83</td>
<td>-Et</td>
<td>Resolved</td>
<td>88</td>
<td>NH₂</td>
<td>Unresolved</td>
</tr>
<tr>
<td>84</td>
<td>-Bu</td>
<td>Resolved</td>
<td>89</td>
<td></td>
<td>Unresolved</td>
</tr>
<tr>
<td>85</td>
<td>-Ph</td>
<td>Resolved</td>
<td>90</td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>86</td>
<td>CO₂H</td>
<td>Unresolved</td>
<td>91</td>
<td>HO</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Resolved: electron density for the ligand could be accurately defined. Unresolved: electron density for the ligand could not be accurately defined. n.d.: no density observed on CK2α.

Of the ten fragments submitted for X-ray crystallography, eight fragments (82–89) were observed to bind CK2α. Electron density for chiral fragments 90 and 91 was not detected at any binding site on the α subunit. Fragments 82–89 were selective for the αD pocket and the biaryl core was well defined in each case. Small, aliphatic amines 82–84 showed good electron density at the opening of the αD pocket, but larger aliphatic or aromatic moieties 85–89 had weak or unresolved electron density for the amine portion.

Based on these unpromising results, it was decided that an alternative peptide-based approach to the linker would be taken in this project. It was hypothesised that a linker resembling the natural peptide substrates might interact with the binding channel more successfully. Literature studies showed that the substrate-binding channel of CK2α is highly basic and therefore complementary Asp and Glu residues would be the focus for linker design.295
2.3.4.2 Development of a Peptide Linker

2.3.4.2.1 Initial Peptide Linker Test Set

The peptide linker strategy began with computational modelling of αD-site fragments bearing up to a tetramer peptide chain. Carboxylate fragment 78 was extended by one amino acid at a time, starting with the addition of a single Ala residue, 92 (Figure 58, a). For each additional amino acid, the predicted polar contacts between the peptide and protein were examined and only fragments predicted to enhance ligand binding were considered further. All modelled fragments overlaid well with parent carboxylate 78 and were predicted to maintain the polar interactions at ring A. The most promising results from the in silico screen are shown in Figure 58.

Addition of a single Ala residue (92) was predicted to form a HBA interaction between the terminal acid group and His160 in the αD pocket opening (Figure 58, a). A HBA contact between the amide carbonyl of fragment 92 and a substrate-binding channel water was also predicted. Extension by a second Ala (93) maintained the interaction to the water molecule and the new terminal acid group was predicted to contact both His160 and Lys121 (Figure 58, b). Adding an Asp residue gave tripeptide 94 which was predicted to lie along the substrate-binding channel (Figure 58, c). The Asp sidechain was anchored in place by a HBA interaction with Ser194 and the terminal acid group was within contact distance of the His160 backbone. The interaction to a water at the mouth of the pocket was maintained. The final round of fragment growth added a second Asp residue and both terminal acid (95) and amide (96) were modelled (Figure 58, d and e respectively). The tetramer was expected to project along the substrate-binding channel with a similar predicted binding mode for both molecules. In both cases, the HBA interaction to water was maintained and His160 was predicted to contact the peptide backbone and first Asp residue. The terminal Asp was shown to contact Ser194 and the backbone of Arg195, and the terminal acid/amide groups were predicted to interact with Lys158 as HBAs.
Figure 58: Computational modelling results for initial peptide linkers docked onto monochloro carboxylate 78 protein structure (grey). a) Ala derivative 92 (magenta) with Pro159 and Val162 highlighted (white) to show ring A interactions maintained (omitted from other images for simplicity). The αD helix is highlighted in green. b) AA derivative 93 (yellow). c) AAD derivative 94 (pink). d) AADD with terminal acid 95 (blue). e) AADD with terminal amide 96 (purple). Predicted polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.

Following analysis of the modelling data, fragment-peptides 92 and 93 were synthesised by traditional manual Fmoc-Solid-Phase Peptide Synthesis (Fmoc-SPPS). Tripeptide 94 and tetramers 95 and 96 were synthesised by microwave Fmoc-SPPS (Table 15).

While the peptides were still resin-bound, the N-terminal amino acid was Fmoc-deprotected and coupled with monochloro carboxylate 78. The fragment-capped peptide was then fully deprotected, cleaved from the resin and purified by semi-
preparative HPLC to provide the desired product. Fragments 92 – 96 were all isolated in low yields ranging from 19% for Ala derivative 92 down to 6% for di-Ala species 93.

Table 15: Fmoc-SPPS of initial peptide linker species 92 – 96 and X-ray crystallography results for the peptide chain.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Fmoc-SPPS method</th>
<th>Yield (%)</th>
<th>Peptide chain resolved?</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>78-A-OH</td>
<td>Manual</td>
<td>19</td>
<td>Y</td>
</tr>
<tr>
<td>93</td>
<td>78-AA-OH</td>
<td>Manual</td>
<td>6</td>
<td>N</td>
</tr>
<tr>
<td>94</td>
<td>78-AAD-OH</td>
<td>Microwave</td>
<td>17</td>
<td>Y</td>
</tr>
<tr>
<td>95</td>
<td>78-AADD-OH</td>
<td>Microwave</td>
<td>7</td>
<td>N</td>
</tr>
<tr>
<td>96</td>
<td>78-AADD-NH₂</td>
<td>Microwave</td>
<td>12</td>
<td>N</td>
</tr>
</tbody>
</table>

Y: yes, electron density of peptide chain strong enough to resolve accurately.
N: no, electron density of peptide chain too weak to resolve accurately.

Next, test linker species 92 – 96 were submitted to Dr. Paul Brear for structural determination by X-ray crystallography. In all cases, electron density for the αD-site biaryl moiety was strong and showed selective binding within the αD pocket. Overlay of the test species biaryl cores with parent carboxylate 78 also showed a consistent positioning within the αD pocket, and ring A interactions were maintained (Figure 59, a). Unfortunately, the peptide chain could only be resolved for two structures: Ala derivative 92 and tripeptide 94 (Figure 59).

Upon closer inspection of Ala derivative 92, it was observed that the ligand bound to the protein via two modes (Figure 59, b). Ring B of the biaryl core was accommodated with the 3-position chlorine pointing to the back of the pocket, but also towards the front of the pocket. The Ala residue projected out of the αD pocket opening and either towards the substrate-binding channel or towards the αD helix. In both binding modes, Glu230 is involved in a HBA interaction with the fragment amide. Tripeptide 94 was reported to bind CK2α with strong electron density for an unexpected binding mode (Figure 59, c and d). The tripeptide chain was not shown to lie within the substrate-binding channel, but instead project away from the protein surface and towards the αD helix. Contacts between the Asp residue and Lys122 were detected, as well as multiple H-bonding interactions between the peptide backbone and water molecules. The C-terminal end of the ligand was highly solvent exposed in this binding mode.

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In summary, five linker test species 92–96 were designed, synthesised and tested by X-ray crystallography. While only two species (92 and 94) could be fully resolved, electron density was observed in the αD pocket for all five ligands. This validated the use of αD-binding fragment 78 as a molecular ‘anchor’ for the development of the linker.

2.3.4.2.2 Polyanionic Tripeptide Linkers

To move forward with linker development, it was decided that tripeptides would be investigated further. Test fragment 94, bearing the sequence AAD, provided the X-ray crystal structure with best electron density in the previous round. In contrast, dimer 93 and tetramers 95 and 96 were unresolved. Unfortunately, tripeptide 94 did not present the binding mode desired, but with rational structure-guided design it was believed that influence over the binding pose may be possible. To this end, a library of polyanionic tripeptide ligands was constructed and screened in silico. The results were analysed to determine the impact of different residues at each position of the peptide chain. The most promising amino acids are reported below.
Ligands bearing an Asp residue adjacent to the αD-site fragment were consistently predicted to bind in one of two modes. The first suggested a HBA interaction between the Asp residue and His160, while the second showed a HBA contact to Lys122 (Figure 60, a and b respectively). Both binding modes were predicted to secure the ligand at the mouth of the αD pocket and position the peptide chain towards the substrate-binding channel. Of all residues examined, an Asp at this position was best.

Next, the central position of the tripeptide chain was considered. An Ala residue at this position was observed to efficiently fill space along the substrate channel, but was not predicted to make any polar contacts to the protein. Substituting for a Lys showed a potential HBD interaction to Glu230, while Gln might contact Lys122 as a HBA (Figure 61, a and b respectively). Finally, a Trp residue in the middle of the chain was predicted to occupy a shallow pocket within the substrate-binding channel, but no polar or π-stacking interactions could be identified (Figure 61, c).
The C-terminal position of the tripeptide was interrogated using amino acids bearing carboxylate and amide sidechains. The two best results were Asp and Glu residues, which were predicted to contact Lys158/Ser194 and Lys158/His160 respectively (Figure 62, a and b respectively). Substituting Glu for Asn maintained the two contacts to Lys158 and His160 (Figure 62, c). It was hypothesised that the electrostatic interactions between the terminal amino acid and substrate-binding site would anchor the peptide within the channel.
In summary, from the computational modelling it was learnt that:

1. An Asp residue directly adjacent to the αD-site fragment looked most promising for orienting the peptide chain towards the substrate-channel.
2. An Ala, Lys, Gln or Trp residue at the middle position of the tripeptide could be beneficial for binding.
3. An Asp, Glu or Asn residue at the C-terminus might hold the chain within the channel by polar interactions.

Based on these guidelines, six tripeptides (97 – 102) hosting combinations of the above residues were synthesised (Table 16). The first two species, DAD 97 and DKD 98, were prepared by microwave Fmoc-SPPS without complication and in moderate yields. Ligands DKE 99 and DQD 100 were also prepared using microwave irradiation but a substantial by-product was observed in both cases (99a and 100a respectively). Isolation and characterisation of the by-product revealed fragment scaffold 103. This species was believed to form during the TFA peptide deprotection and resin cleavage step, performed under microwave irradiation for 30 minutes. To prevent future by-product formation, the microwave reactor was no longer used for this step and instead the cleavage cocktail was left to shake at room temperature. Pleasingly, scaffold 103 was not observed in any of the following reactions. Fifth tripeptide DKN 101 was synthesised in better yield but alternative by-product 101a was isolated. Characterisation of this minor species provided a structure consistent with fragment scaffold 104 which could form during the overnight coupling of carboxylate fragment 78. Future synthesis reduced fragment 78 coupling time to 3.5 minutes and removed DIPEA from the reaction mixture. Following
these changes, scaffold 104 was not detected again. Final ligand DWD 102 was synthesised by manual Fmoc-SPPS. Analysis of the crude reaction mixture by analytical HPLC showed a much cleaner trace than for earlier tripeptides 97 – 101. Furthermore, the desired product was isolated in good yield. At this point, it was decided that all future short peptides would be prepared by manual Fmoc-SPPS.

Table 16: Fmoc-SPPS of tripeptide library 97 – 102 and isolation of by-product species 99a, 100a and 101a.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Fmoc-SPPS method</th>
<th>Yield (%)</th>
<th>By-product</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>78-DAD-OH</td>
<td>Microwave</td>
<td>15</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>98</td>
<td>78-DKD-NH₂</td>
<td>Microwave</td>
<td>30</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>99</td>
<td>78-DKE-OH</td>
<td>Microwave</td>
<td>16</td>
<td>103-DKE-OH (99a)</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>78-DQD-OH</td>
<td>Microwave</td>
<td>12</td>
<td>103-DQD-OH (100a)</td>
<td>10</td>
</tr>
<tr>
<td>101</td>
<td>78-DKN-OH</td>
<td>Microwave</td>
<td>39</td>
<td>104-DKN-OH (101a)</td>
<td>4</td>
</tr>
<tr>
<td>102</td>
<td>78-DWD-OH</td>
<td>Manual</td>
<td>21</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

The six tripeptides 97 – 102 were submitted to Dr. Paul Brear for X-ray crystallography. Two fragments, DKE 99 and DQD 100, did not bind to CK2α, but the remaining four fragments were observed to bind selectively in the αD pocket. The fragments overlaid well with carboxylate fragment 78 and the ring A interactions with Pro159, Val162 and the αD pocket water were maintained (Figure 63, a). In all four cases, 97, 98, 101 and 102, the Asp residue adjacent to the αD-site fragment was well resolved and showed consistent positioning (Figure 63, b). A HBA interaction with His160 and a water molecule was detected. However, the orientation of the Asp residue differed to the computational modelling (Figure 60). As a result, the tripeptide chains of fragments 97, 98, 101 and 102 projected away from the substrate-binding channel and towards the αD helix, as observed for earlier AAD tripeptide 94 (Figure 59, d).
Figure 63: X-ray crystallography results for tripeptide linker species in complex with CK2α (grey). a) Overlay of carboxylate 78 (blue), DAD derivative 97 (yellow), DKD derivative 98 (purple) and DKN derivative 101 (orange) to show relative positioning within the αD pocket. b) Overlay of first Asp residue for DAD derivative 97 (yellow), DKD derivative 98 (purple) and DKN derivative 101 (orange) to show consistent position at the mouth of the αD pocket. c) DAD derivative 97 (yellow). d) DKD derivative 98 (purple). e) DKN derivative 101 (orange). Polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.

For DAD fragment 97, the Ala sidechain was observed to fill space at the protein surface and the backbone contacted a water molecule (Figure 63, c). The terminal Asp made a HBA interaction with water but no contacts to the protein were detected. DKD derivative 98 showed greater interaction with the protein (Figure 63, d). The Lys sidechain contacted Tyr125 as a HBD and the backbone contacted Glu230. The C-terminus was not observed to interact with the protein or solvent. Fragment DKN 101 lost the Lys-Tyr125 interaction but instead the C-terminal acid group formed a HBA interaction with Lys122 (Figure 63, e). Final fragment DWD 102 could not be resolved past the first Asp residue.

Overall, tripeptide library 97 – 102 did not bind to CK2α as predicted. Rational structure-based peptide design was not sufficient to influence the binding mode towards the substrate-binding channel as
hoped. Analysis of the X-ray crystal structures for 97, 98 and 101 showed that H-bonding to His160 held the linker in the wrong orientation for substrate channel binding. HBA interactions with Lys122 on the αD helix may have also prevented the peptide chain from adopting the desired binding position.

2.3.4.2.3 Alternative Peptide Linkers

In an effort to achieve the goal of directing the peptide linker towards the substrate-binding channel, it was hypothesised that disrupting the aforementioned Asp-His160 interaction, at the mouth of the αD pocket, could be sufficient to change the binding mode. From this, three new strategies were considered:

1. Substitution of the first Asp residue by a moiety with greater structural rigidity and no H-bonding ability.
2. Introduction of greater ligand flexibility within the mouth of the αD pocket.
3. Maintain the current Asp-His160 interaction but change the chirality of the Asp residue.

In each case, the theory was tested by design and in silico screening of relevant peptides. Promising candidates were synthesised by manual Fmoc-SPPS and submitted for X-ray crystallography. The results from each investigation are reported herein.

Investigation 1: Rigidifying the peptide chain

It was surmised that rigidifying the peptide chain at the opening of the αD pocket might lock the linker in a conformation towards the substrate-binding channel. The residue directly adjacent to αD fragment 78 was in the most appropriate position to achieve this aim, and thus Asp was substituted for Pro. Two tetrapeptides, 78-PGGD (105) and 78-PGGN (106), were designed and modelled using the Schrödinger package. The software predicted both species to lie within the substrate-binding channel and maintain the optimised αD pocket binding pose (Figure 64).
Figure 64: Computational modelling results for investigation into rigidifying the peptide chain. 

- **a)** PGGD derivative 105 (magenta).
- **b)** PGGN derivative 106 (brown).

Predicted polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.

The Pro residue at the αD pocket opening was predicted to direct the peptide chain down the substrate channel (Figure 64). H-bonding interactions involving the C-terminal Asp/Asn then anchored the linker to the protein surface. The Asp of PGGD derivative 105 was predicted to make HBA contacts with Ser194 and the backbone of Arg195 (Figure 64, a). The Asn of PGGN species 106 was shown to interact with Ser194, Tyr196 and the backbone of Arg195 (Figure 64, b).

Following modelling, tetramers 105 and 106 were synthesised by manual Fmoc-SPPS (Table 17). No by-products were observed by LCMS or HPLC analysis of the crude reaction mixtures and both products were isolated in moderate yields.

**Table 17: Manual Fmoc-SPPS and X-ray crystallography results for tetramers 105 and 106 to investigate rigidifying the peptide chain.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Yield (%)</th>
<th>X-ray cryst. results</th>
</tr>
</thead>
<tbody>
<tr>
<td>105</td>
<td>78-PGGD-OH</td>
<td>30</td>
<td>d.b.</td>
</tr>
<tr>
<td>106</td>
<td>78-PGGN-OH</td>
<td>18</td>
<td>d.b.</td>
</tr>
</tbody>
</table>

d.b.: did not bind to CK2α
X-ray crystallography for tetramer species 105 and 106 in complex with CK2α was conducted, but unfortunately no electron density for either ligand was observed. From this it was concluded that Pro residue substitution may not be the solution and was not considered further.

**Investigation 2: Increasing the flexibility of the peptide chain**

Structural data for the tripeptides suggested that the amide bond linking αD-site fragment 78 to the tripeptide chain locked the angle at which the chain exited the αD pocket. Amide bonds are known to reduce free rotation and thus it was hypothesised that the optimal binding mode may be prevented due to lack of freedom. To test this, we envisaged linking a tripeptide sequence to aldehyde fragment 63 via reductive amination. The resulting ligand would then have a flexible benzylamine moiety at the opening of the αD pocket.

To this end, species 107 bearing the optimised αD-site biaryl core and a DAD tripeptide sequence, was designed and docked onto CK2α (Figure 65). Computational modelling predicted the peptide would lie in the substrate-binding channel and ring A interactions would be maintained. The first Asp was expected to contact His160, as previously observed, and the C-terminal Asp was predicted to interact with Lys158 and Ser194.

![Figure 65: Computational modelling result for investigation into increasing flexibility of the peptide chain. Reductive amination product 107 (pink) is shown on CK2α (grey). Predicted polar interactions between the fragment, protein residues (white) and water molecules are highlighted by yellow dashed lines.](image)

Synthesis of species 107 by literature Fmoc-SPPS reductive amination provided the desired test species in poor yield, but sufficient quantity for X-ray crystallography (Scheme 17). Inspection of the crystallography data showed clear electron density for the αD-site portion of derivative 107, but the peptide chain could not be resolved. This investigation was terminated here.
Investigation 3: Changing the chirality of the peptide chain

The final strategy was to change the chirality of the first peptide residue from an L-Asp to a D-Asp. It was envisaged that this would maintain the Asp-His160 interaction but the peptide chain would be forced to change direction. In order to test this theory, tripeptides bearing a D-Asp adjacent to the αD-site fragment were modelled onto CK2α. Two peptides, dAD 108 and dKD 109, provided the best predicted results (Figure 66).

In both cases, the ring A and Asp-His160 H-bonding interactions were maintained (Figure 66). The remaining two residues of the tripeptide chain were also predicted to bind along the substrate channel. For dAD species 108, the Ala residue filled space and the C-terminal Asp made two HBA interactions with Lys158 and the backbone of His160 (Figure 66, a). dKD derivative 109 was not
predicted to make any electrostatic interactions other than Asp-His160, but the peptide chain was accommodated neatly within the substrate channel, thus this ligand was considered for synthesis (Figure 66, b).

Manual Fmoc-SPPS of D-Asp fragments 108 and 109 proceeded without complication to provide the desired linkers in moderate yields (Table 18). The samples were then submitted for structural determination by X-ray crystallography.

Table 18: Manual Fmoc-SPPS of linkers 108 and 109 to investigate changing amino acid chirality.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>108</td>
<td>78-dAD-OH</td>
<td>23</td>
</tr>
<tr>
<td>109</td>
<td>78-dKD-OH</td>
<td>22</td>
</tr>
</tbody>
</table>

where "d" denotes D-Aspartic acid

To our delight, X-ray crystallography for both tripeptides 108 and 109 gave binding modes within the substrate-binding channel (Figure 67). Both ligands were observed to bind selectively within the αD pocket and the ring A position was maintained. In further detail, dAD species 108 was seen to bind in two modes, both of which projected along the substrate-binding channel (Figure 67, a). In one mode, the D-Asp interaction with His160 was maintained, but in the second it was lost. In both modes, the Ala residue filled space and the C-terminal Asp contacted Lys158 and the backbones of His160 and Tyr196. The tripeptide backbone was observed to interact with two water molecules and Glu230. The second ligand, dKD 109, was found to bind CK2α with one binding mode (Figure 67, b). The D-Asp was not reported to interact with His160. The Lys residue in the middle of the tripeptide chain was not shown to contact the protein either, but the C-terminal Asp interacted with Ser194 and the backbone of His160. Two water molecules and Glu230 were reported to contact the tripeptide backbone in a similar manner to dAD species 108.
In order to show ligands 108 and 109 protruding from the αD pocket, and projecting towards the substrate-binding channel, the protein surface was laid over the X-ray crystal structures (Figure 67, c and d respectively). The αD helix is shown as a cartoon so that the pocket opening is better revealed. In both cases, the tripeptide chain lies on the surface of the protein and within the narrow groove at the terminus of the substrate-binding channel.

Following the successful X-ray crystallography, D-Asp fragments 108 and 109 were submitted for biochemical assessment by kinase inhibition assay (Table 19). Pleasingly, dAD linker 108 performed well, reporting 96% kinase activity inhibition. Within statistical error, this was comparable to αD-site lead fragment 67 which inhibited activity by 95%. Additionally, an IC$_{50}$ value of 23 μM was recorded for linker species 108, which is weaker, but still comparable, to lead 67 (9 μM IC$_{50}$). Unfortunately, dKD
performed poorly in the assay with only 9% activity inhibition, and as a result this linker was not considered further.

Table 19: Biochemical assessment of D-Asp linkers 108 and 109 and comparison to lead benzonitrile fragment 67.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>% inhib. @ 100 (µM) ± SEM[a][b]</th>
<th>IC50 ± SEM (µM)[a][b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td><img src="Image" alt="Structure" /></td>
<td>95 ± 6</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>108</td>
<td>78-dAD-OH</td>
<td>96 ± 1</td>
<td>23 ± 15</td>
</tr>
<tr>
<td>109</td>
<td>78-dKD-OH</td>
<td>9 ± 6</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

[a] Measured by CK2α ADP-glc kinase assay. [b] Tests carried out in triplicate and reported as the mean. [c] Tests carried out in duplicate and reported as the mean. n.d.: no data

In summary, three alternative strategies for linker development were investigated. The first two strategies focussed on linker rigidity and linker flexibility respectively. However, despite promising computational data no X-ray crystal structures could be resolved for these species. The third strategy substituted an L-Asp for a D-Asp within the tripeptide linker. Two ligands, dAD 108 and dKD 109, were synthesised and showed the desired binding mode in X-ray crystallography. To the best of our knowledge, this is the first example of a fully resolved X-ray crystal structure of CK2α with a small molecule co-crystallised in the substrate-binding channel. Subsequent biochemical assessment of the D-Asp species showed dAD derivative 108 as far superior to dKD 109, and comparable to previous lead fragment, benzonitrile 67.

To conclude, an αD-site anchored tripeptide was successfully designed, synthesised and tested. Ligand 108, bearing the sequence dAD, displayed strongest electron density along the substrate-binding channel and performed best in the CK2α inhibition assay. Following this result, the project moved on to the final step: fragment linking to hexamer peptide 75.

2.3.5 Fragment Linking

With dAD tripeptide 108 in hand, project focus turned towards joining this fragment to hexamer 75. To assess the feasibility of this aim, modelled hexamer 75 was overlaid with the X-ray crystal structure of tripeptide 108 (Figure 68). CK2α residues that are crucial for substrate recognition were inspected and pleasingly multiple electrostatic contacts between the protein and peptide 75 were predicted. This confirmed that the binding of ligand 108 to CK2α did not significantly disrupt the conformation of the substrate-binding channel.
In detail, the \( n-2 \) Asp was expected to contact Arg195 and the \( n-1 \) Asp was expected to interact with the backbone of Lys49. The Asp at \( n+1 \) was within range to contact Arg191 and Lys198, which matched reports in the literature.\(^{142,143}\) The two C-terminal Asp residues were not predicted to contact the protein, but this is due to the Lys74-Lys76 Ala mutant used for crystallography.

![Figure 68: Overlay of modelled hexamer 75 (green) and dAD linker 108 (blue) protein crystal structure (grey) with ADP (yellow), phosphate (orange) and Mg\(^{2+}\) (purple) in the ATP-site. CK2\(\alpha\) residues involved with substrate recognition are highlighted (white) and predicted polar contacts are shown as dashed yellow lines. This protein is a K74-K76 alanine mutant. The relative positioning of dAD linker 108 and the N-terminal tail of hexamer 75 (red circle) implied that directly linking the two species may be possible.](image)

Visual inspection of the C-terminal Asp of tripeptide 108 relative to the N-terminal Asp of hexamer 75, showed significant overlap of these two moieties (Figure 68). This suggested that direct linkage of these two species \( \text{via} \) an amide bond may provide the chemical probe desired in the project aim (Section 2.3.2). This proposed ligand would be nine amino acids long with an \( \alpha \)D anchoring fragment at the N-terminus. The peptide sequence would be: dADDDSDDD.

To this end, automated Fmoc-SPPS using a Liberty Blue Peptide Synthesiser was performed to provide proposed chemical probe 110 in moderate yield (Table 20). 3,5-Dichloro carboxylate 74 was also available, so synthesis was repeated using this \( \alpha \)D-site fragment to give second probe 111 also in moderate yield.
Following synthesis of probes 110 and 111, X-ray crystallography was performed to determine the binding modes. Monochloro derivative 110 was observed to bind selectively within the αD pocket with good electron density, but the peptide chain could not be resolved (Table 20). Unfortunately, no electron density was detected at any site on CK2α for 3,5-dichloro derivative 111.

In conclusion, a tripeptide linker with αD-site anchor (108) was successfully designed and synthesised. Overlay of this structure with hexamer peptide 75, derived from molecular dynamics, showed potential for the two species to be directly linked via an amide bond. Synthesis of two chemical probes, 110 and 111, was performed without complication, however X-ray crystallography failed to accurately reveal the binding modes for the two species. Further work is currently ongoing in the Spring/Hyvönen groups to investigate this issue.

Table 20: Automated Fmoc-SPPS and X-ray crystallography results for chemical probes 110 and 111.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Yield (%)</th>
<th>αD fragment resolved?</th>
<th>Peptide chain resolved?</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>78-dADDSSDDD-OH</td>
<td>13</td>
<td>Y</td>
<td>N</td>
</tr>
</tbody>
</table>

d: D-Aspartic acid. Y: yes, electron density strong enough to resolve accurately. N: no, electron density too weak to resolve accurately. n.d.: no density observed
Chapter Three: Conclusions and Future Work

3.1 Conclusions

The project reported in this thesis was a continuation of the work published by Brear et al. on the αD pocket of protein kinase CK2.\textsuperscript{265–267} It was based on the elaboration of Brear et al.’s lead fragment \textbf{34} at the 3-position of ring A, in order to investigate the flexibility of the αD pocket.

A compound library of biaryl, benzylamine fragments was designed and screened \textit{in silico} against CK2α, using the X-ray crystal structure for lead species \textbf{34} (Figure 69, a). Docking results were analysed, and fragments predicted to maintain the binding pose of lead \textbf{34}, while efficiently occupying the pocket space adjacent to the 3-position of ring A, were considered for synthesis. A robust and modular synthetic route to access the benzylamine scaffold was determined, and a target-oriented fragment library consisting 11 members was synthesised. X-ray crystallography revealed seven fragments bound selectively in the αD pocket, and of these, three fragments (\textbf{55}, \textbf{56} and \textbf{59}) were observed to maintain the binding mode of lead benzylamine \textbf{34}. Biochemical assessment reported poor binding affinities for these fragments ($K_d >750 \mu M$) and no activity inhibition in the CK2α kinase assay. The remaining four fragments bound in the αD pocket via two unpredicted, and previously unreported, binding modes. Dibenzylamine derivative \textbf{44} created a new, solvent-exposed αD pocket opening, but had poor binding affinity ($K_d >750 \mu M$) and was not observed to inhibit kinase activity. The final three fragments (\textbf{46}, \textbf{57} and \textbf{58}) adopted a binding mode perpendicular to Brear et al.’s lead \textbf{34}, and forced a pocket opening into the substrate-binding channel (Figure 69, b). Derivatives \textbf{46}, \textbf{57} and \textbf{58} were reported to inhibit CK2α kinase activity (19, 28 and 32% respectively) despite the weak binding affinities (>750, >750 and 750 $\mu M$ respectively). Analysis of the new binding mode revealed crucial rearrangement of three protein residues (Phe121, Tyr125 and Glu230) relative to Brear et al.’s published binding pose.

Trifluoro derivative \textbf{58} was the most potent of the fragments adopting the new binding mode, and thus provided a hit species for elaboration into more effective CK2α inhibitors. Subsequent work modified ring A at the 6-position to introduce a H-bonding group (Figure 69, c). X-ray crystallography of alcohol derivative \textbf{61} revealed interactions between the alcohol moiety, Pro159 and an αD pocket water molecule. Improved CK2α inhibition relative to hit \textbf{58} was reported (44% compared to 32%) and an $IC_{50}$ value of 100 $\mu M$ was recorded. Next, the amine head group was substituted for less polar moieties, which led to the development of benzonitrile \textbf{61a} (Figure 69, c). Structural analysis by X-ray crystallography showed ring A interactions were maintained and that polar contacts involving the
Figure 69: Project overview. a) Brear et al. lead fragment 34 (cyan) bound in the αD pocket of CK2α (grey) (PDB: 5CSH). b) Hit fragment 58 (yellow) bound in the αD pocket (grey) via a novel binding mode. c) Hit-to-lead generation of 3,5-dichloro fragment 67 from hit 58 and optimised linker species 108 with biochemical data. d) Lead fragment 67 (brown) bound in the αD pocket (grey). e) Anchored linker species 108 (blue) in complex with CK2α (grey), showing both reported binding modes. Polar interactions between the fragment, protein residues (white) and water molecules are showed as dashed yellow lines.
nitrile of 61a were reduced relative to the amine of 61. Following biochemical assessment, benzonitrile 61a was observed to inhibit kinase activity by 80% and provided an IC$_{50}$ value of 51 μM – an improvement on prior benzylamine 61 in both cases. A GI$_{50}$ value of 69 μM was also determined. Finally, the substitution pattern of ring B was optimised in order to better fill this portion of the binding pocket. A library of eight fragments was synthesised and 3,5-dichloro derivative 67 performed best in the kinase assay (95% relative to 80% for benzonitrile 61a) (Figure 69, c). Analysis of the X-ray crystal structure for 3,5-dichloro fragment 67 revealed that the ring A and nitrile binding positions were maintained and that the additional chlorine occupied a previously vacant space towards the front of the pocket (Figure 69, d). Relative to benzonitrile 61a, superior IC$_{50}$ and GI$_{50}$ values of 9 and 16 μM were recorded for 3,5-dichloro species 67. Furthermore, this GI$_{50}$ activity level was comparable to that of Brear et al.’s best lead, pro-CAM4066 37, which was reported as 9 μM.265 The SBDD programme thus resulted in the discovery of lead fragment 3,5-dichloro benzonitrile 67.

Following development of optimised lead benzonitrile inhibitor 67, three mechanisms by which it could induce negative allosteric control over CK2α were presented. These included disruption of the ATP- and substrate-binding sites and inhibition of the transition between the open and closed forms of CK2α. Analysis of overlaid X-ray crystal structures and literature precedent provided grounding for each hypothesis, but confirmation of these theories was beyond the scope of this project.

To the best of our knowledge, lead fragment 3,5-dichloro 67 represented a first-in-class allosteric inhibitor, binding selectively in the αD pocket of CK2. Furthermore, this was the first observation of fragments binding in the αD pocket and adopting this binding mode. An αD pocket opening towards the substrate-binding channel had not previously been reported in the literature, and presented an exploitable exit vector for investigation of the substrate-binding site. To this end, a linker strategy was envisaged, in which optimised αD-site fragment 67 could be linked to CK2-selective pseudosubstrate 75. It was postulated that the αD-site portion of the molecule would anchor the species to the protein and enable X-ray crystallography of the pseudosubstrate in complex with the substrate-binding channel.

To begin linker development, the nitrile head groups of lead fragment 67 and earlier derivative 61a were exchanged for lithium carboxylate groups. A variety of tri- and tetra-peptide linkers with αD-site fragment 61a anchors were then designed, synthesised and tested by X-ray crystallography. Analysis of the structural data revealed that the peptidic portions of these molecules did not lie within the substrate-binding channel as hoped. Instead, they formed H-bonding interactions with Lys122 on the αD helix, or were disordered within the solvent and thus unresolved. Introduction of an L-Asp residue to the peptide chain led to the development of tripeptide linker species dAD 108 which crystallised in
the αD pocket and substrate-binding channel as desired (Figure 69, e). Biochemical data comparable to lead benzonitrile fragment 67 was collected for dAD derivative 108, with 96% kinase inhibition and an IC₅₀ of 23 μM. Amide bond fragment linking between anchored dAD linker 108 and hexamer 75 was performed to provide final chemical probe 110. This species was observed to bind the αD pocket of CK2α, however the peptide chain was disordered and could not be resolved.

In conclusion, this project validated the use of FBDD against protein kinase CK2 in the search for a novel binding pocket and allosteric mode of action. A first in class negative allosteric inhibitor, fragment 67, binding in the αD pocket of protein kinase CK2α was developed. Lead fragment 67 was then elaborated into anchored tripeptide 108 which provided the first fully resolved X-ray crystal structure of CK2α with a small molecule accommodated in the substrate-binding site. This work could be used towards the future development of αD-site selective inhibitor fragments, and provides a starting point for further investigation of the substrate-binding channel.

3.2 Future Work

The future work for this project can be divided into four areas: the binding mode, the negative allosteric mode of action, further development of the αD-site fragment and the substrate-binding channel probe.

With respect to the novel binding mode, the future work would be to better understand the factors promoting this fragment pose over that reported by Brear et al. Since numerous X-ray crystal structures for both binding modes are available, molecular dynamics simulations could be performed in order to determine the contribution of the trifluoro group towards binding.

The negative allosteric mode of action for lead fragment 67 is yet to be fully investigated. Three mechanisms for the proposed allosteric inhibition were presented, but the hypotheses were not experimentally verified. This work would require CK2α residue mutation studies to ascertain the impact of lead fragment 67 on the ATP- and substrate-binding sites. A study into the ability of CK2α to recognise different substrates would also be necessary in order to analyse the predicted disruption to the substrate-binding channel. The effect of fragment 67 on the transition between the open and closed forms of CK2α would require molecular dynamics simulations and NMR studies of labelled CK2α. An improved understanding of the mechanism of action would aid future development of αD-site inhibitors.

When considering lead benzonitrile 67, an additional round of optimisation could be performed. This would focus on interrogating the flexibility of the αD pocket around the trifluoro group by substitution
with bulkier moieties (Figure 70). As reported previously, the αD helix has been observed to move by as much as 23.8 Å from its position in the closed Apo protein,\textsuperscript{266} and thus fragments larger than lead 67 might be accommodated. If fragments are able to bind deeper within the αD pocket, then they may display enhanced potency. The X-ray crystal structure of lead benzonitrile 67 in complex with CK2α could be used as a starting point for the design of new fragments.

![Chemical structures](image)

Figure 70: Future work to investigate the flexibility of the αD pocket around the trifluoro group of lead fragment 67. Analysis of the X-ray crystal structure of fragment 67 in complex with CK2α showed the pocket is lined by hydrophobic residues, thus non-polar substituents would be appropriate for testing.

Finally, the linking strategy adopted to investigate the substrate-binding channel did not return an effective chemical probe. Molecular dynamics simulations could be performed using αD-site anchored tripeptide 108 and pseudosubstrate 75 in order to better understand the relative binding positions of these two species. From this, a more informative decision towards linking the two molecules could be made.
Chapter Four: Experimental

4.1 General Experimental

Toluene, hexane, diethyl ether, ethyl acetate, methanol, THF and dichloromethane were dried and distilled using standard methods. All reagents and other solvents were purchased at the highest commercial quality and used without further purification. Reactions were carried out under an atmosphere of nitrogen and in oven-dried glassware unless otherwise stated. All reactions were monitored by TLC, LCMS and $^1$H NMR spectra taken from the reaction.

Room temperature (r.t.) refers to ambient temperature. All temperatures below 0 °C are that of the external bath. Temperatures of 0 °C were maintained using an ice-water bath.

Microwave irradiation was performed in a Biotage® microwave reactor.

Analytical thin layer chromatography (TLC) was performed using pre-coated Merck glass backed silica gel plates (Silicagel 60 F254). Flash column chromatography was undertaken on Fluka or Material Harvest silica gel (230–400 mesh) under a positive pressure of nitrogen unless otherwise stated. Visualization was achieved using ultraviolet light (254 nm) and chemical staining with basic potassium permanganate solution or ninhydrin as appropriate. Retention factors ($R_f$) are quoted to 0.01.

Liquid chromatography-mass spectra (LCMS) were measured on a Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode ionization Waters SQ Detector 2 spectrometer (LC system: solvent A: 2mM ammonium acetate in water/acetonitrile (95:5); solvent B: 100% acetonitrile; column: AQUITY UPLC CSH C18, 2.1 x 50 mm, 1.7 µm, 130 Å; gradient: 5-95% B over 3 min with constant 0.1% formic acid). Retention times ($t_r$) are quoted to 0.01 min.

Analytical high pressure liquid chromatography (HPLC) was performed on an Agilent 1260 Infinity system fitted with a Supelcosil ABZ+Plus column (150 mm x 4.6 mm, 3 µm) using linear gradient systems (solvent A: 0.05% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in acetonitrile) over 15 min at a flow rate of 1 mL min$^{-1}$ and UV detection ($\lambda_{max} = 220$ nm and 254 nm). Retention times ($t_r$) are quoted to 0.01 min.
Semi-preparative HPLC purification was performed on an Agilent 1260 Infinity system fitted with a Supelcosil ABZ+Plus column (250 mm x 21.2 mm, 5 µm) using linear gradient systems (solvent A: 0.1% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in acetonitrile) over 20 min at a flow rate of 20 mL min\(^{-1}\) and UV detection (\(\lambda_{\text{max}} = 220\) nm and 254 nm).

Melting points were obtained on a Buchi B-545 melting point apparatus and are uncorrected.

Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum One FT-IR Spectrometer fitted with an ATR sampling accessory as either solids or neat films, either through direct application or deposited in CDCl\(_3\). Selected absorptions are reported in wavenumbers (cm\(^{-1}\)) with the following abbreviations: w, weak; m, medium; s, strong; br, broad.

Magnetic resonance spectra were processed using MestReNova v. 6.0.2-5475 or TopSpin v. 3.5 (Bruker). An aryl, quaternary, or two or more possible assignments were given when signals could not be distinguished by any means. Measured coupling constants are reported uncorrected or as a multiplet when coupling constant cannot be determined.

Proton magnetic resonance spectra were recorded using an internal deuterium lock (at 298 K unless stated otherwise) on Bruker DPX (400 MHz; 1H-13C DUL probe), Bruker Avance III HD (400 MHz; Smart probe), Bruker Avance III HD (500 MHz; Smart probe) and Bruker Avance III HD (500 MHz; DCH Cryoprobe) spectrometers. Proton assignments are supported by \(^1\)H-\(^1\)H COSY, \(^1\)H-\(^{13}\)C HSQC or \(^1\)H-\(^{13}\)C HMBC spectra, or by analogy. Chemical shifts (\(\delta\)) are quoted in ppm to the nearest 0.01 ppm and are referenced to the residual non-deuterated solvent peak. Discernible coupling constants for mutually coupled protons are reported as measured values in Hertz, rounded to the nearest 0.1 Hz. Data are reported as: chemical shift, number of nuclei, multiplicity (br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; or a combination thereof), coupling constants and assignment. Diastereotopic protons are assigned as \(X\) and \(X'\), where \(X'\) designates the lower-field proton.

Carbon magnetic resonance spectra were recorded using an internal deuterium lock (at 298 K unless stated otherwise) on Bruker DPX (101 MHz), Bruker Avance III HD (101 MHz) and Bruker Avance III HD (126 MHz) spectrometers with broadband proton decoupling. Carbon spectra assignments are supported by DEPT editing, \(^1\)H-\(^{13}\)C HSQC or \(^1\)H-\(^{13}\)C HMBC spectra, or by analogy. Chemical shifts (\(\delta\)) are quoted in ppm to the nearest 0.1 ppm and are referenced to the deuterated solvent peak. Data are reported as: chemical shift, number of nuclei (if not one), multiplicity (if not a singlet), coupling constants and assignment.
For F-containing compounds, the presence of F substituent(s) was confirmed from splitting patterns in $^{13}$C NMR spectra.

The numbering of molecules used for $^{13}$C and $^1$H NMR assignments does not conform to IUPAC standards.

High-resolution mass spectra (HRMS) were measured on a Micromass Q-TOF or a Waters LCT Premier Mass Spectrometer using electrospray ionisation [ESI]. Masses are quoted within the 5ppm error limit.

4.2 General Methods

4.2.1 General Method A: Phenol triflation

To a solution of phenol (1.0 eq.) in anhydrous CH$_2$Cl$_2$ (~0.3 M) was added pyridine (3.0 eq.) or NEt$_3$ (2.25 eq.). The solution was cooled to 0 °C and trifluoromethanesulfonic anhydride (1.1 – 1.4 eq.) was added dropwise over 30 minutes. The reaction was allowed to warm to room temperature and stirred overnight. The volatiles were removed under reduced pressure and the residue was diluted with H$_2$O and extracted with EtOAc three times. The organic layer was washed with 10% aqueous HCl, 5% aqueous Na$_2$CO$_3$, a saturated aqueous solution of NaCl and H$_2$O, then dried over MgSO$_4$, filtered and concentrated in vacuo. The crude product was then purified by flash column chromatography to yield the desired product.

Conditions adapted from literature protocol.$^{259}$

4.2.2 General Method B: Suzuki coupling of unhindered substrates

The aryl bromide (1.10 eq.), appropriate boronic acid (1.00 eq.), PdCl$_2$(dppf).CH$_2$Cl$_2$ (0.05 eq.) and K$_3$PO$_4$ (1.20 eq.) were weighed into a microwave tube and solvated with DME (0.3 – 0.5 M), EtOH (1.3 – 1.5 M) and H$_2$O (2.1 – 2.3 M). The reaction mixture was degassed by bubbling nitrogen through the solution for 5 minutes and then heated to 110 °C under microwave irradiation for 1-2 hours, until completion by TLC monitoring. The reaction was allowed to cool to room temperature, filtered through celite washing with Et$_2$O and the solvent removed under reduced pressure. The residue was dissolved in Et$_2$O/H$_2$O and extracted three times with Et$_2$O. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over MgSO$_4$, filtered and concentrated in vacuo. The crude product was then purified by flash column chromatography to yield the desired product.
4.2.3 General Method C: Benzonitrile reduction

To a stirred suspension of LiAlH₄ (2.0 – 4.0 eq.) in Et₂O (~0.25 M) was added AlCl₃ (2.0 – 4.0 eq.) and the reaction mixture cooled to 0 °C for 10 minutes. The reaction was allowed to warm to room temperature and the nitrile (1.0 eq.) was added portionwise. The reaction was stirred at room temperature for 30 minutes and then heated at 50 °C overnight. After cooling to room temperature, a saturated aqueous solution of potassium sodium tartrate tetrahydrate and Et₂O were added and the mixture stirred for 1 hour. The reaction mixture was diluted with 2.0 M aqueous Na₂CO₃ and extracted three times with Et₂O. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over MgSO₄, filtered and concentrated in vacuo. The crude product was then purified by flash column chromatography or semi-preparative HPLC, as determined necessary by ¹H NMR and LCMS analysis of the crude reaction mixture, to yield the desired product.

Conditions adapted from literature protocol.²⁶¹

4.2.4 General Method D: Suzuki coupling of hindered substrates

The aryl bromide (1.0 eq.), appropriate boronic acid (1.2 eq.), Pd(OAc)₂ (0.05 eq.), S-Phos (0.10 eq.) and K₃PO₄ (2.00 eq.) were weighed into an oven-dried RBF and solvated with toluene (0.5 M) and H₂O (3.5 M). The reaction mixture was degassed and back-filled with nitrogen before heating at 60 °C for 18 hours. The reaction was allowed to cool to room temperature, filtered through celite washing with Et₂O and the solvent removed under reduced pressure. The residue was dissolved in Et₂O/H₂O and extracted three times with Et₂O. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over MgSO₄, filtered and concentrated in vacuo. The crude product was then purified by flash column chromatography to yield the desired product.

Conditions adapted from literature protocol.²⁷²

4.2.5 Manual Fmoc Solid-Phase Peptide Synthesis (Fmoc-SPPS)

Peptides were synthesised using Rink amide MBHA resin (loading: 0.40 mmol/g), pre-loaded Asp Wang resin (loading: 0.70 mmol/g), pre-loaded Glu Wang resin (loading: 0.65 mmol/g) or pre-loaded Asn Wang resin (loading: 0.58 mmol/g) and N-terminal capping was performed with lithium carboxylate ⁷⁸. The side-chain protecting groups used for the different amino acids were as follows: 'Bu for
aspartic acid, glutamic acid and serine; Boc for lysine and tryptophan; and Trt for asparagine and glutamine.

Reactions were conducted in disposable 12 mL syringes with 20 μm frits on a Vac-Man Laboratory Vacuum Manifold.

Resin swelling was carried out with DMF (10 mL) for 30 minutes and the resin was then drained and rinsed with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH₂Cl₂ (3 x 6 mL).

Fmoc deprotactions were performed with a solution of 6% (w/w) piperazine in DMF with 0.1 M HOBt additive (6 mL, 2 x 1 minute) with vigorous shaking and washing with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH₂Cl₂ (3 x 6 mL) after the second deprotection step.

Fmoc-protected amino acid couplings were performed by first pre-activating the fmoc amino acid (2 eq.) with HATU (2 eq.) in DMF (3 mL) for 5 minutes. This solution was added to the resin and shaken vigorously for 30 seconds before the addition of DIPEA (4 eq.) and vigorous shaking for 3 minutes. The resin was drained and rinsed with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH₂Cl₂ (3 x 6 mL).

Deprotection and coupling completion was monitored by the Chloranil test. A saturated solution of chloranil in toluene (50 μL) was mixed with acetaldehyde (200 μL) in a clean vial and a small portion of dry resin added. The vial was capped and shaken vigorously for 30 seconds. No discolouration of the beads indicated a completed coupling step whereas a blue discolouration of the beads indicated incomplete coupling. In the case of incomplete coupling the resin was subjected to a second round of coupling conditions.

N-terminal capping with lithium carboxylate 78 was performed following the same procedure as for fmoc-protected amino acid coupling except without the addition of DIPEA.

Peptide-resin cleavage and side-chain deprotection was carried out by treatment with a TFA/H₂O/TIPS 95:2.5:2.5 cleavage cocktail (10 mL) for 30 minutes. The mixture was filtered, the resin washed with MeCN and the solvent removed under a stream of nitrogen. The crude peptide residue was then triturated with ice-cold Et₂O before LCMS analysis and semi-preparative HPLC purification.

4.2.6 Microwave Fmoc Solid-Phase Peptide Synthesis (Fmoc-SPPS)

Peptides were synthesised using Rink amide MBHA resin (loading: 0.40 mmol/g), pre-loaded Asp Wang resin (loading: 0.70 mmol/g), pre-loaded Glu Wang resin (loading: 0.65 mmol/g) or pre-loaded Asn Wang resin (loading: 0.58 mmol/g) and N-terminal capping was performed with lithium carboxylate 78. The side-chain protecting groups used for the different amino acids were as follows: 'Bu for
aspartic acid, glutamic acid and serine; Boc for lysine and tryptophan; and Trt for asparagine and glutamine.

Reactions were conducted in 25 mL glass microwave vials using a Biotage® microwave reactor. The resin was transferred into disposable 12 mL syringes with 20 μm frits on a Vac-Man Laboratory Vacuum Manifold for drainage and washing steps.

Resin swelling was carried out with DMF (10 mL) for 30 minutes and the resin was then drained and rinsed with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH$_2$Cl$_2$ (3 x 6 mL).

Fmoc deprotections were performed with a solution of 6% (w/w) piperazine in DMF with 0.1 M HOBt additive (10 mL) for 3 minutes at 70 °C and 35 W followed by drainage and washing with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH$_2$Cl$_2$ (3 x 6 mL).

Fmoc-protected amino acid couplings were performed by first pre-activating the fmoc amino acid (4 eq.) with HATU (4 eq.) in DMF (6 mL) for 5 minutes. This solution was added to the resin along with DIPEA (12 eq.) and the microwave vial sealed rapidly. The microwave coupling reaction was performed for 5 minutes at 72 °C and 25 W. The resin was drained and rinsed with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH$_2$Cl$_2$ (3 x 6 mL).

Deprotection and coupling completion was monitored by the Chloranil test. A saturated solution of chloranil in toluene (50 μL) was mixed with acetaldehyde (200 μL) in a clean vial and a small portion of dry resin added. The vial was capped and shaken vigorously for 30 seconds. No discolouration of the beads indicated a completed coupling step whereas a blue discolouration of the beads indicated incomplete coupling. In the case of incomplete coupling the resin was subjected to a second round of coupling conditions.

N-terminal capping with lithium carboxylate 78 was not performed using the microwave reactor. Instead lithium carboxylate 78 (2 eq.) was pre-activated with HATU (2 eq.) in DMF (6 mL) for 5 minutes. This solution was added to the resin followed by DIPEA (4 eq.) and the reaction put on the shaker for 18 hours. The resin was drained and rinsed with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH$_2$Cl$_2$ (3 x 6 mL).

Peptide-resin cleavage and side-chain deprotection was carried out by treatment with a TFA/H$_2$O/TIPS 95:2.5:2.5 cleavage cocktail (10 mL) for 30 minutes at 40 °C and 15 W. The mixture was filtered, the resin washed with MeCN and the solvent removed under a stream of nitrogen. The crude peptide residue was then triturated with ice-cold Et$_2$O before LCMS analysis and semi-preparative HPLC purification.
Conditions adapted from literature protocol.\textsuperscript{291}

4.2.7 Automated Fmoc Solid-Phase Peptide Synthesis (Fmoc-SPPS)

Automated peptide synthesis was carried out on a CEM Liberty Blue Automated Microwave Peptide Synthesiser fitted with a HT-12 resin loader using Rink amide MBHA resin (loading: 0.40 mmol/g) or pre-loaded Asp Wang resin (loading: 0.70 mmol/g). All peptides were synthesised using Fmoc-protected amino acids in DMF (5 eq.), DIC in DMF (1.0 M, 5 eq.) and activator Oxyma Pure in DMF (1.0 M, 10 eq.). Amino acids were coupled for 2 min with microwave irradiation using 30 W power at 90 °C. Fmoc-deprotection was achieved by microwave heating with 20 W power at 90 °C with 6% (w/w) piperazine in DMF with 0.1 M HOBt additive for 1 min.

The side-chain protecting groups used for the different amino acids were as follows: ‘\textsuperscript{t}Bu for aspartic acid, glutamic acid and serine.

$N$-terminal capping with lithium carboxylate 74 or 78 was performed manually, as described in Section 4.2.5, except with shaking for 24 hours. The final deprotection and cleavage was performed manually, as described in Section 4.2.5. The crude peptide residue was then triturated with ice-cold Et$_2$O before LCMS analysis and semi-preparative HPLC purification.

4.3 Experimental Details for Target-Oriented Fragment Library

2'-Chloro-[1,1'-biphenyl]-3,4'-dicarbonitrile (44a)

\begin{center}
\includegraphics[width=0.2\textwidth]{image.png}
\end{center}

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-cyanophenylboronic acid (93 mg, 0.63 mmol), PdCl$_2$(dppf).CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_3$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 20:80 Et$_2$O:Hexane to 30:70 Et$_2$O:Hexane) to provide the title compound 44a as a white solid (113 mg, 0.47 mmol, 75%):
**Ry** 0.19 (2:3 Et₂O:Hexane); **m.p.** 153-154 °C; **IR** \( \nu_{\text{max}} \) 3064 (w, C-H), 2971 (w, C-H), 2232 (s, C≡N), 1579 (w, C=C), 1539 (w, C=C), 1493 (w, C=C); **H NMR** (500 MHz, CDCl₃) \( \delta \) 7.83 (1H, d, \( J = 2.5 \) Hz, H₇), 7.77 – 7.73 (2H, m, H₁₁/H₁₃), 7.70 – 7.76 (2H, m, H₃/H₉), 7.61 (1H, td, \( J = 7.5, 1.0 \) Hz, H₁₀), 7.45 (1H, d, \( J = 8.0 \) Hz, H₄); **C NMR** (125 MHz, CDCl₃) \( \delta \) 142.8 (C₅), 138.7 (C₈), 133.6 (C₇/C₉), 133.54 (C₆), 133.49 (C₇/C₉), 132.6 (C₁₃), 132.2 (C₁₁), 131.8 (C₄), 130.7 (C₃), 129.4 (C₁₀), 118.2 (C₁₄), 117.0 (C₁), 113.7 (C₁₂), 112.9 (C₁₂); **HRMS** (ESI) calcd for \([\text{C}_{14}\text{H}_{12}\text{N}_2\text{Cl} + \text{H}]^+\): 239.0376, found: 239.0381.

**2'-Chloro-[1,1'-biphenyl]-3,4'-diyl]dimethanamine TFA salt (44)**

Prepared by General Method C using LiAlH₄ (55 mg, 1.44 mmol), Et₂O (4.0 mL), AlCl₃ (59 mg, 1.44 mmol) and 44a (85 mg, 0.36 mmol). The crude amine was purified by semi-preparative HPLC (5-35% B) to provide title compound 44 as a yellow oil (78 mg, 0.16 mmol, 46%):

**HPLC** \( t_r = 6.65 \) mins (5-35% B); **IR** \( \nu_{\text{max}} \) 2988 (br s, NH₃⁺), 1667 (s, C=C), 1526 (m, C=C), 1345 (w, C=C); **H NMR** (500 MHz, d₆-DMSO) \( \delta \) 8.44 – 8.24 (6H, m, H₁₅/H₁₆), 7.73 (1H, d, \( J = 1.5 \) Hz, H₇), 7.55 – 7.50 (4H, m, H₃/H₁₀/H₁₁/H₁₃), 7.48 – 7.44 (2H, m, H₄/H₉), 4.15 – 4.07 (4H, m, H₁/H₁₄); **C NMR** (125 MHz, d₆-DMSO) \( \delta \) 139.3 (C₅), 138.4 (C₈), 135.7 (C₂), 134.6 (C₁₂), 131.6 (C₄), 131.3 (C₆), 130.4 (C₇), 129.7 (C₁₀), 129.4 (C₉), 128.6 (C₁₁/C₁₃), 128.5 (C₁₁/C₁₃), 128.2 (C₃), 42.2 (C₁₄), 41.4 (C₁); **HRMS** (ESI) calcd for \([\text{C}_{14}\text{H}_{12}\text{N}_2\text{Cl} + \text{H}]^+\): 247.1002, found: 247.1000.

**2-Chloro-3'-methyl-[1,1'-biphenyl]-4-carbonitrile (45a)**

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-methylphenylboronic acid (86 mg, 0.63 mmol), PdCl₂(dpff).CH₂Cl₂ (26 mg, 0.032 mmol), K₃PO₄ (161
mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H2O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, elution: 5:95 Et2O:Hexane) to provide the title compound 45a as a white solid (120 mg, 0.53 mmol, 84%):

RF 0.25 (1:19 Et2O:Hexane); m.p. 72–73 °C; IR \( \nu_{max} \) 3073 (w, C-H), 2971 (w, C-H), 2230 (m, C≡N), 1589 (w, C=C), 1539 (w, C=C), 1475 (m, C=C); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 7.78 (1H, d, \( J = 1.0 \) Hz, H7), 7.61 (1H, dd, \( J = 10.0, 1.0 \) Hz, H3), 7.45 (1H, d, \( J = 10.0 \) Hz, H4), 7.37 (1H, t, \( J = 8.0 \) Hz, H10), 7.28 - 7.25 (1H, m, H11), 7.25 – 7.25 (2H, m, H9/H13), 2.43 (3H, s, H14); \(^1\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) 145.5 (C5), 138.1 (C12), 137.6 (C8), 133.5 (C6), 133.3 (C7), 132.0 (C4), 130.3 (C3), 129.7 (C13), 129.4 (C11), 128.2 (C10), 126.1 (C9), 117.5 (C1), 112.3 (C2), 21.4 (C14); HRMS (ESI) calcd for \([C_{14}H_{10}N_{35}Cl + H]^+\): 228.0580, found: 228.0577.

(2-Chloro-3'-methyl-[1,1'-biphenyl]-4-yl)methanamine TFA salt (45)

Prepared by General Method C using LiAlH\(_4\) (33 mg, 0.88 mmol), Et\(_2\)O (2.0 mL), AlCl\(_3\) (116 mg, 0.88 mmol) and 45a (100 mg, 0.44 mmol). The crude amine was purified by semi-preparative HPLC (20-70% B) to provide title compound 45 as a white solid (70 mg, 0.20 mmol, 46%):

HPLC \( t_r \) = 7.73 mins (20-70% B); m.p. 143–144 °C; IR \( \nu_{max} \) 2988 (br s, NH\(_3^+\)), 1673 (s, C=C), 1596 (m, C=C), 1530 (m, C=C), 1482 (m, C=C); \(^1\)H NMR (500 MHz, d\(_6\)-DMSO) \( \delta \) 8.33 (3H, br s, H15), 7.70 (1H, d, \( J = 1.5 \) Hz, H7), 7.49 (1H, dd, \( J = 8.0, 1.5 \) Hz, H3), 7.45 (1H, d, \( J = 8.0 \) Hz, H4), 7.36 (1H, t, \( J = 7.5 \) Hz, H10), 7.26 – 7.19 (3H, m, H9/H11/H13), 4.11 (2H, s, H1), 2.37 (3H, s, H14); \(^1\)C NMR (125 MHz, d\(_6\)-DMSO) \( \delta \) 140.0 (C5), 138.2 (C8), 137.6 (C12), 135.3 (C2), 131.7 (C4), 131.4 (C6), 130.3 (C7), 129.7 (C13), 128.6 (C11), 128.3 (C10), 128.1 (C3), 126.3 (C9), 41.4 (C1), 21.1 (C14); HRMS (ESI) calcd for \([C_{14}H_{14}N_{35}Cl + H]^+\): 232.0893, found: 232.0897.
2-Chloro-3'-vinyl-[1,1'-biphenyl]-4-carbonitrile (46a)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-vinylphenylboronic acid (93 mg, 0.63 mmol), PdCl₂(dppf).CH₂Cl₂ (26 mg, 0.032 mmol), K₃PO₄ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H₂O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, elution: 10:90 Et₂O:Hexane) to provide the title compound 46a as a white solid (128 mg, 0.53 mmol, 85%):

R_f 0.27 (1:19 Et₂O:Hexane); m.p. 93-94 °C; IR ν max 3066 (w, C-H), 2232 (m, C≡N), 1597 (m, C=C), 1489 (s, C=C), 1380 (s, C=C); ¹H NMR (400 MHz, CDCl₃) δ 7.79 (1H, d, J = 1.6 Hz, H7), 7.63 (1H, dd, J = 8.0, 1.6 Hz, H3), 7.52 - 7.41 (4H, m, H4/H10/H11/H13), 7.32 (1H, dt, J = 7.6, 1.6 Hz, H9), 6.77 (1H, dd, J = 17.6, 10.8 Hz, H14), 5.81 (1H, dd, J = 17.6, 0.4 Hz, H15), 5.33 (1H, dd, J = 10.8, 0.4 Hz, H15'); ¹³C NMR (100 MHz, CDCl₃) δ 145.2 (C5), 137.9 (C8/C12), 137.8 (C8/C12), 136.2 (C14), 133.6 (C6), 133.4 (C7), 132.0 (C4), 130.4 (C3), 128.6 (C10), 128.4 (C9), 126.9 (C13), 126.4 (C11), 117.5 (C1), 114.9 (C15), 112.5 (C2); HRMS (ESI) calcd for [C₁₅H₁₀N₃Cl + H]⁺: 240.0580, found: 240.0583.

(2-Chloro-3'-vinyl-[1,1'-biphenyl]-4-yl)methanamine TFA salt (46)

Prepared by General Method C using LiAlH₄ (32 mg, 0.84 mmol), Et₂O (2.0 mL), AlCl₃ (112 mg, 0.84 mmol) and 46a (100 mg, 0.42 mmol). The crude amine was purified by semi-preparative HPLC (20-70% B) to provide title compound 46 as a yellow solid (72 mg, 0.20 mmol, 47%):

HPLC t_r = 7.92 mins (20-70% B); m.p. 122–123 °C; IR ν max 2922 (br s, NH₃⁺), 1599 (w, C=C), 1476 (m, C=C); ¹H NMR (500 MHz, d₆-DMSO) δ 7.54 (1H, d, J = 0.5 Hz, H7), 7.51 (1H, dt, J = 7.5, 1.5 Hz, H11), 7.48 (1H, t, J = 1.5 Hz, H13), 7.43 (1H, t, J = 7.5 Hz, H10), 7.37 – 7.33 (2H, m, H3/H4), 7.31 (1H, dt, J =
7.5, 1.5 Hz, H9), 6.79 (1H, dd, J = 17.5, 11.0 Hz, H14), 5.89 (1H, dd, J = 17.5, 1.0 Hz, H15), 5.30 (1H, dd, J = 11.0, 1.0 Hz, H15′), 3.75 (2H, s, H1); **^13C NMR** (125 MHz, d$_6$-DMSO) δ 146.1 (C2), 139.2 (C8), 137.4 (C5), 137.2 (C12), 136.5 (C14), 131.2 (C4), 131.1 (C6), 128.9 (C9), 128.5 (C10), 128.2 (C7), 127.1 (C13), 126.2 (C3), 125.3 (C11), 114.9 (C15), 44.9 (C1); **HRMS** (ESI) calcd for [C$_{15}$H$_{14}$N$_3$Cl + H]$^+$: 244.0893, found: 244.0895.

NH$_3$ peak (H16) too broad to be observed in $^1$H NMR.

**2-Chloro-4-cyanophenyl trifluoromethanesulfonate (48)**

![Image of molecule](image)

Prepared by General Method A using 3-chloro-4-hydroxybenzonitrile (3.00 g, 19.54 mmol), anhydrous CH$_2$Cl$_2$ (60 mL), anhydrous pyridine (4.78 mL, 58.62 mmol) and trifluoromethanesulfonic anhydride (3.61 mL, 21.45 mmol). The crude residue was purified by flash column chromatography (silica gel, gradient elution: 5:95 Et$_2$O:Hexane to 15:85 Et$_2$O:Hexane) to provide the title compound 48 as a white solid (5.24 g, 18.35 mmol, 94%):

R$_f$ 0.31 (1:4 Et$_2$O:Hexane); **IR** $\nu_{max}$ 3076 (w, C-H), 2245 (w, C≡N), 1575 (w, C=C), 1478 (w, C=C); **$^1$H NMR** (400 MHz, CDCl$_3$) δ 7.87 (1H, d, J = 2.0 Hz, H7), 7.69 (1H, dd, J = 8.8, 2.0 Hz, H3), 7.52 (1H, d, J = 8.8 Hz, H4); **$^{13}$C NMR** (100 MHz, CDCl$_3$) δ 148.5 (C5), 134.9 (C7), 132.2 (C3), 128.9 (C6), 124.2 (C4), 118.5 (C1, q, J = 318.8 Hz, C8), 116.0 (C11), 113.7 (C2); **HRMS** (ESI) calcd for [C$_8$H$_3$NO$_3$S$_3$ClF$_3$ + H]$^+$: 285.9553, found: 285.9555.

Spectroscopic data consistent with that reported in the literature.$^{250,251}$

**2-Chloro-3'-hydroxy-[1,1'-biphenyl]-4-carbonitrile (51a)**

![Image of molecule](image)
Prepared by General Method B using 3-chloro-4-bromobenzonitrile (2.16 g, 10.00 mmol), 3-hydroxyphenylboronic acid pinacol ester (2.00 g, 9.08 mmol), PdCl$_2$(dpff).CH$_2$Cl$_2$ (371 mg, 0.45 mmol), K$_3$PO$_4$ (2.30 g, 10.84 mmol), DME (20 mL), EtOH (6 mL) and H$_2$O (4 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 5:95 Et$_2$O:Hexane to 40:60 Et$_2$O:Hexane) to provide the title compound 51a as a white solid (1.93 g, 8.40 mmol, 93%):

$R_f$ 0.19 (2:3 Et$_2$O:Hexane); m.p. 141-142 °C; IR $\nu_{max}$ 3355 (br, O-H), 2988 (w, C-H), 2246 (m, C≡N), 1616 (w, C=C), 1582 (s, C=C), 1469 (s, C=C); $^1$H NMR (400 MHz, d$_6$-DMSO) $\delta$ 9.69 (1H, s, H14), 8.15 (1H, d, $J$ = 1.6 Hz, H7), 7.88 (1H, dd, $J$ = 8.0, 1.6 Hz, H3), 7.57 (1H, d, $J$ = 8.0 Hz, H4), 7.29 (1H, t, $J$ = 8.0 Hz, H10), 6.87 – 6.80 (3H, m, H9/H11/H13); $^{13}$C NMR (100 MHz, d$_6$-DMSO) $\delta$ 157.3 (C12), 144.8 (C5), 138.6 (C8), 133.4 (C7), 132.3 (C4), 132.3 (C6), 131.3 (C3), 129.7 (C10), 119.7 (C9), 117.6 (C1), 115.9 (C11), 115.6 (C13), 111.8 (C2); HRMS (ESI) calcd for [C$_{13}$H$_8$NO$_3$Cl + H]$^+$: 229.0289, found: 229.0286.

4’-(Aminomethyl)-2’-chloro-[1,1’-biphenyl]-3-ol TFA salt (51)

Prepared by General Method C using LiAlH$_4$ (83 mg, 2.18 mmol), Et$_2$O (10 mL), AlCl$_3$ (291 mg, 2.18 mmol) and 51a (90 mg, 0.39 mmol). The crude amine was purified by semi-preparative HPLC (20-70% B) to provide title compound 51 as a white solid (63 mg, 0.18 mmol, 45%):

HPLC $t_r$ = 6.87 mins (5-95% B); m.p. 119–120 °C; IR $\nu_{max}$ 3344 (m, NH$_2$), 2923 (br, O-H), 1574 (s, C=C), 1471 (s, C=C); $^1$H NMR (400 MHz, d$_6$-DMSO) $\delta$ 9.55 (1H, br s, H14), 7.51 (1H, d, $J$ = 0.8 Hz, H7), 7.35 – 7.21 (3H, m, H3/H4/H10), 6.81 – 6.76 (3H, m, H9/H11/H13), 3.74 (2H, s, H1); $^{13}$C NMR (100 MHz, d$_6$-DMSO) $\delta$ 157.1 (C12), 145.8 (C2), 140.1 (C8), 137.7 (C5), 131.03 (C4), 130.97 (C6), 129.3 (C10), 128.3 (C7), 126.1 (C3), 120.0 (C9), 116.2 (C11), 114.6 (C13), 44.8 (C1); HRMS (ESI) calcd for [C$_{13}$H$_{12}$NO$_3$Cl + H]$^+$: 234.0686, found: 234.0693.

NH$_3$ peak (H15) too broad to be observed in $^1$H NMR.
2-Chloro-3’-(hydroxymethyl)-[1,1’-biphenyl]-4-carbonitrile (54a)

![Chemical structure]

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-(hydroxymethyl)phenylboronic acid (96 mg, 0.63 mmol), PdCl$_2$(dpf).CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_3$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 30:70 Et$_2$O:Hexane to 50:50 Et$_2$O:Hexane) to provide the title compound 54a as a brown oil (133 mg, 0.55 mmol, 87%):

$R_f$ 0.16 (3:2 Et$_2$O:Hexane); $\text{IR } \nu_{\text{max}}$ 3216 (br, O-H), 2231 (s, C≡N), 1479 (m, C=C), 1429 (m, C=C); $^1$H NMR (500 MHz, d$_6$-DMSO) δ 8.18 (1H, d, $J$ = 1.5 Hz, H7), 7.91 (1H, dd, $J$ = 8.0, 1.5 Hz, H3), 7.60 (1H, d, $J$ = 8.0 Hz, H4), 7.46 (1H, t, $J$ = 8.0 Hz, H10), 7.43 – 7.38 (2H, m, H11/H13), 7.33 (1H, dt, $J$ = 7.5, 1.5 Hz, H9), 5.28 (1H, t, $J$ = 5.5 Hz, H15), 4.57 (2H, d, $J$ = 5.5 Hz, H14); $^{13}$C NMR (125 MHz, d$_6$-DMSO) δ 144.9 (C5), 143.0 (C12), 137.1 (C8), 133.4 (C7), 132.4 (C4), 132.3 (C6), 131.4 (C3), 128.3 (C10), 127.4 (C9), 127.0 (C13), 126.7 (C11), 117.6 (C1), 111.9 (C2), 62.7 (C14); HRMS (ESI) calcd for [C$_{14}$H$_{10}$NO$_3$Cl$^+$ + H$^+$]: 244.0529, found: 244.0539.

(4’-(Aminomethyl)-2’-chloro-[1,1’-biphenyl]-3-yl)methanol TFA salt (54)

![Chemical structure]

Prepared by General Method C using LiAlH$_4$ (62 mg, 1.64 mmol), Et$_2$O (2.5 mL), AlCl$_3$ (219 mg, 1.64 mmol) and 54a (100 mg, 0.41 mmol). The crude amine was purified by semi-preparative HPLC (5-95% B) to provide title compound 54 as a clear oil (9 mg, 0.02 mmol, 6%):

HPLC $t_r$ = 9.12 mins (5-95% B); $\text{IR } \nu_{\text{max}}$ 2988 (br s, O-H/NH$_3^+$), 1674 (s, C=C), 1644 (m, C=C), 1622 (m, C=C), 1509 (w, C=C), 1483 (w, C=C); $^1$H NMR (500 MHz, d$_6$-DMSO) δ 8.28 (3H, br s, H16), 7.70 (1H, s, H7), 7.52 – 7.41 (3H, m, H3/H4/H10), 7.39 – 7.34 (2H, m, H11/H13), 7.30 – 7.26 (1H, m, H9), 5.27 (1H,
t, J = 5.0 Hz, H15), 5.56 (2H, d, J = 5.0 Hz, H14), 4.11 (2H, br s, H1); $^{13}$C NMR (125 MHz, d$_6$-DMSO) δ 142.8 (C12), 140.1 (C5), 138.0 (C8), 135.8 (C2), 131.7 (C3), 131.4 (C6), 130.3 (C7), 128.1 (2C, C4/C10), 127.6 (C9), 127.1 (C13), 126.1 (C11), 62.8 (C14), 41.5 (C1); HRMS (ESI) calcd for [C$_{14}$H$_{14}$N$_{35}$Cl + H]$^+$: 248.0842, found: 248.0843.

2-Chloro-3'-ethyl-[1,1'-biphenyl]-4-carbonitrile (55a)

Prepared by General Method B using 3-chloro-4-bromobenzonitride (150 mg, 0.69 mmol), 3-ethylphenylboronic acid (95 mg, 0.63 mmol), PdCl$_2$(dppf).CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_2$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, elution: 5:95 Et$_2$O:Hexane) to provide the title compound 55a as a yellow oil (123 mg, 0.51 mmol, 81%): 

$R_f$ 0.54 (1:4 Et$_2$O:Hexane); IR $\nu_{max}$ 2970 (s, C-H), 2901 (m, C-H), 2232 (s, C=N), 1597 (w, C=C), 1538 (w, C=C), 1473 (m, C=C); $^1$H NMR (500 MHz, CDCl$_3$) δ 7.78 (1H, d, J = 2.0 Hz, H7), 7.61 (1H, dd, J = 7.0, 2.0 Hz, H3), 7.47 (1H, d, J = 8.0 Hz, H4), 7.39 (1H, td, J = 7.5, 1.0 Hz, H10), 7.31 - 7.27 (1H, m, H9/H11), 7.27 – 7.24 (2H, m, H9/H11/H13), 2.73 (2H, q, J = 7.5 Hz, H14), 1.29 (3H, t, J = 7.5 Hz, H15); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 144.6 (C5), 144.4 (C12), 137.6 (C8), 133.6 (C6), 133.3 (C7), 132.1 (C4), 130.3 (C3), 128.6 (C13), 128.3 (C10), 128.2 (C11), 126.4 (C9), 117.6 (C1), 112.3 (C2), 28.8 (C14), 15.5 (C15); HRMS (ESI) calcd for [C$_{15}$H$_{14}$N$_{35}$Cl + H]$^+$: 242.0731, found: 242.0770.

(2-Chloro-3'-ethyl-[1,1'-biphenyl]-4-yl)methanamine TFA salt (55)

$^{16}$NH$_3^+$•CF$_3$CO$_2^-$
Prepared by General Method C using LiAlH$_4$ (25 mg, 0.66 mmol), Et$_2$O (1.5 mL), AlCl$_3$ (88 mg, 0.66 mmol) and 55a (80 mg, 0.33 mmol). The crude amine was purified by semi-preparative HPLC (20-70% B) to provide title compound 55 as a white solid (84 mg, 0.23 mmol, 71%):

**HPLC** $t_r = 8.37$ mins (20-70% B); **m.p.** 121–122 °C; **IR** $\nu_{\text{max}}$ 2963 (br s, NH$_3$), 1675 (s, C=C), 1595 (m, C=C), 1524 (w, C=C), 1476 (w, C=C); $^1$H NMR (400 MHz, d$_6$-DMSO) δ 8.32 (3H, br s, H16), 7.70 (1H, d, $J = 1.2$ Hz, H7), 7.51 – 7.45 (2H, m, H3/H4), 7.39 (1H, t, $J = 7.6$ Hz, H10), 7.29 – 7.20 (3H, m, H9/H11/H13), 4.11 (2H, s, H1), 2.67 (2H, q, $J = 8.0$ Hz, H14), 1.21 (3H, t, $J = 8.0$ Hz, H15); $^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 143.9 (C12), 140.1 (C5), 138.2 (C8), 135.3 (C2), 131.7 (C4), 131.4 (C6), 130.3 (C7), 128.6 (C13), 128.3 (C10), 128.1 (C3), 127.5 (C11), 126.6 (C9), 41.5 (C1), 28.2 (C14), 15.7 (C15); **HRMS** (ESI) calcd for [C$_{15}$H$_{16}$N$_3$5Cl + H$^+$(] + : 246.1044, found: 246.1047.

2-Chloro-3'-fluoro-[1,1'-biphenyl]-4-carbonitrile (56a)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-fluorophenylboronic acid (88 mg, 0.63 mmol), PdCl$_2$(dpff),CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_3$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 1:99 Et$_2$O:Hexane to 2:98 Et$_2$O:Hexane) to provide the title compound 56a as a white solid (130 mg, 0.56 mmol, 89%):

**R$_f$** 0.37 (1:4 Et$_2$O:Hexane); **m.p.** 73-74 °C; **IR** $\nu_{\text{max}}$ 3072 (w, C-H), 2971 (w, C-H), 2232 (m, C≡N), 1616 (w, C≡N), 1581 (s, C=C), 1541 (w, C=C), 1500 (w, C=C); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.80 (1H, d, $J = 1.2$ Hz, H7), 7.63 (1H, dd, $J = 8.0$, 1.2 Hz, H3), 7.49 – 7.42 (2H, m, H4/H10), 7.21 (1H, dt, $J = 8.0$, 1.2 Hz, H9), 7.19 - 7.13 (2H, m, H11/H13); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 162.5 (1C, d, $J = 240$ Hz, C12), 144.0 (1C, d, $J = 2.4$ Hz, C5), 140.0 (1C, d, $J = 8.3$ Hz, C8), 133.54 (C6), 133.46 (C7), 131.9 (C4), 130.5 (C3), 130.1 (1C, d, $J = 8.3$ Hz, C10), 124.9 (1C, d, $J = 3.0$ Hz, C9), 117.3 (C1), 116.3 (1C, d, $J = 22.5$ Hz, C11/C13), 115.7 (1C, d, $J = 20.8$ Hz, C11/C13), 113.0 (C2); **HRMS** (ESI) calcd for [C$_{13}$H$_7$N$_3$5Cl$^{19}$F + H$^+$(] + : 232.0329, found: 232.0340.
(2-Chloro-3′-fluoro-[1,1′-biphenyl]-4-yl)methanamine TFA salt (56)

Prepared by General Method C using LiAlH₄ (33 mg, 0.87 mmol), Et₂O (2.0 mL), AlCl₃ (116 mg, 0.87 mmol) and 56a (100 mg, 0.43 mmol). The crude amine was purified by semi-preparative HPLC (5-55% B) to provide title compound 56 as a white solid (116 mg, 0.33 mmol, 77%):

**HPLC t_r = 11.61 mins (5-55% B); m.p. 152–153 °C; IR ν_max 2988 (br s, NH₃⁺), 1675 (s, C=C), 1589 (s, C=C), 1527 (m, C=C), 1474 (m, C=C); ¹H NMR (500 MHz, d₆-DMSO) δ 8.26 (3H, br s, H14), 7.73 (1H, br s, H7), 7.56 – 7.50 (3H, m, H3/H4/H10), 7.31 – 7.25 (3H, m, H9/H11/H13), 4.11 (2H, s, H1); ¹³C NMR (125 MHz, d₆-DMSO) δ 161.9 (1C, d, J = 242.6 Hz, C12), 140.4 (1C, d, J = 8.0 Hz, C8), 138.5 (C5), 135.9 (C2), 131.7 (C4), 131.3 (C6), 130.4 (1C, d, J = 8.5 Hz, C10), 130.5 (C7), 128.2 (C3), 125.6 (C9), 116.1 (1C, d, J = 21.9 Hz, C11/C13), 114.9 (1C, d, J = 20.7 Hz, C11/C13), 41.4 (C1); HRMS (ESI) calcd for [C₁₃H₁₁N⁺Cl⁻F + H]⁺: 236.0642, found: 236.0645.

2-Chloro-3′-ethynyl-[1,1′-biphenyl]-4-carbonitrile (57a)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-ethynylphenylboronic acid pinacol ester (144 mg, 0.63 mmol), PdCl₂(dpff).CH₂Cl₂ (26 mg, 0.032 mmol), K₃PO₄ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H₂O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 5:95 Et₂O:Hexane to 30:70 Et₂O:Hexane) to provide the title compound 57a as a brown solid (131 mg, 0.55 mmol, 88%):

R_f 0.34 (1:4 Et₂O:Hexane); m.p. 96–97 °C; IR ν_max 3277 (m, C≡C-H), 2231 (m, C≡N), 1578 (w, C=C), 1472 (s, C=C); ¹H NMR (400 MHz, CDCl₃) δ 7.79 (1H, d, J = 1.6 Hz, H7), 7.63 (1H, dd, J = 8.0, 1.6 Hz, H3), 7.59
- 7.54 (2H, m, H11/H13), 7.47 - 7.40 (3H, m, H4/H9/H10), 3.14 (1H, s, H16); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 144.3 (C5), 137.8 (C8), 133.6 (C6), 133.4 (C7), 132.6 (C11/C13), 132.3 (C11/C13), 131.9 (C4), 130.5 (C3), 129.5 (C9), 128.5 (C10), 122.5 (C12), 117.4 (C1), 112.9 (C2), 82.9 (C14), 77.2 (C15); HRMS (ESI) calcd for [C$_{15}$H$_8$N$_3$Cl + H]$^+$: 238.0424, found: 238.0419.

(2-Chloro-3'-ethynyl-[1,1'-biphenyl]-4-yl)methanamine TFA salt (57)

Prepared by General Method C using LiAlH$_4$ (16 mg, 0.42 mmol), Et$_2$O (0.84 mL), AlCl$_3$ (56 mg, 0.42 mmol) and 57a (50 mg, 0.21 mmol). The crude amine was purified by semi-preparative HPLC (25-40% B) to provide title compound 57 as a yellow oil (13 mg, 0.04 mmol, 19%): HPLC $t_r = 13.10$ mins (20-35% B); IR $\nu_{max}$ 3299 (w, C≡C-H), 2907 (br, NH$_3^+$), 1666 (s, C=C), 1532 (m, C=C), 1473 (m, C=C); $^1$H NMR (500 MHz, d$_6$-DMSO) δ 8.27 (3H, br s, H17), 7.72 (1H, br s, H7), 7.55 - 7.49 (4H, m, H3/H4/H10/H13), 7.46 (1H, dt, $J = 7.5$, 1.5 Hz, H9), 4.27 (1H, s, H16), 4.12 (2H, br s, H1); $^{13}$C NMR (125 MHz, d$_6$-DMSO) δ 138.8 (C5), 138.6 (C8), 135.8 (C2), 132.2 (C13), 131.7 (C4), 131.35 (C11), 131.32 (C6), 130.3 (C7), 130.0 (C9), 128.9 (C10), 128.2 (C3), 121.9 (C12), 83.1 (C14), 81.5 (C15), 41.4 (C1); HRMS (ESI) calcd for [C$_{15}$H$_{12}$N$_3$Cl + H]$^+$: 242.0737, found: 242.0737.

2-Chloro-3'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (58a)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-(trifluoromethyl)phenylboronic acid (120 mg, 0.63 mmol), PdCl$_2$(dppf).CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_3$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL). The crude product was
purified by flash column chromatography (silica gel, gradient elution: 2:98 Et₂O:Hexane to 4:96 Et₂O:Hexane) to provide the title compound 58a as a white solid (169 mg, 0.60 mmol, 95%):

**Rf** 0.32 (1:4 Et₂O:Hexane); **m.p.** 76–77 °C; **IR** \( \nu_{\text{max}} \) 3077 (w, C-H), 2236 (m, C≡N), 1539 (w, C=C), 1483 (m, C=C), 1432 (m, C=C); **\(^1^H\) NMR** (500 MHz, CDCl₃) δ 7.82 (1H, d, \( J = 1.0 \) Hz, H7), 7.74 – 7.71 (1H, m, H11), 7.71 – 7.69 (1H, m, H13), 7.66 (1H, dd, \( J = 8.0, 1.0 \) Hz, H3), 7.64 - 7.60 (2H, m, H9/H10), 7.48 (1H, d, \( J = 8.0 \) Hz, H4); **\(^{13}C\) NMR** (125 MHz, CDCl₃) δ 143.7 (C5), 138.2 (C8), 133.6 (C6), 133.5 (C7), 132.5 (C9), 131.9 (C4), 131.0 (1C, q, \( J = 32.9 \) Hz, C12), 130.6 (C3), 129.0 (C10), 125.9 (1C, q, \( J = 3.5 \) Hz, C13), 125.5 (1C, q, \( J = 3.4 \) Hz, C11), 121.7 (1C, q, \( J = 268.1 \) Hz, C14), 117.2 (C1), 113.3 (C2); **HRMS** (ESI) calcd for [C₁₄H₁₇N₃ClF₃ + H]⁺: 282.0297, found: 282.0306.

(2-Chloro-3′-(trifluoromethyl)-[1,1′-biphenyl]-4-yl)methanamine TFA salt (58)

Prepared by General Method C using LiAlH₄ (27 mg, 0.70 mmol), Et₂O (1.75 mL), AlCl₃ (93 mg, 0.70 mmol) and 58a (100 mg, 0.35 mmol). The crude amine was purified by semi-preparative HPLC (20-60% B) to provide title compound 58 as a white solid (109 mg, 0.27 mmol, 78%):

**HPLC** \( t_r \) = 9.55 mins (20-60% B); **m.p.** 154–155 °C; **IR** \( \nu_{\text{max}} \) 2902 (br s, NH₃⁺), 1670 (s, C=C), 1632 (w, C=C), 1480 (w, C=C); **\(^1^H\) NMR** (500 MHz, d₆-DMSO) δ 8.37 (3H, br s, H15), 7.83 – 7.80 (1H, m, H7), 7.77 – 7.71 (4H, m, H7/H9/H10/H13), 7.58 – 7.53 (2H, m, H3/H4), 4.13 (2H, s, H1); **\(^{13}C\) NMR** (125 MHz, d₆-DMSO) δ 139.1 (C8), 138.2 (C5), 136.2 (C2), 133.5 (C9), 131.8 (C4), 131.3 (C6), 130.4 (C7), 129.6 (C10), 129.2 (1C, q, \( J = 31.9 \) Hz, C12), 128.3 (C3), 125.7 (1C, q, \( J = 4.3 \) Hz, C13), 124.8 (1C, q, \( J = 4.3 \) Hz, C11), 124.1 (1C, q, \( J = 272.1 \) Hz, C14), 41.3 (C1); **HRMS** (ESI) calcd for [C₁₄H₁₃NCl₁₉F₃ + H]⁺: 286.0605, found: 286.0595.
3-Chloro-4-(thiophen-2-yl)benzonitrile (59a)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 2-thienylboronic acid (81 mg, 0.63 mmol), PdCl$_2$(dpff).CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_3$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL) with microwave irradiation for 4 hours at 110 °C. The crude product was purified by flash column chromatography (silica gel, elution: 6:94 Et$_2$O:Hexane) to provide the title compound 59a as a white solid (120 mg, 0.55 mmol, 79%):

R$_f$ 0.34 (1:4 Et$_2$O:Hexane); m.p. 174-175 °C; IR $\nu$$_{max}$ 3098 (m, C-H), 2233 (m, C≡N), 1595 (m, C=C), 1538 (w, C=C), 1477 (m, C≡C); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.78 (1H, d, $J$ = 2.0 Hz, H7), 7.67 (1H, d, $J$ = 7.5 Hz, H4), 7.58 (1H, dd, $J$ = 7.5, 2.0 Hz, H3), 7.53 – 7.50 (2H, m, H9/H11), 7.17 (1H, dd, $J$ = 5.0, 3.5 Hz, H10); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 138.1 (C8), 137.8 (C5), 134.0 (C7), 132.8 (C6), 131.6 (C4), 130.3 (C3), 128.1 (C9/C11), 128.1 (C9/C11), 127.6 (C10), 117.4 (C1), 111.9 (C2); HRMS (ESI) calcd for [C$_{11}$H$_6$N$_3$ClS + H]$^+$: 219.9988, found: 219.9986.

(3-Chloro-4-(thiophen-2-yl)phenyl)methanamine HCl salt (59)

Prepared by General Method C using LiAlH$_4$ (35 mg, 0.92 mmol), Et$_2$O (2.0 mL), AlCl$_3$ (123 mg, 0.92 mmol) and 59a (100 mg, 0.46 mmol). The crude amine was dissolved in CH$_2$Cl$_2$ (0.5 mL) and HCl (2M in Et$_2$O) (2.3 mL, 4.6 mmol) added dropwise. The reaction was stirred for 1 hour before the precipitate was filtered, washed with cold Et$_2$O and dried to provide the title compound 59 as a yellow solid (64 mg, 0.25 mmol, 53%):

HPLC $t_r$ = 7.54 mins (5-95% B); m.p. 246-247 °C; IR $\nu$$_{max}$ 2923 (br s, NH$_3^+$), 1526 (m, C=C), 1415 (m, C=C); $^1$H NMR (400 MHz, d$_6$-DMSO) $\delta$ 8.60 (3H, br s, H12), 7.78 (1H, d, $J$ = 1.6 Hz, H7), 7.72 (1H, dd, $J$ = 5.2, 0.8 Hz, H11), 7.69 (1H, d, $J$ = 8.4 Hz, H4), 7.54 (1H, dd, $J$ = 78.4, 1.6 Hz, H3), 7.48 (1H, dd, $J$ = 3.6, 0.8 Hz,
H9), 7.19 (1H, dd, J = 5.2, 3.5 Hz, H10), 4.06 (2H, s, H1); $^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 138.6 (C8), 135.6 (C2), 132.3 (C5), 131.3 (C4), 131.0 (C7), 130.7 (C6), 128.4 (C3), 128.4 (C9), 127.8 (C11), 127.6 (C10), 41.2 (C1); HRMS (ESI) calcd for [C$_{11}$H$_{10}$N$_3$ClS + H$^+$]: 224.0301, found: 224.0293.

3-Chloro-4-(thiophen-3-yl)benzonitrile (60a)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 3-thienylboronic acid (81 mg, 0.63 mmol), PdCl$_2$(dpdf).CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_3$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, elution: 5:95 Et$_2$O:Hexane) to provide the title compound 60a as a white solid (120 mg, 0.55 mmol, 87%):

R$_f$ 0.34 (1:4 Et$_2$O:Hexane); m.p. 178-179 °C; IR $\nu_{max}$ 3118 (w, C-H), 2232 (m, C≡N), 1596 (m, C=C), 1521 (w, C=C), 1478 (m, C=C); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.77 (1H, dd, J = 1.6, 0.4 Hz, H7), 7.61 – 7.57 (2H, m, H3/H11), 7.55 (1H, dd, J = 8.0, 0.4 Hz, H4), 7.44 (1H, dd, J = 5.2, 3.2 Hz, H9), 7.34 (1H, dd, J = 5.2, 1.3 Hz, H10); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 140.0 (C5), 137.6 (C8), 133.6 (C7), 133.2 (C6), 131.6 (C4), 130.4 (C3), 128.3 (C10), 125.8 (C9/C11), 125.7 (C9/C11), 117.5 (C1), 112.0 (C2); HRMS (ESI) calcd for [C$_{11}$H$_6$N$_3$ClS + H$^+$]: 219.9988, found: 219.9987.

(3-Chloro-4-(thiophen-3-yl)phenyl)methanamine HCl salt (60)

Prepared by General Method C using LiAlH$_4$ (35 mg, 0.92 mmol), Et$_2$O (2.0 mL), AlCl$_3$ (123 mg, 0.92 mmol) and 60a (100 mg, 0.46 mmol). The crude amine was dissolved in CH$_2$Cl$_2$ (0.5 mL) and HCl (2M in Et$_2$O) (2.3 mL, 4.6 mmol) added dropwise. The reaction was stirred for 1 hour before the precipitate was filtered, washed with cold Et$_2$O and dried to provide the title compound 60 as a yellow solid (73 mg, 0.29 mmol, 63%):
**HPLC** $t_r = 7.53$ mins (5-95% B); **m.p.** 235-236 °C; **IR** $\nu_{max}$ 2913 (br s, NH$_3^+$), 1605 (m, C=C), 1523 (m, C=C), 1493 (m, C=C); **$^1$H NMR** (500 MHz, d$_6$-DMSO) $\delta$ 8.59 (3H, br s, H12), 7.77 – 7.74 (2H, m, H7/H11), 7.67 (1H, dd, $J = 4.5, 3.0$ Hz, H9), 7.57 (1H, d, $J = 7.5$ Hz, H4), 7.53 (1H, dd, $J = 7.5, 1.5$ Hz, H3), 7.36 (1H, dd, $J = 4.5, 1.0$ Hz, H10), 4.06 (2H, s, H1); **$^{13}$C NMR** (125 MHz, d$_6$-DMSO) $\delta$ 138.5 (C8), 135.6 (C2), 135.0 (C5), 131.8 (C4), 131.5 (C6), 131.0 (C7), 129.0 (C10), 128.6 (C3), 126.6 (C9), 125.6 (C11), 41.6 (C1); **HRMS** (ESI) calcd for [C$_{11}$H$_{10}$N$_3$S + H]$^+$: 224.0301, found: 224.0309.

4.4 Experimental Details for Structure-Based Optimisation of the New Binding Mode

2-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-4-(trifluoromethyl)phenol (61b)

To a flask charged with 2-benzyloxy-5-(trifluoromethyl)phenylboronic acid (1.00 g, 3.38 mmol) and pinacol (600 mg, 5.07 mmol) was added dry Et$_2$O (17 mL) before refluxing overnight. Following cooling to room temperature, the solvent was removed under reduced pressure and the residue dissolved in dry MeOH (24 mL). To the solution was added palladium (10% w.t. on charcoal) (180 mg, 0.17 mmol) at room temperature. The reaction was stirred under a H$_2$ atmosphere overnight and then filtered through Celite and the solvent evaporated to provide the title compound 61b as a white solid in sufficient purity (895 mg, 3.11 mmol, 92%):

**Rf** 0.12 (1:1 EtOAc:Hexane); **m.p.** 79-80 °C; **IR** $\nu_{max}$ 3422 (br s, O-H), 2984 (m, C-H), 1631 (s, C=C), 1595 (m, C=C), 1496 (m, C=C); **$^1$H NMR** (400 MHz, DMSO-d$_6$) $\delta$ 10.00 (1H, s, H10), 7.71 (1H, d, $J = 2.2$ Hz, H3), 7.62 (1H, dd, $J = 8.6, 2.4$ Hz, H5), 6.98 (1H, d, $J = 8.6$ Hz, H6), 1.29 (12H, s, H9); **$^{13}$C NMR** (101 MHz, DMSO-d$_6$) $\delta$ 165.6 (C1), 133.2 (1C, q, $J = 3.8$ Hz, C3), 129.8 (1C, q, $J = 3.4$ Hz, C5), 124.8 (1C, q, $J = 270.8$ Hz, C7), 119.2 (1C, q, $J = 31.9$ Hz, C4), 116.0 (C6), 83.6 (2C, C8), 24.6 (4C, C9); **HRMS** (ESI) calcd for [C$_{13}$H$_{16}$O$_3$F$_3^{13}$B + H]$^+$: 289.1223, found: 289.1226.

C2 signal lost in the baseline.

Conditions adapted from literature protocol.$^{262}$
2-Chloro-2'-hydroxy-5'-{(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (61a)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (165 mg, 0.76 mmol), boronic ester 61b (199 mg, 0.69 mmol), PdCl₂(dppf).CH₂Cl₂ (29 mg, 0.035 mmol), K₃PO₄ (177 mg, 0.83 mmol), DME (2.2 mL), EtOH (0.5 mL) and H₂O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 5:95 Et₂O:Hexane to 20:80 Et₂O:Hexane) to provide the title compound 61a as a white solid (160 mg, 0.54 mmol, 77%):

R_f 0.28 (1:1 Et₂O:Hexane); m.p. 93-94 °C; IR ν_max 3340 (s, O-H), 2245 (m, C≡N), 1614 (m, C=C), 1592 (m, C=C), 1479 (s, C=C); ¹H NMR (500 MHz, DMSO-d₆) δ 10.70 (1H, s, H₁₅), 8.15 (1H, d, J = 1.5 Hz, H₇), 7.88 (1H, dd, J = 7.9, 1.6 Hz, H₃), 7.64 (1H, dd, J = 8.6, 2.0 Hz, H₁₁), 7.59 (1H, d, J = 7.9 Hz, H₄), 7.48 (1H, d, J = 1.9 Hz, H₁₃), 7.13 (1H, d, J = 8.6 Hz, H₁₀); ¹³C NMR (126 MHz, DMSO-d₆) δ 157.8 (C₉), 141.5 (C₅), 133.9 (C₆), 133.0 (C₄), 132.7 (C₇), 130.9 (C₃), 127.5 (1C, q, J = 3.1 Hz, C₁₃), 127.4 (1C, q, J = 2.9 Hz, C₁₁), 125.2 (C₈), 124.5 (1C, q, J = 271.0 Hz, C₁₄), 119.5 (1C, q, J = 32.2 Hz, C₁₂), 117.5 (C₁), 116.2 (C₁₀), 112.1 (C₂); HRMS (ESI) calcd for [C₁₄H₇NO₃ClF₃ + H]⁺: 298.0247, found: 298.0242.

4’-{(Aminomethyl)-2’-chloro-5’-{(trifluoromethyl)-[1,1’-biphenyl]-2-ol TFA salt (61)

Prepared by General Method C using LiAlH₄ (33 mg, 0.88 mmol), Et₂O (1.1 mL) and 61a (67 mg, 0.22 mmol) and stirred at 0 °C for 30 minutes before allowed to warm to room temperature for 1h and quenched. The crude amine was purified by semi-preparative HPLC (5-95% B) to provide title compound 61 as a white solid (21 mg, 0.05 mmol, 23%):

HPLC t_r = 9.58 mins (5-95% B); m.p. 172-173 °C; IR ν_max 3176 (br s, O-H/NH₃⁺), 2989 (m, C-H), 1669 (m, C=C), 1625 (m, C=C), 1511 (w, C=C); ¹H NMR (400 MHz, DMSO-d₆) δ 10.65 (1H, s, H₁₅), 8.30 (3H, s br,
H16), 7.68 (1H, d, J = 1.4 Hz, H7), 7.61 (1H, dd, J = 8.6, 2.0 Hz, H11), 7.47 (1H, dd, J = 7.9, 1.6 Hz, H3), 7.43 (1H, d, J = 7.8 Hz, H4), 7.37 (1H, d, J = 2.1 Hz, H13), 7.12 (1H, d, J = 8.5 Hz, H10), 4.11 (2H, s, H1); 

$^{13}$C NMR (101 MHz, DMSO-d$_6$) δ 158.2 (C9), 136.4 (C5), 135.5 (C6), 132.9 (C2), 132.1 (C4), 129.6 (C7), 127.8 – 127.4 (1C, m, C13), 127.6 (C3), 127.0 – 126.7 (1C, m, C11), 126.1 (C8), 124.6 (1C, q, J = 271.0 Hz, C14), 119.3 (1C, q, J = 32.1 Hz, C12) 116.1 (C10), 41.5 (C1); HRMS (ESI) calcd for [C$_{14}$H$_{11}$NO$_3$ClF$_3$ + H]$^+$: 302.0554, found: 302.0551.

2-Chloro-2'-hydroxy-[1,1'-biphenyl]-4-carbonitrile (62a)

![Chemical Structure](image)

Prepared by General Method B using 3-chloro-4-bromobenzonitrile (150 mg, 0.69 mmol), 2-hydroxyphenylboronic acid (87 mg, 0.63 mmol), PdCl$_2$(dpff).CH$_2$Cl$_2$ (26 mg, 0.032 mmol), K$_3$PO$_4$ (161 mg, 0.76 mmol), DME (2.0 mL), EtOH (0.5 mL) and H$_2$O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 5:95 Et$_2$O:Hexane to 20:80 Et$_2$O:Hexane) to provide the title compound 62a as a white solid (100 mg, 0.44 mmol, 69%).

R$_f$ 0.33 (1:1 Et$_2$O:Hexane); m.p. 141–142 °C; IR $v_{max}$ 3340 (s, O-H), 2245 (m, C≡N), 1614 (m, C=C), 1592 (m, C=C); $^1$H NMR (500 MHz, DMSO-d$_6$) δ 9.72 (1H, s, H14), 8.10 (1H, d, J = 1.6 Hz, H7), 7.83 (1H, dd, J = 7.9, 1.7 Hz, H3), 7.52 (1H, d, J = 7.9 Hz, H4), 7.26 (1H, td, J = 8.2, 1.7 Hz, H11), 7.10 (1H, dd, J = 7.6, 1.6 Hz, H13), 6.95 (1H, dd, J = 8.2, 0.7 Hz, H10), 6.89 (1H, td, J = 7.5, 1.0 Hz, H12); $^{13}$C NMR (126 MHz, DMSO-d$_6$) δ 154.3 (C9), 143.2 (C5), 133.9 (C6), 133.0 (C4), 132.6 (C7), 130.6 (C3), 130.2 (C13), 130.0 (C11), 124.7 (C8), 118.9 (C12), 117.7 (C1), 115.7 (C10), 111.5 (C2); HRMS (ESI) calcd for [C$_{13}$H$_8$NO$_3$Cl + H]$^+$: 230.0373, found: 230.0373.
4′-(Aminomethyl)-2′-chloro-[1,1′-biphenyl]-2-ol HCl salt (62)

Prepared by General Method C using LiAlH₄ (33 mg, 0.88 mmol), Et₂O (1.1 mL), AlCl₃ (117 mg, 0.88 mmol) and 62a (50 mg, 0.22 mmol). The crude amine was dissolved in CH₂Cl₂ (0.5 mL) and HCl (2M in Et₂O) (1.1 mL, 2.2 mmol) added dropwise. The reaction was stirred for 1 hour before the precipitate was filtered, washed with cold Et₂O and dried to provide the title compound 62 as a brown solid (35 mg, 0.13 mmol, 59%):

HPLC tᵣ = 7.63 mins (5-95% B); m.p. 314-315 °C; IR ν max 3204 (br s, O-H/NH₃⁺), 2919 (m, C-H), 1606 (s, C=C), 1485 (m, C=C); ¹H NMR (400 MHz, DMSO-d₆) δ 9.61 (1H, s, H14), 8.52 (3H, s br, H15), 7.68 (1H, d, J = 1.5 Hz, H7), 7.47 (1H, dd, J = 7.9, 1.6 Hz, H3), 7.34 (1H, d, J = 7.8 Hz, H4), 7.21 (1H, ddd, J = 8.1, 7.5, 1.7 Hz, H11), 7.05 (1H, dd, J = 7.5, 1.7 Hz, H13), 6.95 (1H, dd, J = 8.1, 0.8 Hz, H10), 6.85 (1H, td, J = 7.4, 1.0 Hz, H12), 4.06 (2H, s, H1); ¹³C NMR (101 MHz, DMSO-d₆) δ 154.6 (C9), 137.9 (C5), 134.9 (C2), 132.9 (C6), 132.1 (C4), 130.6 (C13), 129.6 (C7), 129.3 (C11), 127.4 (C3), 125.6 (C8), 118.7 (C12), 115.6 (C10), 41.4 (C1); HRMS (ESI) calcd for [C₁₃H₁₂NO₃Cl + H]⁺: 234.0680, found: 234.0671.

2-Chloro-2′-hydroxy-5′-(trifluoromethyl)-[1,1′-biphenyl]-4-carbaldehyde (63)

Prepared by General Method B using 3-chloro-4-bromobenzaldehyde (150 mg, 0.69 mmol), boronic ester 61b (181 mg, 0.63 mmol), PdCl₂(dppf).CH₂Cl₂ (29 mg, 0.035 mmol), K₃PO₄ (177 mg, 0.83 mmol), DME (2.0 mL), EtOH (0.5 mL) and H₂O (0.3 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 15:85 Et₂O:Hexane to 35:65 Et₂O:Hexane) to provide the title compound 63 as a white solid (124 mg, 0.41 mmol, 60%):
HPLC $t_r = 13.25$ mins (5-95% B); m.p. 159-160 °C; IR $v_{\text{max}}$ 3258 (s, O-H), 2988 (m, C-H), 1683 (s, C=O), 1618 (w, C=C), 1599 (m, C=C), 1554 (s, C=C), 1520 (m, C=C); $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 10.71 (1H, s br, H15), 10.04 (1H, s, H1), 8.05 (1H, d, $J = 1.3$ Hz, H7), 7.91 (1H, dd, $J = 7.8, 1.4$ Hz, H3), 7.67 – 7.57 (2H, m, H4/H11), 7.47 (1H, d, $J = 2.1$ Hz, H13), 7.13 (1H, d, $J = 8.6$ Hz, H10); $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 192.0 (C1), 158.0 (C9), 142.1 (C5), 136.9 (C2), 133.9 (C6), 132.9 (C4), 130.1 (C7), 127.5 (C3), 127.5 (1C, q, $J = 6.4$ Hz, C13), 127.2 (1C, q, $J = 3.7$ Hz, C11), 125.7 (C8), 124.6 (1C, q, $J = 271.0$ Hz, C14), 119.4 (1C, q, $J = 32.2$ Hz, C12), 116.2 (C10); HRMS (ESI) calcd for [C$_{14}$H$_{16}$O$_2$ClF$_3$ + H]$^+$: 301.0243, found: 301.0239.

2'-Chloro-4'-(hydroxymethyl)-5-(trifluoromethyl)-[1,1'-biphenyl]-2-ol (64)

To a stirred solution of aldehyde 63 (50 mg, 0.16 mmol) in dry MeOH (3.2 mL) at 0 °C was added NaBH$_4$ (19 mg, 0.50 mmol) as a single portion. The mixture was allowed to warm to room temperature with stirring overnight before removal of the solvent in vacuo. The residue was dissolved in Et$_2$O/H$_2$O and extracted three times with Et$_2$O. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over MgSO$_4$, filtered and concentrated in vacuo. The crude alcohol was then purified by semi-preparative HPLC (5-95% B) to provide title compound 64 as a white solid (31 mg, 0.10 mmol, 61%): HPLC $t_r = 12.23$ mins (5-95% B); m.p. 150-151 °C; IR $v_{\text{max}}$ 3451 (br s, O-H), 3078 (s, O-H), 1675 (m, C-H), 1608 (m, C=C), 1533 (m, C=C); $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 10.26 (1H, s br, H15), 7.58 (1H, dd, $J = 8.6, 2.0$ Hz, H11), 7.46 (1H, s, H7), 7.37 (1H, d, $J = 2.2$ Hz, H13), 7.34 – 7.28 (2H, m, H3/H4), 7.09 (1H, d, $J = 8.5$ Hz, H10), 5.36 (1H, s br, H16), 4.54 (2H, d, $J = 3.5$ Hz, H1); $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 158.2 (C9), 144.3 (C2), 134.4 (C5), 132.5 (C6), 131.6 (C4), 127.8 (1C, q, $J = 3.7$ Hz, C13), 126.8 (C7), 126.6 (C8), 126.5 (1C, q, $J = 3.8$ Hz, C11), 124.9 (C3), 124.7 (1C, q, $J = 270.9$ Hz, C14), 119.3 (1C, q, $J = 32.1$ Hz, C12), 116.0 (C10), 62.0 (C1); HRMS (ESI) calcd for [C$_{14}$H$_{16}$O$_2$ClF$_3$]: 302.0321, found: 302.0319.

Conditions adapted from literature protocol.
2'-Hydroxy-2-methyl-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (65)

Prepared by General Method B using 3-methyl-4-bromobenzonitrile (56 mg, 0.28 mmol), boronic ester 61b (75 mg, 0.26 mmol), PdCl$_2$(dppf).CH$_2$Cl$_2$ (11 mg, 0.013 mmol), K$_3$PO$_4$ (66 mg, 0.31 mmol), DME (0.85 mL), EtOH (0.25 mL) and H$_2$O (0.15 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 10:90 EtOAc:Hexane to 25:75 EtOAc:Hexane) to provide the title compound 65 as a white solid (31 mg, 0.11 mmol, 43%):

R$_f$ 0.22 (3:7 EtOAc:Hexane); m.p. 125-126°C; IR $\nu_{\text{max}}$ 3364 (s, O-H), 2235 (m, C≡N), 1618 (s, C=C), 1605 (m, C=C), 1518 (w, C=C); $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 10.57 (1H, s, H$_{16}$), 7.77 (1H, s br, H$_7$), 7.69 (1H, d, $J$ = 7.8 Hz, H$_3$), 7.61 (1H, d, $J$ = 8.5 Hz, H$_{11}$), 7.46 – 7.30 (2H, m, H$_4$/H$_{13}$), 7.12 (1H, d, $J$ = 8.5 Hz, H$_{10}$), 2.15 (3H, s, H$_{15}$); $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 157.6 (C$_9$), 142.5 (C$_5$), 138.3 (C$_8$), 133.0 (C$_7$), 131.1 (C$_4$), 129.4 (C$_3$), 127.3 (2C, br s, C$_6$/C$_{13}$), 126.7 (1C, q, $J$ = 3.1 Hz, C$_{11}$), 124.6 (1C, q, $J$ = 270.9 Hz, C$_{14}$), 119.7 (1C, q, $J$ = 32.1 Hz, C$_{12}$), 118.9 (C$_1$), 116.1 (C$_{10}$), 110.4 (C$_2$), 19.2 (C$_{15}$); HRMS (ESI) calcd for [C$_{15}$H$_{10}$NOF$_3$ + H]$^+$: 278.0793, found: 278.0800.

3-Amino-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (66)

Prepared by General Method B using 2-amino-4-bromobenzonitrile (56 mg, 0.28 mmol), boronic ester 61b (75 mg, 0.26 mmol), PdCl$_2$(dppf).CH$_2$Cl$_2$ (11 mg, 0.013 mmol), K$_3$PO$_4$ (66 mg, 0.31 mmol), DME (0.85 mL), EtOH (0.25 mL) and H$_2$O (0.15 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 10:90 EtOAc:Hexane to 30:70 EtOAc:Hexane) to provide the title compound 66 as a white solid (40 mg, 0.14 mmol, 56%):
Rf 0.37 (1:1 EtOAc:Hexane); m.p. 210-211 °C; IR \( \nu_{\text{max}} \) 3363 (s, N-H), 3302 (s, N-H), 2988 (m, C-H), 2221 (m, C≡N), 1613 (m, C=C), 1562 (s, C=C); \(^1\)H NMR (500 MHz, DMSO-d\(_6\)) \( \delta \) 10.61 (1H, s, H15), 7.55 (1H, d br, \( J = 8.5 \) Hz, H11), 7.48 (1H, s br, H13), 7.41 (1H, d, \( J = 8.1 \) Hz, H3), 7.11 (1H, d, \( J = 8.5 \) Hz, H10), 6.99 (1H, s br, H6), 6.79 (1H, d br, \( J = 8.1 \) Hz, H4), 6.06 (2H, s, H16); \(^{13}\)C NMR (126 MHz, DMSO-d\(_6\)) \( \delta \) 157.8 (C9), 151.4 (C1), 142.6 (C5), 132.1 (C3), 127.4 (C8), 126.9 (1C, q, \( J = 3.0 \) Hz, C13), 126.4 (1C, q, \( J = 2.9 \) Hz, C11), 124.6 (1C, q, \( J = 271.0 \) Hz, C14), 119.9 (1C, q, \( J = 32.1 \) Hz, C12), 118.2 (C7), 117.2 (C4), 116.6 (C10), 115.7 (C6), 92.3 (C2); HRMS (ESI) calcd for [C\(_{14}\)H\(_9\)N\(_2\)OF\(_3\) - H]: 277.0589 found: 277.0589.

4-Bromo-3,5-dichlorobenzonitrile (67a)

![4-Bromo-3,5-dichlorobenzonitrile](image)

To a stirred solution of 4-amino-3,5-dichlorobenzonitrile (1.38 g, 7.38 mmol) in conc. HBr (14 mL) at 0 °C was added sodium nitrite (560 mg, 8.11 mmol) in H\(_2\)O (2.5 mL) followed by copper (I) bromide (2.12 g, 14.76 mmol) in conc. HBr (2.5 mL) and H\(_2\)O (13.3 mL). The mixture was allowed to warm to room temperature before heating at 50 °C for 3 hours. After cooling to room temperature the reaction mixture was stirred for a further 18 hours before the residue was dissolved in CH\(_2\)Cl\(_2\)/H\(_2\)O and extracted three times with CH\(_2\)Cl\(_2\). The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over MgSO\(_4\), filtered and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, gradient elution: 2.5:97.5 EtOAc:Hexane to 10:90 EtOAc:Hexane) to provide the title compound 67a as a white solid (1.04 g, 4.16 mmol, 56%): Rf 0.64 (1:1 EtOAc:Hexane); m.p. 137-138 °C; IR \( \nu_{\text{max}} \) 3061 (w, C-H), 2235 (m, C≡N), 1533 (m, C=C), 1411 (w, C=C); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 7.65 (2H, s, H3); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 138.0 (2C, C4), 131.3 (2C, C3), 130.1 (C5), 116.0 (C1), 113.0 (C2); HRMS (ESI) calcd for [C\(_7\)H\(_2\)NCl\(_2\)Br]: 248.8748, found: 248.8746.

Conditions adapted from literature protocol. Spectroscopic data consistent with that reported for commercially available 4-bromo-3,5-dichlorobenzonitrile (CAS: 1160574-40-2).
2,6-Dichloro-4-cyanophenyl trifluoromethanesulfonate (67c)

![Chemical Structure Image]

Prepared by General Method A using 3,5-dichloro-4-hydroxybenzonitrile (966 mg, 5.14 mmol), anhydrous CH$_2$Cl$_2$ (16 mL), anhydrous pyridine (1.29 mL, 15.96 mmol) and trifluoromethanesulfonic anhydride (0.96 mL, 5.85 mmol). The crude residue was used without further purification. Title compound 67c was a white solid (1.68 g, 5.14 mmol, 100%):

R$_f$ 0.46 (1:4 EtOAc:Hexane); m.p. = 95-96 °C; IR $\nu_{max}$: 2238 (m, C≡N), 1204 (w, C=C); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.76 (2H, s, H3); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 145.9 (C5), 133.1 (2C, C3), 131.1 (2C, C4), 118.5 (1C, q, J = 321.4 Hz, C6), 115.2 (C1), 114.0 (C2); LCMS t$_r$ = 4.07 min, calcd for [C$_9$H$_2$Cl$_2$F$_3$NO$_3$S + K]$^+$: 359.16, found: 358.21.

Spectroscopic data consistent with that reported in the literature.$^{252}$

2,6-Dichloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (67)

![Chemical Structure Image]

Prepared by General Method D using bromide 67a (103 mg, 0.41 mmol), boronic ester 61b (142 mg, 0.49 mmol), Pd(OAc)$_2$ (5 mg, 0.02 mmol), S-Phos (16 mg, 0.04 mmol), K$_3$PO$_4$ (174 mg, 0.82 mmol), toluene (0.82 mL) and H$_2$O (0.12 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 10:90 EtOAc:Hexane to 30:70 EtOAc:Hexane) to provide the title compound 67 as a white solid (47 mg, 0.14 mmol, 35%).
**Rf** 0.49 (1:1 EtOAc:Hexane); **m.p.** 198-199 °C; IR $\nu_{\text{max}}$ 3295 (s, O-H), 2988 (m, C-H), 1618 (w, C=C), 1536 (s, C=C), 1518 (m, C=C); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.73 (1H, s, H13), 8.21 (2H, s, H3), 7.66 (1H, dd, $J = 8.6, 2.0$ Hz, H9), 7.49 (1H, $d$, $J = 2.1$ Hz, H11), 7.14 (1H, $d$, $J = 8.6$ Hz, H8); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 157.7 (C7), 140.5 (C5), 135.7 (2C, C4), 131.6 (2C, C3), 127.8 (1C, q, $J = 3.6$ Hz, C9), 127.4 (1C, q, $J = 3.8$ Hz, C11), 124.5 (1C, q, $J = 271.0$ Hz, C12), 123.0 (C6), 119.7 (1C, q, $J = 32.3$ Hz, C10), 116.5 (C2), 116.4 (C8), 113.2 (C1); HRMS (ESI) calcd for [C$_{14}$H$_8$NOClF$_3$ + H]$^+$: 331.9857 found: 331.9853.

2'-Hydroxy-2,5'-bis(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (68)

Prepared by General Method B using 3-trifluoromethyl-4-bromobenzonitrile (95 mg, 0.38 mmol), boronic ester 61b (100 mg, 0.34 mmol), PdCl$_2$(dpf).CH$_2$Cl$_2$ (14 mg, 0.017 mmol), K$_3$PO$_4$ (87 mg, 0.41 mmol), DME (1.12 mL), EtOH (0.3 mL) and H$_2$O (0.2 mL) with 1 hour microwave irradiation. The crude product was purified by flash column chromatography (silica gel, gradient elution: 5:95 EtOAc:Hexane to 12.5:87.5 EtOAc:Hexane) to provide the title compound 68 as a white solid (39 mg, 0.12 mmol, 35%):  

**Rf** 0.56 (1:1 EtOAc:Hexane); **m.p.** 195-196 °C; IR $\nu_{\text{max}}$ 3288 (s, O-H), 2988 (m, C-H), 1622 (s, C=C), 1529 (m, C=C), 1500 (s, C=C); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.64 (1H, s br, H15), 8.39 (1H, $d$, $J = 1.1$ Hz, H7), 8.19 (1H, dd, $J = 8.0, 1.3$ Hz, H3), 7.66 – 7.60 (2H, m, H4/H11), 7.42 (1H, $d$, $J = 1.3$ Hz, H13), 7.10 (1H, $d$, $J = 8.6$ Hz, H10); $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 157.9 (C9), 141.0 (C5), 135.8 (C3), 133.9 (C4), 130.3 (1C, q, $J = 4.9$ Hz, C7), 129.2 (1C, q, $J = 30.6$ Hz, C6), 127.4 (1C, q, $J = 3.0$ Hz, C11), 127.1 (1C, q, $J = 3.1$ Hz, C13), 125.1 (C8), 124.5 (1C, q, $J = 270.9$ Hz, C14), 123.0 (1C, q, $J = 274.5$ Hz, C16), 119.0 (1C, q, $J = 32.3$ Hz, C12), 117.6 (C1), 115.8 (C10), 111.6 (C2); HRMS (ESI) calcd for [C$_{15}$H$_7$NOF$_6$]$: 331.0432$, found: 331.0431.
2-Amino-2'-hydroxy-5'-{trifluoromethyl}-[1,1'-biphenyl]-4-carbonitrile (69)

Prepared by General Method B using 3-amino-4-bromobenzonitrile (75 mg, 0.38 mmol), boronic ester 61b (100 mg, 0.34 mmol), PdCl₂(dppf).CH₂Cl₂ (14 mg, 0.017 mmol), K₃PO₄ (87 mg, 0.41 mmol), DME (1.12 mL), EtOH (0.3 mL) and H₂O (0.2 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 10:90 EtOAc:Hexane to 25:75 EtOAc:Hexane) to provide the title compound 69 as a beige solid (37 mg, 0.13 mmol, 39%):

R₉ 0.31 (1:1 EtOAc:Hexane); m.p. 212-213 °C; IR ν max 3660 (s, N-H), 3358 (s, N-H/O-H), 2987 (m, C-H), 2228 (m, C≡N), 1580 (s, C=C); ¹H NMR (500 MHz, DMSO-d₆) δ 10.56 (1H, s br, H₁₅), 7.58 (1H, dd, J = 8.6, 2.0 Hz, H₁₁), 7.39 (1H, d, J = 2.1 Hz, H₁₃), 7.14 - 7.09 (2H, m, H₄/H₁₀), 7.07 (1H, d, J = 1.6 Hz, H₇), 6.98 (1H, dd, J = 7.7, 1.7 Hz, H₃), 5.14 (2H, s br, H₁₆); ¹³C NMR (126 MHz, DMSO-d₆) δ 158.0 (C₉), 146.9 (C₆), 131.9 (C₄), 128.0 (1C, q, J = 3.2 Hz, C₁₃), 127.0 (C₅), 126.5 (1C, q, J = 3.1 Hz, C₁₁), 125.4 (C₈), 123.6 (1C, q, J = 270.9 Hz, C₁₄), 119.9 (1C, q, J = 32.0 Hz, C₁₂), 119.4 (C₁), 119.0 (C₃), 117.3 (C₇), 116.5 (C₁₀), 110.8 (C₂); HRMS (ESI) calcd for [C₁₄H₉N₂OF₃]: 278.0667, found: 278.0665.

2',3-Dihydroxy-5'-{trifluoromethyl}-[1,1'-biphenyl]-4-carbonitrile (70)

Prepared by General Method B using 2-hydroxyl-4-bromobenzonitrile (56 mg, 0.28 mmol), boronic ester 61b (75 mg, 0.26 mmol), PdCl₂(dppf).CH₂Cl₂ (11 mg, 0.013 mmol), K₃PO₄ (66 mg, 0.31 mmol), DME (0.85 mL), EtOH (0.25 mL) and H₂O (0.15 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 10:90 EtOAc:Hexane to 30:70 EtOAc:Hexane) to provide the title compound 70 as a white solid (42 mg, 0.15 mmol, 58%).
Rf 0.25 (1:1 EtOAc:Hexane); m.p. 201-202 °C; IR \( \nu_{\text{max}} \) 3355 (s, O-H), 3122 (s, O-H), 2987 (m, C-H), 2243 (m, C≡N), 1615 (s, C=C), 1579 (m, C=C), 1450 (m, C=C); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \( \delta \) 11.09 (1H, s br, H15/H16), 10.76 (1H, s br, H15/H16), 7.64 (1H, d, \( J = 8.1 \) Hz, H3), 7.58 (1H, dd, \( J = 8.6, 2.1 \) Hz, H11), 7.55 (1H, d, \( J = 2.0 \) Hz, H13), 7.24 (1H, d, \( J = 1.3 \) Hz, H6), 7.17 - 7.09 (2H, m, H4/H10); \(^{13}\)C NMR (101 MHz, DMSO-d\(_6\)) \( \delta \) 159.8 (C7), 157.8 (C9), 143.2 (C5), 132.9 (C3), 127.1 (1C, q, \( J = 3.6 \) Hz, C13), 126.8 (1C, q, \( J = 3.6 \) Hz, C11), 126.6 (C8), 124.6 (1C, q, \( J = 271.0 \) Hz, C14), 120.5 (2C, s br, C2/C10), 120.1 (1C, q, \( J = 32.1 \) Hz, C12), 117.1 (C1), 116.8 (1C, C4/C6), 116.7 (1C, C4/C6); HRMS (ESI) calcd for \([\text{C}_{14}\text{H}_8\text{NO}_2\text{F}_3 - \text{H}]^+\): 278.0429, found: 278.0468.

2'-Hydroxy-3-(methylsulfonyl)-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (71)

Prepared by General Method B using 2-methylsulfonyl-4-bromobenzonitrile (74 mg, 0.28 mmol), boronic ester 61b (75 mg, 0.26 mmol), PdCl\(_2\) (11 mg, 0.013 mmol), K\(_3\)PO\(_4\) (66 mg, 0.31 mmol), DME (0.85 mL), EtOH (0.25 mL) and H\(_2\)O (0.15 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 10:90 EtOAc:Hexane to 30:70 EtOAc:Hexane) to provide the title compound 71 as a clear oil (35 mg, 0.10 mmol, 40%).

Rf 0.23 (1:1 EtOAc:Hexane); IR \( \nu_{\text{max}} \) 2988 (s, O-H), 2933 (m, C-H), 2233 (m, C≡N), 1619 (s, C=C), 1598 (m, C=C); \(^1\)H NMR (500 MHz, DMSO-d\(_6\)) \( \delta \) 11.07 (1H, s, H15), 8.34 (1H, d, \( J = 1.6 \) Hz, H6), 8.23 (1H, d, \( J = 8.0 \) Hz, H3), 8.16 (1H, dd, \( J = 8.0, 1.7 \) Hz, H4), 7.78 (1H, d, \( J = 2.0 \) Hz, H13), 7.66 (1H, dd, \( J = 8.6, 2.1 \) Hz, H11), 7.18 (1H, d, \( J = 8.6 \) Hz, H10), 3.43 (3H, s, H16); \(^{13}\)C NMR (126 MHz, DMSO-d\(_6\)) \( \delta \) 158.0 (C9), 142.3 (C5), 141.9 (C7), 135.8 (C3), 134.4 (C4), 129.9 (C6), 127.8 (1C, q, \( J = 3.1 \) Hz, C11), 127.7 (1C, q, \( J = 3.1 \) Hz, C13), 124.8 (C8), 124.5 (1C, q, \( J = 271.2 \) Hz, C14), 120.4 (1C, q, \( J = 32.4 \) Hz, C12), 117.0 (C10), 115.9 (C1), 108.0 (C2), 43.1 (C15); HRMS (ESI) calcd for \([\text{C}_{15}\text{H}_{10}\text{NO}_2\text{F}_3 - \text{H}]^+\): 240.0255, found: 240.0263.
2'-Hydroxy-2,6-dimethyl-5'(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (72)

Prepared by General Method D using 4-bromo-3,5-dimethylbenzonitrile (86 mg, 0.41 mmol), boronic ester 61b (142 mg, 0.49 mmol), Pd(OAc)$_2$ (5 mg, 0.02 mmol), S-Phos (16 mg, 0.04 mmol), K$_3$PO$_4$ (174 mg, 0.82 mmol), toluene (0.82 mL) and H$_2$O (0.12 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 10:90 EtOAc:Hexane to 20:80 EtOAc:Hexane) to provide the title compound 72 as a yellow solid (76 mg, 0.26 mmol, 64%):

$R_f$ 0.59 (1:1 EtOAc:Hexane); m.p. 128-129 °C; IR $\nu_{max}$ 3372 (s, O-H), 2987 (m, C-H), 2244 (m, C≡N), 1624 (s, C=C), 1589 (m, C=C), 1508 (s, C=C); $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 10.43 (1H, s, H14), 7.64 – 7.56 (3H, m, H3/H9), 7.30 (1H, d, $J$ = 2.1 Hz, H11), 7.13 (1H, d, $J$ = 8.5 Hz, H8), 1.99 (6H, s, H13); $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 157.4 (C7), 142.5 (C5), 138.1 (2C, C4), 130.5 (2C, C3), 126.9 (1C, q, $J$ = 3.7 Hz, C11), 126.6 (1C, q, $J$ = 3.7 Hz, C9), 125.9 (C6), 124.7 (1C, q, $J$ = 270.9 Hz, C12), 120.0 (1C, q, $J$ = 32.1 Hz, C10), 119.0 (C1), 116.3 (C8), 110.1 (C2), 19.8 (2C, C13); HRMS (ESI) calcd for [C$_{16}$H$_{12}$NOF$_3$ - H]: 290.0793 found: 290.0798.

4-Ethoxybenzoic acid (73)

Isolated as a by-product (73) and characterised:

$R_f$ 0.43 (1:1 EtOAc:Hexane); $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 10.30 (1H, s, H8), 7.81 (2H, d, $J$ = 8.7 Hz, H3), 6.84 (2H, d, $J$ = 8.7 Hz, H4), 4.24 (2H, q, $J$ = 7.1 Hz, H6), 1.29 (3H, t, $J$ = 7.1 Hz, H7); $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 165.6 (C5), 161.9 (C1), 131.4 (2C, C3), 120.5 (C2), 115.3 (2C, C4), 60.1 (C6), 14.3 (C7); HRMS (ESI) calcd for [C$_9$H$_{10}$O$_3$]: 166.0630 found: 166.0630.
Spectroscopic data consistent with that reported for commercially available 4-ethoxybenzoic acid (CAS: 619-86-3).

4.5 Experimental Details for αD Pocket to Substrate-Binding Channel Linker Investigation

**Ethyl 4-amino-3,5-dichlorobenzoate (74b)**

To a stirred suspension of 4-amino-3,5-dichlorobenzoic acid (1.00 g, 4.85 mmol) in EtOH (20 mL) was added conc. H₂SO₄ (1 mL) dropwise before refluxing for 18 hours. The mixture was allowed to cool to room temperature before neutralising with a saturated aqueous solution of NaHCO₃ and extracting with EtOAc three times. The combined organic extracts were washed with a saturated aqueous solution of NaHCO₃ and a saturated aqueous solution of NaCl before drying over MgSO₄, filtering and concentration in vacuo. The crude product was then purified by flash column chromatography using a short (~5 cm) silica gel plug with 10:90 EtOAc:Hexane to provide the title compound 74b as a cream solid (1.13 g, 4.82 mmol, 99%): 

\[ R_f 0.60 \ (1:20:80 \text{NEt}_3: \text{EtOAc}: \text{Cyclohexane}); \text{m.p.} \ 85–86 \ ^\circ\text{C}; \text{IR} \ \nu_{\text{max}} \ (\text{s, C-H}), 3375 \ (\text{s, C-H}), 1699 \ (\text{s, C=O}), 1610 \ (m, C=C), 1554 \ (s, C=C), 1495 \ (m, C=C); ^1\text{H NMR} \ (500 \text{MHz, DMSO-}d_6) \ \delta \ 7.72 \ (2H, s, H3), \ 6.39 \ (2H, s, H8), \ 4.24 \ (2H, q, J = 7.1 \text{ Hz, H6}), \ 1.28 \ (3H, t, J = 7.1 \text{ Hz, H7}); ^13\text{C NMR} \ (126 \text{MHz, DMSO-}d_6) \ \delta \ 164.1 \ (C1), \ 145.5 \ (C2), \ 129.1 \ (2C, C3), \ 117.5 \ (C5), \ 117.2 \ (2C, C4), \ 60.7 \ (C6), \ 14.3 \ (C7); \text{HRMS} \ (\text{ESI}) \ \text{calcd for } [\text{C}_9\text{H}_9\text{NO}_2^{35}\text{Cl}_2 + \text{H}]^+: \ 234.0089, \text{found: } 234.0083. \]

Spectroscopic data consistent with that reported for commercially available ethyl 4-amino-3,5-dichlorobenzoate (CAS: 74878-31-2).

**Ethyl 4-bromo-3,5-dichlorobenzoate (74a)**
To a stirred suspension of 74b (1.07 g, 4.55 mmol) in conc. HBr (4.55 mL) at 0 °C was added sodium nitrite (409 mg, 5.92 mmol) in H2O (2.96 mL). Following warming to room temperature, a solution of copper (I) bromide (1.31 g, 9.10 mmol) in conc. HBr (4.55 mL) was added and the reaction heated at 105 °C for 4 hours. After cooling to 0 °C the reaction was quenched with ice cold H2O and extracted into EtOAc three times. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over MgSO4, filtered and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, gradient elution: 0:100 EtOAc:Cyclohexane to 1.5:98.5 EtOAc:Cyclohexane) to provide the title compound 74a as a white solid (526 mg, 1.77 mmol, 39%):

RF 0.34 (5:95 EtOAc:Cyclohexane); m.p. 113-114 °C; IR vmax 1713 (s, C=O), 1548 (m, C=C), 1368 (s, C=C); 1H NMR (500 MHz, DMSO-d6) δ 8.02 (2H, s, H3), 4.34 (2H, q, J = 7.1 Hz, H6), 1.33 (3H, t, J = 7.1 Hz, H7); 13C NMR (126 MHz, DMSO-d6) δ 163.3 (C1), 136.0 (2C, C4), 131.2 (C2), 128.9 (2C, C3), 128.2 (C5), 62.0 (C6), 14.1 (C7); HRMS (ESI) calcd for [C9H7O2Cl2Br + H]+: 296.9085, found: 296.9079.

Conditions adapted from literature protocol and compound referenced but no spectroscopic data reported.

Ethyl 4-iodo-3,5-dichlorobenzoate (74e)

To a stirred solution of 74b (500 mg, 2.14 mmol) in acetone (2.68 mL) and conc. HCl (21.40 mL) at 0 °C was added sodium nitrite (193 mg, 1.40 mmol) in H2O (2.5 mL) dropwise. The solution was stirred at 0 °C for 2 hours before a solution of potassium iodide (1.07 g, 6.42 mmol) in H2O (3.21 mL) was added. The mixture was allowed to warm to room temperature and stirred for a further 72 hours before extraction into EtOAc three times. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over MgSO4, filtered and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, gradient elution: 0:100 EtOAc:Cyclohexane to 5:95 EtOAc:Cyclohexane) to provide the title compound 74e as a white solid (455 mg, 1.32 mmol, 62%):

RF 0.73 (1:9 EtOAc:Cyclohexane); m.p. 114-115 °C; IR vmax 1707 (s, C=O), 1541 (m, C=C), 1365 (s, C=C); 1H NMR (500 MHz, DMSO-d6) δ 7.90 (2H, s, H3), 4.32 (2H, q, J = 7.1 Hz, H6), 1.32 (3H, t, J = 7.1 Hz, H7); 13C NMR (126 MHz, DMSO-d6) δ 163.5 (C1), 140.5 (2C, C4), 131.2 (C2), 128.9 (2C, C3), 128.2 (C5), 61.9, (C6), 14.1 (C7); HRMS (ESI) calcd for [C9H12I2Cl2 + H]+: 344.8946, found: 344.8942.
Conditions adapted from literature protocol and spectroscopic data consistent with that reported.  

**Ethyl 3,5-dichloro-4-(((trifluoromethyl)sulfonyl)oxy)benzoate (74g)**

![Structure of 74g]

Prepared by General Method A using ethyl 3,5-dichloro-4-hydroxybenzoate (2.00 g, 8.51 mmol), anhydrous CH₂Cl₂ (28.0 mL), anhydrous NEt₃ (2.65 mL, 19.2 mmol) and trifluoromethanesulfonic anhydride (2.00 mL, 11.9 mmol). The crude residue was purified by flash column chromatography (silica gel, gradient elution: 0:100 EtOAc:cyclohexane to 5:95 EtOAc:Cyclohexane) to provide the title compound 74g as a white solid (2.31 g, 6.29 mmol, 74%):

- **Rf** 0.35 (2:98 EtOAc:Cyclohexane); **m.p.** 89-90 °C; **IR** νmax 1717 (s, C=O), 1569 (m, C=C), 1541 (s, C=C), 1431 (w, C=C);
- **1H NMR** (400 MHz, DMSO-d₆) δ 8.18 (2H, s, H3), 4.34 (2H, q, J = 7.1 Hz, H6), 1.32 (3H, t, J = 7.1 Hz, H7);
- **13C NMR** (126 MHz, DMSO-d₆) δ 162.9 (C1), 144.6 (C5), 132.3 (C3), 130.8 (2C, C3), 128.8 (2C, C4), 118.1 (1C, q, J = 321.2 Hz, C8), 62.5 (C6), 14.2 (C7); **HRMS** (ESI) calcd for [C₁₀H₇O₅S₃Cl₂F₃ + H]⁺: 366.9422, found: 366.9416.

**Ethyl 2,6-dichloro-2'-hydroxy-5'-{(trifluoromethyl)-[1,1'-biphenyl]-4-carboxylate (74d)**

![Structure of 74d]

A solution of triflate 74g (920 mg, 2.50 mmol), boronic ester 61b (1.80 g, 6.25 mmol) and Pd(PPh₃)₄ (580 mg, 0.50 mmol) in MeOH (16.7 mL) and saturated aqueous NaHCO₃ (2.50 mL) was degassed by bubbling argon through the stirred mixture for 10 minutes before heating at 110 °C for 20 hours. The reaction was allowed to cool to room temperature, filtered through celite washing with EtOAc and the solvent removed under reduced pressure. The residue was dissolved in EtOAc/H₂O and extracted with EtOAc three times. The combined organic extracts were washed with a saturated aqueous solution of
NaCl, dried over MgSO₄, filtered and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, gradient elution: 0:100 EtOAc:Cyclohexane to 10:90 EtOAc:Cyclohexane) to provide the title compound 74d as a white solid (772 mg, 2.04 mmol, 81%): \( R_f \) 0.24 (1:4 EtOAc:Cyclohexane); \textbf{m.p.} 153-154 °C; \textbf{IR} \( \nu_{\text{max}} \) 3217 (br s, O-H), 1685 (s, C=O), 1615 (m, C=C), 1546 (m, C=C), 1517 (w, C=C), 1475 (w, C=C); \textbf{H NMR} (500 MHz, DMSO-\( d_6 \)) \( \delta \) 10.70 (1H, s, H13), 8.01 (2H, s, H3), 7.66 (1H, dd, \( J = 8.7, 2.3 \) Hz, H9), 7.46 (1H, d, \( J = 2.3 \) Hz, H11), 7.14 (1H, d, \( J = 8.5 \) Hz, H8), 4.37 (2H, q, \( J = 7.1 \) Hz, H14), 1.34 (3H, t, \( J = 7.1 \) Hz, H15); \textbf{C NMR} (126 MHz, DMSO-\( d_6 \)) \( \delta \) 163.6 (C1), 157.8 (C7), 139.8 (C5), 135.4 (2C, C4), 131.9 (C2), 128.3 (2C, C3), 127.8 (1C, q, \( J = 3.7 \) Hz, C11), 127.6 (1C, q, \( J = 4.0 \) Hz, C9), 124.6 (1C, q, \( J = 271.0 \) Hz, C12), 123.5 (C6), 119.73 (1C, q, \( J = 32.2 \) Hz, C10), 116.4 (C8), 61.8 (C14), 14.1 (C15); \textbf{HRMS} (ESI) calcd for \([\text{C}_{16}\text{H}_{11}\text{O}_{3}\text{Cl}_{2}\text{F}_{3} + \text{H}]^{+}\): 379.0116, found: 379.0106.

**Lithium 2,6-dichloro-2'-hydroxy-5'-{(trifluoromethyl)-[1,1'-(biphenyl)-4-carboxylate (74)}**

To a stirred solution of ester 74d (607 mg, 1.60 mmol) in THF (3.55 mL) and MeOH (1.78 mL) was added a 3.0 M aqueous solution of NaOH (1.60 mL, 4.80 mmol) dropwise. After stirring for 18 hours the reaction mixture was quenched with 2.0 M aqueous HCl and extracted into EtOAc three times. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over Na₂SO₄ for 10 minutes, filtered and concentrated in vacuo to provide the crude carboxylic acid of 74 (694 mg). The crude material was dissolved in minimal CH₂Cl₂ with stirring and a 0.5 M aqueous solution of LiOH·H₂O (3.20 mL, 1.60 mmol) added dropwise. After 18 hours the excess CH₂Cl₂ and H₂O was blown off and the lithium salt was freeze dried to provide the title compound 74 as a white solid in sufficient purity for the next step (566 mg, 1.59 mmol, 99%): \( R_f \) 0.51 (1:50:50 AcOH:EtOAc:Cyclohexane); \textbf{m.p.} 222-223 °C; \textbf{IR} \( \nu_{\text{max}} \) 3377 (br s, O-H), 1620 (s, C=O), 1572 (m, C=C), 1529 (s, C=C), 1436 (w, C=C); \textbf{H NMR} (500 MHz, DMSO-\( d_6 \)) \( \delta \) 7.89 (2H, s, H3), 7.56 (1H, dd, \( J = 8.7, 2.4 \) Hz, H9), 7.33 (1H, d, \( J = 2.3 \) Hz, H11), 7.14 (1H, d, \( J = 8.6 \) Hz, H8); \textbf{C NMR} (126 MHz, DMSO-\( d_6 \)) \( \delta \) 166.1 (C1), 159.1 (C7), 142.7 (C2), 135.6 (C5), 133.8 (2C, C4), 128.4 (2C, C3), 127.8 (1C, q,
Ethyl 4-bromo-3-chlorobenzoate (76b)

To a stirred suspension of 4-bromo-3-chlorobenzoic acid (1.00 g, 4.25 mmol) in EtOH (10 mL) was added conc. H₂SO₄ (1 mL) dropwise before refluxing for 18 hours. The mixture was allowed to cool to room temperature before neutralising with a saturated aqueous solution of NaHCO₃ and extracting with EtOAc three times. The combined organic extracts were then washed with a saturated aqueous solution of NaHCO₃ and a saturated aqueous solution of NaCl before drying over MgSO₄, filtering and concentration in vacuo. The crude product was then purified by flash column chromatography using a short (~5 cm) silica gel plug with 10:90 EtOAc:Hexane to provide the title compound 76b as a white solid (838 mg, 3.18 mmol, 75%):

**Rf** 0.49 (1:4 EtOAc:Hexane); m.p. 40-41 °C; IR \( \nu_{max} \) 1719 (s, C=O), 1586 (m, C=C), 1458 (m, C=C); \(^1\)H NMR (400 MHz, CDCl₃) \( \delta \) 8.11 (1H, d, \( J = 1.9 \) Hz, H7), 7.77 (1H, dd, \( J = 8.3, 2.0 \) Hz, H3), 7.70 (1H, d, \( J = 8.3 \) Hz, H4), 4.38 (2H, q, \( J = 7.1 \) Hz, H8), 1.40 (3H, t, \( J = 7.1 \) Hz, H9); \(^13\)C NMR (101 MHz, CDCl₃) \( \delta \) 165.0 (C1), 135.0 (C6), 134.0 (C4), 131.4 (C7), 131.2 (C2), 128.8 (C3), 128.0 (C5), 61.8 (C8), 14.4 (C9); HRMS (ESI) calcd for \([C_{9}H_{8}O_{2}^{35}Cl_{79}Br + H]^{+}\): 262.9474, found: 262.9474.

Spectroscopic data consistent with that reported for commercially available ethyl 4-bromo-3-chlorobenzoate (CAS: 120077-67-0).

Ethyl 2-chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carboxylate (76c)

Prepared by General Method B with refluxing for 4 hours using ester 76b (1.14 g, 4.34 mmol), boronic ester 61b (1.50 g, 5.21 mmol), PdCl₂(dppf).CH₂Cl₂ (177 mg, 0.22 mmol), K₃PO₄ (1.11 g, 5.21 mmol),
DME (14.5 mL), EtOH (4.30 mL) and H$_2$O (2.20 mL). The crude product was purified by flash column chromatography (silica gel, gradient elution: 5:95 EtOAc:Hexane to 25:75 EtOAc:Hexane) to provide the title compound 76c as a white solid (1.40 g, 4.05 mmol, 93%): 

R$_f$ 0.48 (1:1 EtOAc:Hexane); m.p. 128-129 °C; IR $\nu$$_{max}$ 3264 (br s, O-H), 1686 (s, C=O), 1617 (m, C=C); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.63 (1H, s, H15), 8.02 (1H, d, $J$ = 1.6 Hz, H7), 7.94 (1H, dd, $J$ = 8.0, 1.7 Hz, H3), 7.63 (1H, dd, $J$ = 8.8, 2.2 Hz, H11), 7.54 (1H, d, $J$ = 7.9 Hz, H4), 7.46 (1H, d, $J$ = 2.1 Hz, H13), 7.12 (1H, d, $J$ = 8.5 Hz, H10), 4.36 (2H, q, $J$ = 7.1 Hz, H16), 1.34 (3H, t, $J$ = 7.1 Hz, H17); $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 164.5 (C1), 157.9 (C9), 141.0 (C5), 133.3 (C6), 132.5 (C4), 130.9 (C2), 129.4 (C7), 127.8 – 127.4 (1C, m, C13), 127.6 (C3), 127.3 – 126.9 (1C, m, C11), 125.7 (C8), 124.6 (1C, q, $J$ = 273.2 Hz, C14), 119.4 (1C, q, $J$ = 32.2 Hz, C12), 116.2 (C10), 61.3 (C16), 14.1 (C17); HRMS (ESI) calcd for [C$_{16}$H$_{12}$O$_3$ClF$_3$ + H]$^+$: 345.0505, found: 345.0514.

2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carboxylic acid (76)

Prepared by General Method B using 4-bromo-3-chlorobenzoic acid (162 mg, 0.69 mmol), boronic ester 61b (240 mg, 0.83 mmol), PdCl$_2$(dpdpf).CH$_2$Cl$_2$ (28 mg, 0.035 mmol), K$_3$PO$_4$ (177 mg, 0.83 mmol), DME (2.30 mL), EtOH (0.69 mL) and H$_2$O (0.35 mL). The crude product was purified by flash column chromatography (silica gel, 1:2:98 AcOH:MeOH:CH$_2$Cl$_2$) to provide the title compound 76 as a yellow oil (146 mg, 0.46 mmol, 67%): 

R$_f$ 0.37 (1:10:90 AcOH:MeOH:CH$_2$Cl$_2$); IR $\nu$$_{max}$ 2923 (br s, O-H), 1680 (s, C=O), 1536 (m, C=C); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.68 (1H, s br, H16), 10.64 (1H, s, H15), 10.64 (1H, s, H15), 8.00 (1H, s, H7), 7.92 (1H, d, $J$ = 7.8 Hz, H3), 7.62 (1H, d, $J$ = 7.2 Hz, H11), 7.51 (1H, d, $J$ = 7.9 Hz, H4), 7.45 (1H, d, $J$ = 1.4 Hz, H13), 7.12 (1H, d, $J$ = 8.4 Hz, H10); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 166.1 (C1), 157.9 (C9), 140.6 (C5), 133.2 (C2/C6), 132.3 (C4), 132.0 (C2/C6), 129.7 (C7), 127.7 (C3), 127.6 (1C, q, $J$ = 3.3 Hz, C13), 127.1 (1C, q, $J$ = 4.3 Hz, C11), 125.8 (C8), 124.6 (1C, q, $J$ = 271.0 Hz, C14), 119.4 (1C, q, $J$ = 32.1 Hz, C12), 116.1 (C10); HRMS (ESI) calcd for [C$_{16}$H$_{12}$O$_3$ClF$_3$ + H]$^+$: 316.0114, found: 316.0099.
To a stirred solution of nitrile 61a (155 mg, 0.52 mmol) in EtOH (2.1 mL) was added a 6.0 M aqueous solution of NaOH (2.10 mL, 12.6 mmol) and the reaction heated at 80 °C for 18 hours. Following cooling to room temperature and then to 0 °C, the reaction was quenched with a 2.0 M aqueous solution of HCl and extracted into EtOAc three times. The combined organic extracts were then washed with a saturated aqueous solution of NaCl, dried over MgSO$_4$, filtered and concentration in vacuo. The residual oil was then freeze dried to provide the title compound 77 as a beige solid in sufficient purity for analysis (142 mg, 0.49 mmol, 93%):

LCMS t$_r$ 1.22 min, calcd for [C$_{14}$H$_9$O$_5$Cl-H] 291.0, [M-H] 291.2; m.p. decomp. above 350 °C; IR $\nu_{\text{max}}$ 2927 (br s, O-H), 1677 (s, C=O), 1606 (m, C=C), 1552 (m, C=C); $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 10.54 (1H, s, H15), 8.00 (1H, d, $J$ = 1.6 Hz, H7), 7.92 (1H, dd, $J$ = 7.9, 1.7 Hz, H3), 7.85 (1H, d, $J$ = 2.2 Hz, H11), 7.68 (1H, d, $J$ = 2.2 Hz, H13), 7.49 (1H, d, $J$ = 7.9 Hz, H4), 7.02 (1H, d, $J$ = 8.6 Hz, H10), 3.51 (2H, s br, H16/H17); $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 166.9 (C14), 166.1 (C1), 158.7 (C9), 141.3 (C5), 133.0 (C2), 132.3 (C4), 132.1 (C13), 131.7 (C6), 131.5 (C11), 129.7 (C7), 127.7 (C3), 125.2 (C8), 121.3 (C12), 115.6 (C10); HRMS (ESI) calcd for [C$_{14}$H$_9$O$_5$Cl-H]: 291.0060, found: 291.0062.

Conditions adapted from literature protocol.$^{281}$

Lithium 2-chloro-2'-hydroxy-5'-{(trifluoromethyl)-[1,1'-biphenyl]-4-carboxylate (78)
To a stirred solution of ester \(76c\) (694 mg, 2.01 mmol) in THF (4.78 mL) and MeOH (2.39 mL) was added a 3.0 M aqueous solution of NaOH (2.01 mL, 6.03 mmol) dropwise. After stirring for 18 hours the reaction mixture was quenched with 2.0 M aqueous HCl and extracted into EtOAc three times. The combined organic extracts were washed with a saturated aqueous solution of NaCl, dried over Na\(_2\)SO\(_4\) for 10 minutes, filtered and concentrated \textit{in vacuo} to provide the crude carboxylic acid of \(78\). The crude material was dissolved in minimal CH\(_2\)Cl\(_2\) with stirring and a 0.5 M aqueous solution of LiOH-H\(_2\)O (4.02 mL, 2.01 mmol) added dropwise. After 18 hours the excess CH\(_2\)Cl\(_2\) and H\(_2\)O was blown off and the lithium salt was freeze dried to provide the title compound \(78\) as a beige solid in sufficient purity for the next step (645 mg, 2.00 mmol, 99%):

**LCMS**
- \(t_r \ 1.46 \text{ min, calcd for } [C_{14}H_8O_3^{35}ClF_3 - H]^{-} \ 315.0, [M-H]^{-} \ 315.2; m.p. 210-211 \degree C; IR \ \nu_{max} 2923 (m, C-H), 1692 (m, C=O), 1581 (s, C=C), 1536 (s, C=C), 1404 (m, C=C); ^1H NMR (500 MHz, DMSO-d\(_6\)) \delta 11.80 (1H, s, H\(_{15}\)), 7.97 (1H, d, \(J = 1.3 \) Hz, H\(_7\)), 7.85 (1H, dd, \(J = 7.8, 1.4 \) Hz, H\(_3\)), 7.54 (1H, dd, \(J = 8.6, 2.2 \) Hz, H\(_{11}\)), 7.37 (1H, d, \(J = 2.1 \) Hz, H\(_{13}\)), 7.29 (1H, d, \(J = 7.8 \) Hz, H\(_4\)), 7.23 (1H, d, \(J = 8.6 \) Hz, H\(_{10}\)); \(^{13}C\) NMR (126 MHz, DMSO-d\(_6\)) \delta 167.4 (C\(_1\)), 159.0 (C\(_9\)), 141.0 (C\(_2\)), 137.0 (C\(_5\)), 131.9 (C\(_6\)), 131.0 (C\(_4\)), 129.7 (C\(_7\)), 127.6 (1C, q, \(J = 2.5 \) Hz, C\(_{13}\)), 127.4 (C\(_3\)), 126.7 (C\(_8\)), 126.4 (1C, q, \(J = 1.4 \) Hz, C\(_{11}\)), 124.8 (1C, q, \(J = 270.8 \) Hz, C\(_{14}\)), 118.5 (1C, q, \(J = 31.9 \) Hz, C\(_{12}\)), 116.3 (C\(_{10}\)); HRMS (ESI) calcd for [C\(_{14}H_7O_3^{35}ClF_3 + H]^{-}: 316.0114, found: 316.0099.

Conditions adapted from literature protocol.\(^{282}\)

**Lithium 2-chloro-2′-hydroxy-5′-(methoxycarbonyl)-[1,1′-biphenyl]-4-carboxylate (79)**

To a stirred solution of ester \(76c\) (4.72 g, 13.7 mmol) in minimal MeOH was added LiOH·H\(_2\)O (3.0 M in MeOH, 13.7 mL, 41.1 mmol) and the reaction stirred at room temperature for 18 hours. After quenching with 2.0 M aqueous HCl the reaction mixture was extracted into EtOAc three times. The combined organic extracts were dried over MgSO\(_4\), filtered and reduced \textit{in vacuo} to provide the crude carboxylic acid of \(79\). The crude material was dissolved in minimal CH\(_2\)Cl\(_2\) with stirring and a 2.0 M aqueous solution of LiOH-H\(_2\)O (6.84 mL, 13.7 mmol) added dropwise. After 18 hours the excess CH\(_2\)Cl\(_2\) and H\(_2\)O was blown off and the lithium salt was freeze dried to provide the title compound \(79\) as a white solid in sufficient purity for analysis (4.24 g, 13.6 mmol, 99%):
LCMS t: 1.30 min, calcd for [C15H11O535Cl - H]+ 305.0, [M-H]- 305.2; m.p. 305-306 °C; IR νmax 3257 (br s, O-H), 1692 (s, C=O), 1591 (m, C=C), 1538 (m, C=C); 1H NMR (500 MHz, DMSO-d6) δ 7.96 (1H, d, J = 1.3 Hz, H7), 7.86 – 7.78 (2H, m, H3/H11), 7.67 (1H, d, J = 2.3 Hz, H13), 7.28 (1H, d, J = 7.8 Hz, H4), 7.11 (1H, d, J = 8.6 Hz, H10), 3.78 (3H, s, H16); 13C NMR (126 MHz, DMSO-d6) δ 167.1 (C1), 166.0 (C14), 159.9 (C9), 140.5 (C2/C6), 137.4 (C5), 132.2 (C13), 132.0 (C2/C6), 131.0 (C4), 130.8 (C3/C11), 129.6 (C7), 127.4 (C3/C11), 126.3 (C8), 119.5 (C12), 115.9 (C10), 51.7 (C16); HRMS (ESI) calcd for [C15H11O535Cl + H]+: 306.0295, found: 306.0298. H15 is a very broad singlet and lost in the baseline upon spectra processing.

Conditions adapted from literature protocol.

(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-alanine (92)

Prepared by manual Fmoc-SPPS to provide the title compound 92 as a white solid (5 mg, 0.01 mmol, 19%): HPLC tR = 11.22 mins (5-95% B); m.p. 157-158 °C; IR νmax 3292 (br s, O-H), 1710 (s, C=O), 1662 (s, C=O), 1606 (m, C=C), 1532 (m, C=C); 1H NMR (500 MHz, DMSO-d6) δ 12.60 (1H, s br, H20), 10.60 (1H, s, H15), 8.84 (1H, d, J = 7.2 Hz, H19), 8.04 (1H, d, J = 1.7 Hz, H7), 7.89 (1H, dd, J = 8.0, 1.7 Hz, H3), 7.62 (1H, dd, J = 8.6, 2.0 Hz, H11), 7.49 (1H, d, J = 8.0 Hz, H4), 7.44 (1H, d, J = 2.1 Hz, H13), 7.12 (1H, d, J = 8.5 Hz, H10), 4.51 – 4.34 (1H, m, H16), 1.41 (3H, d, J = 7.3 Hz, H17); 13C NMR (126 MHz, DMSO-d6) δ 174.1 (C18), 164.6 (C1), 158.0 (C9), 139.1 (C5), 134.9 (C2/C6), 132.9 (C2/C6), 132.0 (C4), 128.0 (C7), 127.6 (1C, q, J = 3.1 Hz, C13), 127.0 (1C, q, J = 1.8 Hz, C11), 126.1 (C3), 125.9 (C8), 124.6 (1C, q, J = 270.8 Hz, C14), 119.4 (1C, q, J = 32.3 Hz, C12), 116.1 (C10), 48.3 (C16), 16.9 (C17); HRMS (ESI) calcd for [C17H13NO335ClF3 + H]+: 388.0563, found: 388.0565.
(2-Chloro-2'-hydroxy-5'-[trifluoromethyl]-[1,1'-biphenyl]-4-carbonyl)-L-alanyl-L-alanine (93)

Prepared by manual Fmoc-SPPS to provide the title compound 93 as a white solid (4 mg, 0.01 mmol, 6%):

**HPLC** $t_r = 10.60$ mins (5-95% B); **m.p.** 164-165 °C; **IR** $\nu_{\text{max}}$ 3334 (br s, O-H), 1721 (s, C=O), 1637 (s, C=O), 1618 (m, C=C), 1538 (m, C=C), 1455 (s, C=C); **$^1$H NMR** (500 MHz, DMSO-d$_6$) $\delta$ 12.51 (1H, s br, H24), 10.58 (1H, s, H15), 8.69 (1H, d, $J = 7.6$ Hz, H22), 8.23 (1H, d, $J = 7.3$ Hz, H23), 8.06 (1H, d, $J = 1.7$ Hz, H7), 7.89 (1H, dd, $J = 8.0$, 1.8 Hz, H3), 7.62 (1H, dd, $J = 8.6$, 1.9 Hz, H11), 7.47 (1H, d, $J = 8.0$ Hz, H4), 7.44 (1H, d, $J = 2.2$ Hz, H13), 7.11 (1H, d, $J = 8.5$ Hz, H10), 4.59–4.46 (1H, m, H16), 4.23 (1H, quint, $J = 7.3$ Hz, H19), 1.36 (3H, d, $J = 7.2$ Hz, H17), 1.30 (3H, d, $J = 7.3$ Hz, H20); **$^{13}$C NMR** (126 MHz, DMSO-d$_6$) $\delta$ 174.1 (C21), 172.0 (C18), 164.5 (C1), 158.0 (C9), 139.0 (C5), 135.1 (C2/C6), 132.8 (C2/C6), 131.9 (C4), 128.1 (C7), 127.6 (1C, q, $J = 2.5$ Hz, C13), 127.1 – 126.8 (1C, m, C11), 126.1 (C3), 126.0 (C8), 124.6 (1C, q, $J = 271.0$ Hz, C14), 119.4 (1C, q, $J = 32.0$ Hz, C12) 116.1, (C10), 48.7 (C16), 47.5 (C19), 17.8 (C17), 17.1 (C20); **HRMS** (ESI) calcd for [C$_{20}$H$_{18}$N$_2$O$_5$F$_3$ + H]$^+$: 459.0950, found: 459.0935.

(2-Chloro-2'-hydroxy-5'-[trifluoromethyl]-[1,1'-biphenyl]-4-carbonyl)-L-alanyl-L-alanyl-L-aspartic acid (94)

Prepared by microwave Fmoc-SPPS to provide the title compound 94 as a white solid (20 mg, 0.04 mmol, 17%):

**HPLC** $t_r = 9.78$ mins (5-95% B); **m.p.** decomp. above 350 °C; **IR** $\nu_{\text{max}}$ 3308 (br s, O-H), 1731 (s, C=O), 1663 (s, C=O), 1624 (m, C=C), 1598 (s, C=C), 1536 (m, C=C); **$^1$H NMR** (500 MHz, DMSO-d$_6$) $\delta$ 12.50 (2H,
1H, d, J = 8.0 Hz, H26), 8.14–8.01 (3H, m, H7/H27/H28), 7.89 (1H, dd, J = 8.0 Hz, J = 2.2 Hz, H10), 7.11 (1H, d, J = 8.5 Hz, H10), 4.57–4.44 (2H, m, H16/H22), 4.32 (1H, quint, J = 7.1 Hz, H19), 2.68 (1H, dd, J = 16.7, 5.9 Hz, H23), 2.60 (1H, dd, J = 16.7, 6.6 Hz, H23'), 1.35 (3H, d, J = 7.2 Hz, H17), 1.23 (3H, d, J = 7.1 Hz, H20); $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 172.2 (1C, C24/C25), 172.0 (C21), 171.8 (C18), 171.7 (1C, C24/C25), 164.6 (C1), 158.0 (C9), 139.0 (C5), 135.0 (C6), 132.9 (C2), 131.9 (C4), 128.1 (C7), 127.6 (1C, q, J = 2.8 Hz, C13), 127.0 (1C, q, J = 2.5 Hz, C11), 126.1 (C3), 126.0 (C8), 124.6 (1C, q, J = 271.0 Hz, C14), 119.4 (1C, q, J = 32.1 Hz, C12), 116.1 (C10), 49.0 (C16), 48.5 (C22), 47.9 (C19), 36.0 (C23), 18.2 (C20), 17.8 (C17); HRMS (ESI) calcd for [C$_{24}$H$_{23}$N$_3$O$_8$ClF$_3$ + H]$^+$: 574.1204, found: 574.1204.

(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-aspartyl-L-alanyl-L-aspartic acid (97)

Prepared by microwave Fmoc-SPPS to provide the title compound 97 as a white solid (20 mg, 0.03 mmol, 15%):

HPLC $t_r$ = 9.51 mins (5-95% B); m.p. decomp. above 350 °C; IR $\nu_{max}$ 3309 (br s, O-H), 2923 (m, C-H), 1705 (s, C=O), 1663 (s, C=O), 1628 (w, C=C), 1597 (m, C=C); $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 12.56 (3H, s br, H28/H31/H32), 10.58 (1H, s, H15), 8.84 (1H, d, J = 7.6 Hz, H27), 8.13 (1H, d, J = 8.5 Hz, H10), 8.09 (1H, d, J = 7.5 Hz, H29), 8.03 (1H, d, J = 1.7 Hz, H7), 7.87 (1H, dd, J = 8.0, 1.7 Hz, H3), 7.62 (1H, dd, J = 8.6, 2.0 Hz, H11), 7.49 (1H, d, J = 8.0 Hz, H4), 7.45 (1H, d, J = 2.2 Hz, H13), 7.11 (1H, d, J = 8.5 Hz, H10), 4.80 (1H, ddd, J = 9.5, 7.7, 4.6 Hz, H16), 4.49 (1H, dd, J = 13.6, 6.4 Hz, H23), 4.30 (1H, quint, J = 7.1 Hz, H20), 2.83 (1H, dd, J = 16.7, 4.5 Hz, H17), 2.76–2.62 (2H, m, H17'/H24), 2.57 (1H, dd, J = 16.6, 6.4 Hz, H24'), 1.22 (3H, d, J = 7.1 Hz, H21); $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 172.3 (1C, C25/C26), 171.9 (2C, C18/C22), 171.7 (1C, C25/C26), 170.2 (C19), 164.8 (C1), 158.0 (C9), 139.1 (C5), 134.9 (C6), 132.9 (C2), 132.0 (C4), 128.0 (C7), 127.6 (1C, q, J = 2.8 Hz, C13), 127.2–126.6 (1C, m, C11), 126.1 (C3), 126.0 (C8), 124.6 (1C, q, J = 270.9 Hz, C14), 119.4 (1C, q, J = 32.2 Hz, C12), 116.1 (C10), 50.3 (C16), 48.5
(C23), 48.1 (C20), 36.2 (C24), 35.9 (C17), 18.1 (C21); HRMS (ESI) calcd for [C25H23N3O1035ClF3 + H]+: 618.1102, found: 618.1131.

(S)-4-Amino-3-((S)-6-amino-2-((S)-3-carboxy-2-(2-chloro-2'-hydroxy-5'-((trifluoromethyl)-[1,1'-biphenyl]-4-carboxamido)propanamido)hexanamido)-4-oxobutanoic acid TFA salt (98)

![Chemical Structure](image)

Prepared by microwave Fmoc-SPPS to provide the title compound 98 as a white solid (57 mg, 0.07 mmol, 30%):

**HPLC** tr = 8.37 mins (5-95% B); **m.p.** decomp. above 350 °C; **IR** νmax 2974 (br s, O-H/NH3+), 1645 (s, C=O), 1583 (m, C=C), 1532 (m, C=C); **1H NMR** (500 MHz, DMSO-d6) δ 12.40 (2H, s br, H36), 10.64 (1H, s, H15), 8.90 (1H, d, J = 7.2 Hz, H30), 8.19 (1H, d, J = 7.5 Hz, H32), 8.09 – 7.98 (2H, m, H7/H34), 7.87 (1H, dd, J = 8.0, 1.8 Hz, H3), 7.67 (3H, s br, H33), 7.62 (1H, dd, J = 8.6, 2.0 Hz, H11), 7.49 (1H, d, J = 8.0 Hz, H4), 7.44 (1H, d, J = 1.9 Hz, H13), 7.16 – 7.08 (1H, m, H10), 4.83 – 4.72 (1H, m, H16), 4.50 – 4.39 (1H, m, H26), 4.22 – 4.08 (1H, m, H20), 2.90 – 2.51 (6H, m, H17/H24/H27), 1.76 – 1.63 (1H, m, H21), 1.62 – 1.43 (3H, m, H21'/H23), 1.40 – 1.26 (2H, m, H22); **13C NMR** (126 MHz, DMSO-d6) δ 172.4 (1C, C18/C28/C29), 172.0 (1C, C18/C28/C29), 171.9 (1C, C18/C28/C29), 171.2 (C25), 171.0 (C19), 165.0 (C1), 158.0 (C9), 139.2 (C5), 134.8 (C6), 132.9 (C2), 132.0 (C4), 128.1 (C7), 127.6 – 127.4 (1C, m, C13), 127.0 (1C, q, J = 3.3 Hz, C11), 126.2 (C3), 125.9 (C8), 124.6 (1C, q, J = 270.9 Hz, C14), 119.4 (1C, q, J = 32.1 Hz, C12), 116.1 (C10), 52.8 (C20), 50.6 (C16), 49.4 (C26), 38.7 (C24), 36.0 (C27), 35.7 (C17), 30.8 (C21), 26.5 (C23), 22.0 (C22); HRMS (ESI) calcd for [C29H30N6O935ClF3 + H]+: 674.1841, found: 674.1870.
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-aspartyl-L-lysyl-L-glutamic acid TFA salt (99)

Prepared by microwave Fmoc-SPPS to provide the title compound 99 as a white solid (29 mg, 0.04 mmol, 16%):

**HPLC** tᵣ = 8.54 mins (5-95% B); **m.p.** decomp. above 350 °C; **IR** υmax: 3127 (br s, O-H/NH₃⁺), 1710 (s, C=O), 1650 (s, C=O), 1528 (m, C=C), 1453 (w, C=C); **¹H NMR** (500 MHz, DMSO-d₆) δ 12.37 (3H, s br, H₃₂/H₃₆/H₃₇), 10.63 (1H, s, H15), 8.87 (1H, d, J = 7.3 Hz, H31), 8.09 (1H, d, J = 2.9 Hz, H33), 8.07 (1H, d, J = 2.4 Hz, H35), 8.03 (1H, d, J = 1.7 Hz, H7), 7.86 (1H, dd, J = 8.0, 1.7 Hz, H3), 7.67 (3H, s br, H34), 7.62 (1H, dd, J = 8.6, 1.5 Hz, H11), 7.49 (1H, d, J = 8.0 Hz, H4), 7.44 (1H, d, J = 2.2 Hz, H13), 7.12 (1H, d, J = 8.5 Hz, H10), 4.80 – 4.71 (1H, m, H16), 4.28 (1H, td, J = 8.6, 5.0 Hz, H20), 4.23 – 4.15 (1H, m, H26), 2.90 – 2.67 (4H, m, H17/H24), 2.32 – 2.23 (2H, m, H28), 2.03 – 1.92 (1H, m, H27), 1.86 – 1.75 (1H, m, H27'), 1.75 – 1.64 (1H, m, H21), 1.62 – 1.44 (3H, m, H21'/H23), 1.35 – 1.32 (2H, m, H22); **¹³C NMR** (126 MHz, DMSO-d₆) δ 173.7 (C29), 173.1 (C30), 171.9 (C18), 171.5 (C25), 170.6 (C16), 164.9 (C1), 158.0 (C9), 139.2 (C5), 134.8 (C6), 132.9 (C2), 132.0 (C4), 128.0 (C7), 127.6 (1C, q, J = 3.2 Hz, C13), 127.1 – 126.8 (1C, m, C11), 126.1 (C3), 125.9 (C8), 124.6 (1C, q, J = 270.9 Hz, C14), 119.4 (1C, q, J = 32.0 Hz, C12), 116.1 (C10), 52.1 (C20), 51.2 (C26), 50.6 (C16), 38.8 (C24), 35.8 (C17), 31.1 (C21), 30.0 (C28), 26.6 (C23), 26.2 (C27), 22.0 (C22); **HRMS** (ESI) calcd for [C₂₉H₂₂N₄O₁₀³⁵ClF₃]: 688.1759, found: 688.1744.
(2-Chloro-2’-(2,2,2-trifluoroacetoxy)-5’-(trifluoromethyl)-[1,1’-biphenyl]-4-carbonyl)-L-aspartyl-L-lysyl-L-glutamic acid TFA salt (99a)

Prepared by microwave Fmoc-SPPS to provide the title compound 99a as a white solid (17 mg, 0.02 mmol, 8%):

**HPLC** $t_r$ = 7.35 mins (5-95% B); **m.p.** decomp. above 350 °C; **IR** $\nu_{\text{max}}$ 2951 (br s, O-H/NH$_3^+$), 1660 (s, C=O), 1526 (m, C=C), 1420 (m, C=C); **$^1$H NMR** (500 MHz, DMSO-$d_6$) $\delta$ 12.32 (3H, s br, H32/H36/H37), 8.91 (1H, d, $J$ = 7.3 Hz, H31), 8.15 (1H, d, $J$ = 1.3 Hz, H7), 8.10 (1H, d, $J$ = 7.7 Hz, H35), 8.08 (1H, d, $J$ = 8.0 Hz, H33), 8.05 (1H, dd, $J$ = 9.0, 2.1 Hz, H11), 7.97 (1H, dd, $J$ = 8.0, 1.7 Hz, H3), 7.94 (1H, d, $J$ = 2.1 Hz, H13), 7.74 – 7.67 (4H, m, H4/H34), 7.63 (1H, d, $J$ = 8.6 Hz, H10), 4.83 – 4.72 (1H, m, H16), 4.31 – 4.22 (1H, m, H20), 4.22 – 4.13 (1H, m, H26), 2.86 – 2.67 (4H, m, H17/H24), 2.31 – 2.23 (2H, m, H28), 2.03 – 1.93 (1H, m, H27), 1.84 – 1.65 (2H, m, H21/H27’), 1.60 – 1.47 (3H, m, H21’/H23), 1.42 – 1.28 (2H, m, H22); **$^{13}$C NMR** (126 MHz, DMSO-$d_6$) $\delta$ 173.7 (C29), 173.1 (C30), 171.8 (C18), 171.5 (C25), 170.5 (C16), 164.4 (C1), 157.9 (1C, q, $J$ = 31.8 Hz, C38), 152.0 (C9), 136.2 (C5), 136.0 (C6), 132.8 (C2), 131.8 (C4), 129.2 (1C, s br, C13), 129.1 (C8), 128.5 (1C, s br, C11), 128.3 (C7), 126.7 (C3), 126.7 (1C, q, $J$ = 33.1 Hz, C12), 123.6 (1C, q, $J$ = 272.3 Hz, C14), 117.2 (C10), 116.7 (1C, q, $J$ = 355.6 Hz, C39), 52.2 (C20), 51.2 (C26), 50.7 (C16), 38.7 (C24), 35.9 (C17), 31.2 (C21), 30.0 (C28) 26.6 (C23), 26.2 (C27), 22.0 (C22); **LCMS** $t_r$ 0.98 min, calcd for [C$_{31}$H$_{31}$N$_4$O$_{11}$ClF$_6$ - H]$^-$ 785.0, [M-H]$^-$ 785.8.
(2-Chloro-2’-hydroxy-5’-(trifluoromethyl)-[1,1’-biphenyl]-4-carbonyl)-L-aspartyl-L-glutaminyl-L-aspartic acid (100)

Prepared by microwave Fmoc-SPPS to provide the title compound 100 as a white solid (17 mg, 0.03 mmol, 12%):

**HPLC** t_r = 9.00 mins (5-95% B); **m.p.** decomp. above 350 °C; **IR** u_{max} 3333 (br s, O-H/NH), 1716 (s, C=O), 1643 (s, C=O), 1530 (m, C=C), 1410 (w, C=C); **^1HNMR** (500 MHz, DMSO-d_6) δ 12.42 (3H, s br, H30/H34/H35), 10.58 (1H, s, H15), 8.86 (1H, d, J = 7.5 Hz, H29), 8.21 (1H, d, J = 8.0 Hz, H33), 8.08 (1H, d, J = 8.0 Hz, H31), 8.03 (1H, d, J = 1.7 Hz, H7), 7.87 (1H, dd, J = 8.0, 1.7 Hz, H3), 7.62 (1H, dd, J = 8.6, 1.9 Hz, H11), 7.49 (1H, d, J = 8.0 Hz, H4), 7.45 (1H, d, J = 2.1 Hz, H13), 7.19 (1H, s br, H32), 7.12 (1H, d, J = 8.5 Hz, H10), 6.74 (1H, s br, H32’), 4.79 (1H, ddd, J = 9.6, 7.5, 4.5 Hz, H16), 4.53 (1H, dd, J = 14.2, 6.4 Hz, H25), 4.27 (1H, td, J = 8.5, 5.0 Hz, H20), 2.83 (1H, dd, J = 16.7, 4.4 Hz, H17), 2.75 – 2.65 (2H, m, H17’/H26), 2.59 (1H, dd, J = 16.7, 6.7 Hz, H26’), 2.21 – 2.07 (2H, m, H22), 1.96 – 1.87 (1H, m, H21), 1.82 – 1.68 (1H, m, H21’); **^13CNMR** (126 MHz, DMSO-d_6) δ 173.8 (C23), 172.2 (C18/C27), 171.9 (C28), 171.6 (C18/C27), 171.0 (C24), 170.5 (C19), 165.0 (C1), 158.0 (C9), 139.1 (C8), 134.9 (C6), 132.9 (C2), 131.9 (C4), 128.1 (C7), 127.8 – 127.3 (1C, m, C13), 127.2 – 126.7 (1C, m, C11), 126.2 (C3), 126.0 (C8), 124.6 (1C, q, J = 270.8 Hz, C14), 119.4 (1C, q, J = 32.1 Hz, C12), 116.1 (C10), 52.1 (C20), 50.5 (C16), 48.5 (C25), 35.9 (2C, C17/C26), 31.3 (C22), 28.0 (C21); **HRMS** (ESI) calcd for [C_{27}H_{28}N_{11}O_{13}ClF_{3} + H]^+: 675.1302, found: 675.1300.
(2-Chloro-2’-{2,2,2-trifluoroacetoxy}-5’-{trifluoromethyl}-[1,1’-biphenyl]-4-carbonyl)-L-aspartyl-L-glutaminyl-L-aspartic acid (100a)

Prepared by microwave Fmoc-SPPS to provide the title compound 100a as a white solid (17 mg, 0.02 mmol, 10%):

**HPLC** $t_r = 7.67$ mins (5-95% B); **m.p.** decomposed above 350 °C; **IR** $\nu_{\text{max}}$ 3315 (br s, O-H), 1650 (s, C=O), 1527 (m, C=C), 1411 (m, C=C); **$^1$H NMR** (500 MHz, DMSO-$d_6$) $\delta$ 12.49 (3H, s br, H30/H34/H35), 8.92 (1H, d, $J = 7.4$ Hz, H29), 8.25 (1H, d, $J = 8.0$ Hz, H33), 8.15 (1H, d, $J = 1.4$ Hz, H7), 8.08 – 8.00 (2H, m, H11/H31), 7.92 (2H, m, H3/H13), 7.72 (1H, d, $J = 8.0$ Hz, H4), 7.63 (1H, d, $J = 8.7$ Hz, H10), 7.21 (1H, s br, H32), 6.75 (1H, s br, H32'), 4.84 – 4.75 (1H, m, H16), 4.52 (1H, dd, $J = 14.1$, 6.5 Hz, H25), 4.32 – 4.22 (1H, m, H20), 2.84 (1H, dd, $J = 16.7$, 4.1 Hz, H17), 2.77 – 2.64 (2H, m, H17'/H26), 2.59 (1H, dd, $J = 16.7$, 6.7 Hz, H26'), 2.18 – 2.05 (2H, m, H22), 1.98 – 1.87 (1H, m, H21), 1.77 – 1.70 (1H, m, H2); **$^{13}$C NMR** (126 MHz, DMSO-$d_6$) $\delta$ 173.8 (C23), 172.2 (C28), 171.8 (C18), 171.6 (C27), 171.0 (C24), 170.4 (C19), 164.5 (C1), 158.0 (1C, q, $J = 32.8$ Hz, C36), 152.0 (C9), 136.2 9 (C5), 136.0 (C6), 132.8 (C2), 131.8 (C4), 129.5 – 129.2 (1C, m, C13), 129.1 (C8), 128.7 – 128.4 (1C, m, C11), 128.3 (C7), 126.7 (C3), 126.7 (1C, q, $J = 32.6$ Hz, C12), 123.6 (1C, q, $J = 272.5$ Hz, C14), 117.3 (C10), 116.8 (1C, q, $J = 294.3$ Hz, C37), 52.0 (C20), 50.6 (C16), 48.5 (C25), 35.9 (2C, C17/C26), 31.3 (C22), 28.1 (C21); **HRMS** (ESI) calcd for $[C_{29}H_{21}N_4O_{12}^{35}ClF_6]$: 770.1062, found: 770.1038.
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-aspartyl-L-lysyl-L-asparagine TFA salt (101)

Prepared by microwave Fmoc-SPPS to provide the title compound 101 as a white solid (50 mg, 0.06 mmol, 39%):

**HPLC** \( t_r = 8.29 \text{ mins (5-95\% B)} \); **m.p.** decomp. above 350 °C; **IR** \( \nu_{\text{max}} \) 3065 (br s, O-H/NH \(^3\)), 1651 (s, C=O), 1519 (m, C=C), 1429 (w, C=C); \(^1\text{H NMR} \) (500 MHz, DMSO-\(d_6\)) \( \delta \) 12.41 (2H, s br, H31/H36), 10.64 (1H, s, H15), 8.87 (1H, d, \( J = 7.4 \text{ Hz} \), H30), 8.12 (1H, d, \( J = 7.9 \text{ Hz} \), H34), 8.07 (1H, d, \( J = 8.1 \text{ Hz} \), H32), 8.03 (1H, d, \( J = 1.7 \text{ Hz} \), H7), 7.86 (1H, dd, \( J = 8.0, 1.7 \text{ Hz} \), H3), 7.77 – 7.56 (4H, m, H11/H33), 7.49 (1H, d, \( J = 8.0 \text{ Hz} \), H4), 7.44 (1H, d, \( J = 2.2 \text{ Hz} \), H13), 7.40 (1H, s br, H35), 7.12 (1H, d, \( J = 8.5 \text{ Hz} \), H10), 6.92 (1H, s br, H35'), 4.78 (1H, ddd, \( J = 9.7, 7.4, 4.5 \text{ Hz} \), H16), 4.50 (1H, dd, \( J = 13.9, 6.3 \text{ Hz} \), H26), 4.28 (1H, td, \( J = 8.6, 4.9 \text{ Hz} \), H20), 2.84 (1H, d, \( J = 16.8 \), 4.4 Hz, H17), 2.78 – 2.70 (3H, m, H17'/H24), 2.56 (1H, dd, \( J = 15.7, 5.7 \text{ Hz} \), H27), 1.76 – 1.63 (1H, m, H21), 1.60 – 1.45 (3H, m, H20'/H23), 1.41 – 1.27 (2H, m, H22); \(^{13}\text{C NMR} \) (126 MHz, DMSO-\(d_6\)) \( \delta \) 172.6 (C29), 171.9 (C18), 171.2 (C25/C28), 171.1 (C25/C28), 170.5 (C19), 164.9 (C1), 158.0 (C9), 139.2 (C5), 134.9 (C6), 132.9 (C2), 132.0 (C4), 128.0 (C7), 127.6 (1C, s br, C13), 127.0 (1C, s br, C11), 126.1 (C3), 125.9 (C8), 124.6 (1C, q, \( J = 270.9 \text{ Hz} \), C14), 119.38 (1C, q, \( J = 32.3 \text{ Hz} \), C12), 116.1 (C10), 52.0 (C20), 50.6 (C16), 48.7 (C26), 38.8 (C24), 36.5 (C27), 35.8 (C17), 31.3 (C21), 26.6 (C23), 21.9 (C22); **HRMS** (ESI) calcd for \([C_{28}H_{31}N_5O_9^{35}ClF_3]^-\): 673.1762, found: 673.1735.

H27' is hidden under the DMSO solvent peak, confirmed by 2D NMR analysis.
(2-Chloro-2'-(2-chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)oxy)-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-aspartyl-L-lysyl-L-asparagine TFA salt (101a)

Prepared by microwave Fmoc-SPPS to provide the title compound 101a as a white solid (7 mg, 0.01 mmol, 4%):

**HPLC** $t_r = 11.24$ mins (5-95% B); **m.p.** decomp. above 350 °C; **IR** $\nu_{max}$ 3082 (br s, O-H/NH$_3^+$), 1718 (s, C=O), 1662 (br s, C=O), 1535 (m, C=C), 1422 (w, C=O); **$^1$H NMR** (500 MHz, DMSO-d$_6$) $\delta$ 12.33 (2H, s br, H$_{31}$/H$_{36}$), 10.71 (1H, s, H$_{51}$), 8.87 (1H, d, $J = 7.2$ Hz, H$_{30}$), 8.12 (1H, d, $J = 7.7$ Hz, H$_{32}$), 8.09 – 8.04 (2H, m, H$_7$/H$_{34}$), 8.02 (1H, dd, $J = 8.8, 2.0$ Hz, H$_{11}$), 7.95 (1H, d, $J = 1.7$ Hz, H$_{43}$), 7.93 – 7.84 (3H, m, H$_3$/H$_{13}$/H$_{39}$), 7.82 (1H, d, $J = 8.5$ Hz, H$_{10}$), 7.71 – 7.58 (5H, m, H$_4$/H$_{47}$/H$_{47}$), 7.55 (1H, d, $J = 8.0$ Hz, H$_{40}$), 7.49 (1H, d, $J = 2.0$ Hz, H$_{49}$), 7.39 (1H, s br, H$_{35}$), 7.12 (1H, d, $J = 8.5$ Hz, H$_{46}$), 6.91 (1H, s br, H$_{35'}$), 4.74 (1H, ddd, $J = 9.8, 7.3, 4.4$ Hz, H$_{16}$), 4.48 (1H, dd, $J = 13.9, 6.2$ Hz, H$_{26}$), 4.31 – 4.18 (1H, m, H$_2$), 2.80 (1H, dd, $J = 16.8, 4.2$ Hz, H$_{17}$), 2.77 – 2.64 (3H, m, H$_{17'}$/H$_{24}$), 2.55 (1H, dd, $J = 15.8, 5.7$ Hz, H$_{27}$), 1.74 – 1.63 (1H, m, H$_{21}$), 1.57 – 1.43 (3H, m, H$_{21'}$/H$_{23}$), 1.38 – 1.27 (2H, m, H$_{22}$); **$^{13}$C NMR** (126 MHz, DMSO-d$_6$) $\delta$ 172.6 (C$_{29}$), 171.8 (C$_{18}$), 171.2 (C$_{25}$/C$_{28}$), 171.2 (C$_{25}$/C$_{28}$), 170.5 (C$_1$), 164.5 (C$_1$), 162.3 (C$_{37}$), 157.8 (C$_{45}$), 150.6 (C$_9$), 142.2 (C$_{41}$), 137.0 (C$_5$), 135.6 (C$_{2}$/C$_6$), 133.7 (C$_{38}$/C$_{42}$), 132.9 (C$_{40}$), 132.4 (C$_2$/C$_4$), 132.3 (C$_8$), 131.9 (C$_4$), 129.9 (C$_{43}$), 129.0 (C$_{38}$/C$_{42}$), 128.3 (C$_7$), 128.3 (C$_{1}$), 128.0 (C$_{43}$), 127.6 (C$_1$), 126.8 (1C, q, $J = 61.8$ Hz, C$_{12}$), 126.5 (C$_3$), 125.4 (C$_{44}$), 124.6 (1C, q, $J = 271.1$ Hz, C$_{50}$), 124.3 (C$_{10}$), 123.8 (1C, q, $J = 272.5$ Hz, C$_{14}$), 119.5 (1C, q, $J = 32.2$ Hz, C$_{48}$), 116.2 (C$_{46}$), 52.1 (C$_{20}$), 50.5 (C$_{16}$), 48.7 (C$_{26}$), 38.8 (C$_{24}$), 36.5 (C$_{27}$), 35.8 (C$_{17}$), 31.2 (C$_{21}$), 26.6 (C$_{23}$), 21.9 (C$_{22}$); **HRMS** (ESI) calcd for $[C_{42}H_{37}N_3O_{11}Cl_2F_6]$; 971.1771, found: 971.1687.

(H$_{27'}$ is hidden under the DMSO solvent peak, confirmed by 2D NMR analysis)
(2-Chloro-2′-hydroxy-5′-(trifluoromethyl)-[1,1′-biphenyl]-4-carbonyl)-L-aspartyl-L-tryptophyl-L-aspartic acid (102)

Prepared by manual Fmoc-SPPS to provide the title compound 102 as a white solid (32 mg, 0.04 mmol, 21%):

**HPLC** t_r = 6.57 mins (40-60% B); **m.p.** decomp. above 350 °C; **IR** u_{max} 3305 (br s, O-H), 1716 (s, C=O), 1641 (s, C=O), 1521 (s, C=C), 1423, (m, C=C); **^1^H NMR** (500 MHz, DMSO-d_6) δ 12.47 (3H, s br, H36/H40/H41), 10.78 (1H, d, J = 1.9 Hz, H38), 10.59 (1H, s, H15), 8.78 (1H, d, J = 7.6 Hz, H35), 8.30 (1H, d, J = 7.8 Hz, H39), 8.00 (1H, d, J = 8.0 Hz, H37), 7.97 (1H, d, J = 1.7 Hz, H7), 7.81 (1H, dd, J = 8.0, 1.7 Hz, H3), 7.62 (1H, dd, J = 8.6, 2.0 Hz, H11), 7.57 (1H, d, J = 7.9 Hz, H28), 7.47 (1H, d, J = 8.0 Hz, H4), 7.45 (1H, d, J = 2.1 Hz, H13), 7.29 (1H, d, J = 8.1 Hz, H25), 7.15 (1H, d, J = 2.3 Hz, H23), 7.12 (1H, d, J = 8.5 Hz, H10), 7.05 – 6.98 (1H, m, H26), 6.95 – 6.89 (1H, m, H27), 4.78 (1H, ddd, J = 9.6, 7.7, 4.5 Hz, H16), 4.60 – 4.50 (2H, m, H20/H31), 3.15 (1H, dd, J = 14.8, 4.3 Hz, H21), 2.98 (1H, dd, J = 14.9, 8.7 Hz, H21'), 2.78 (1H, dd, J = 16.7, 4.4 Hz, H17), 2.72 – 2.60 (2H, m, H17'/H32), 2.59 – 2.52 (1H, m, H32'); **^13^C NMR** (126 MHz, DMSO-d_6) δ 172.3 (C33/C34), 171.9 (C18), 171.7 (C33/C34), 171.2 (C13), 170.4 (C19), 164.9 (C1), 158.0 (C9), 139.1 (C5), 136.0 (C24), 134.8 (C6), 132.9 (C2), 131.9 (C4), 128.1 (C7), 127.6 (1C, s br, C13), 127.4 (C29), 127.0 (1C, s br, C11), 126.1 (C3), 126.0 (C8), 124.6 (1C, q, J = 270.9 Hz, C14), 123.7 (C23), 120.8 (C26), 119.4 (1C, q, J = 32.2 Hz, C12), 118.3 (C28), 118.2 (C27), 116.1 (C10), 111.2 (C25), 109.8 (C22), 53.4 (C20), 50.4 (C16), 48.7 (C31), 36.1 (C17/C32), 35.8 (C17/C32), 27.5 (C21); **HRMS** (ESI) calcd for [C_{33}H_{28}N_{4}O_{10}^{35}ClF_{3} - H]: 731.1373, found: 731.1363.
(()-2-Chloro-2′-hydroxy-S′-(trifluoromethyl)-[1,1′-biphenyl]-4-yl)methyl-L-aspartyl-L-alanyl-L-aspartic acid TFA salt (107)

Prepared by manual Fmoc-SPPS except following deprotection of the final Asp residue a solution of aldehyde 63 (60 mg, 0.2 mmol) in DMF (1.67 mL) was added and the mixture shaken for 5 minutes. NaBH(OAc)$_3$ (212 mg, 1.0 mmol) was added as one portion and the reaction left to shake for 24 hours before the resin was drained and rinsed with DMF (3 x 6 mL), MeOH (3 x 6 mL) and CH$_2$Cl$_2$ (3 x 6 mL). Final peptide deprotection and resin cleavage was performed as described in the manual Fmoc-SPPS general procedure to provide the title compound 107 as a white solid (4 mg, 0.01 mmol, 6%):

**HPLC** $t_r = 8.90$ mins (5-95% B); **m.p.** decomp. above 350 °C; **$^1$H NMR** (500 MHz, DMSO-d$_6$) $\delta$ 10.49 (1H, s, H15), 8.37 (1H, s br, H29), 8.22 (1H, d, $J = 7.9$ Hz, H30), 7.58 (1H, dd, $J = 8.6$, 2.0 Hz, H11), 7.55 (1H, s br, H7), 7.40 – 7.34 (2H, m, H3/H13), 7.32 (1H, d, $J = 7.8$ Hz, H4), 7.10 (1H, d, $J = 8.5$ Hz, H10), 4.52 (1H, dd, $J = 14.2$, 6.5 Hz, H23), 4.36 (1H, dt, $J = 14.5$, 7.1 Hz, H20), 3.91 – 3.58 (3H, m, H1/H16), 2.73 – 2.55 (4H, m, H17/H24), 1.23 (3H, d, $J = 7.0$ Hz, H21); **$^{13}$C NMR** (126 MHz, DMSO-d$_6$) $\delta$ 172.3 (C25/C26), 171.8 (C22), 171.6 (C25/C26), 158.1 (C9), 135.0 (C5), 132.6 (C6), 131.7 (C4), 129.0 (C7), 127.8 (1C, s br, C13), 127.0 (C3), 126.8 (1C, s br, C11), 126.4 (C8), 124.7 (1C, q, $J = 271.0$ Hz, C14), 119.3 (1C, dd, $J = 64.0$, 31.9 Hz, C12), 116.0 (C10), 57.3 (C16), 49.5 (C1), 48.6 (C23), 47.9 (C20), 36.8 (C17), 36.0 (C24), 18.4 (C21); **LCMS** $t_r = 1.19$ min, calcd for [C$_{25}$H$_{25}$N$_3$O$_9^{35}$ClF$_3$ - H] $^+$ 602.1, [M-H] $^-$ 602.5; **HRMS** (ESI) calcd for [C$_{25}$H$_{25}$N$_3$O$_9^{35}$ClF$_3$]: 603.1231, found: 603.1210.
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-D-aspartyl-L-alanyl-L-aspartic acid (108)

Prepared by manual Fmoc-SPPS to provide the title compound 108 as a white solid (15 mg, 0.02 mmol, 23%):

**HPLC** $t_r = 9.77$ mins (5-95% B); m.p. decom. above 350 °C; **IR** $\nu_{\text{max}}$ 3307 (br s, O-H), 1716 (s, C=O), 1641 (s, C=O), 1530 (m, C=C); **$^1$H NMR** (500 MHz, DMSO-d$_6$) $\delta$ 12.42 (3H, s br, H$_{28}$/H$_{31}$/H$_{32}$), 10.58 (1H, s, H15), 8.88 (1H, d, $J = 7.4$ Hz, H27), 8.20 (1H, d, $J = 8.1$ Hz, H30), 8.09 (1H, d, $J = 7.8$ Hz, H29), 8.04 (1H, d, $J = 1.7$ Hz, H7), 7.87 (1H, dd, $J = 8.0$, 1.7 Hz, H3), 7.62 (1H, dd, $J = 8.6$, 2.0 Hz, H11), 7.49 (1H, d, $J = 8.0$ Hz, H4), 7.45 (1H, d, $J = 2.1$ Hz, H13), 7.12 (1H, d, $J = 6.5$ Hz, H10), 4.77 (1H, ddd, $J = 8.8$, 7.5, 5.3 Hz, H16), 4.54 (1H, dd, $J = 14.3$, 6.8 Hz, H23), 4.41 – 4.27 (1H, m, H20), 2.79 (1H, dd, $J = 16.5$, 5.2 Hz, H17), 2.75 – 2.65 (2H, m, H17'/H24), 2.59 (1H, dd, $J = 16.6$, 7.0 Hz, H24'), 1.21 (3H, d, $J = 7.1$ Hz, H21);

**$^{13}$C NMR** (126 MHz, DMSO-d$_6$) $\delta$ 172.2 (1C, C25/C26), 171.8 (2C, C18/C22), 171.6 (1C, C25/C26), 170.0 (C19), 165.0 (C1), 158.0 (C9), 139.2 (C5), 134.8 (C6), 132.9 (C2), 131.9 (C4), 128.1 (C7), 127.6 (1C, s br, C13), 127.0 (1C, s br, C11), 126.2 (C3), 126.0 (C8), 124.6 (1C, q, $J = 270.9$ Hz, C14), 119.4 (1C, q, $J = 32.4$ Hz, C12), 116.1 (C10), 50.6 (C16), 48.6 (C23), 47.9 (C20), 35.9 (C24), 35.9 (C17), 18.3 (C21); **HRMS** (ESI) calcd for [C$_{25}$H$_{23}$N$_3$O$_{10}$ClF$_3$ + H$^+$]: 618.1102, found: 618.1091.
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-D-aspartyl-L-lysyl-L-aspartic acid TFA salt (109)

Prepared by manual Fmoc-SPPS to provide the title compound 109 as a white solid (18 mg, 0.02 mmol, 22%):

**HPLC** $t_r = 8.85$ mins (5-95% B); **m.p.** decomp. above 350 °C; **IR** $\nu_{max}$ 3072 (br s, O-H/NH$_3$ +), 1715 (s, C=O), 1636 (s, C=O), 1520 (m, C=C), 1428 (w, C=C); **$^1$H NMR** (500 MHz, DMSO-$d_6$) δ 12.46 (3H, s br, H31/H35/H36), 10.63 (1H, s, H15), 8.90 (1H, d, $J = 7.2$ Hz, H30), 8.24 (1H, d, $J = 8.0$ Hz, H32), 8.08 (1H, d, $J = 8.0$ Hz, H34), 8.04 (1H, d, $J = 1.7$ Hz, H7), 7.87 (1H, dd, $J = 8.0$, 1.7 Hz, H3), 7.78 – 7.56 (4H, m, H11/H33), 7.49 (1H, d, $J = 8.0$ Hz, H4), 7.44 (1H, d, $J = 2.2$ Hz, H13), 7.12 (1H, d, $J = 8.5$ Hz, H10), 4.78 (1H, ddd, $J = 8.8$, 7.2, 5.4 Hz, H16), 4.54 (1H, dd, $J = 14.0$, 7.0 Hz, H26), 4.31 (1H, td, $J = 8.7$, 4.7 Hz, H20), 2.83 – 2.65 (5H, m, H17/H24/H27), 2.60 (1H, dd, $J = 16.6$, 7.1 Hz, H27'), 1.77 – 1.65 (1H, m, H21), 1.59 – 1.42 (3H, m, H21'/H23), 1.34 – 1.23 (2H, m, H22); **$^{13}$C NMR** (126 MHz, DMSO-$d_6$) δ 172.2 (1C, C28/C29), 171.8 (C18), 171.6 (1C, C28/C29), 171.1 (C25), 170.4 (C19), 165.1 (C1), 158.0 (C9), 139.2 (C5), 134.8 (C6), 132.9 (C2), 132.0 (C4), 128.1 (C7), 127.6 (1C, s br, C13), 127.0 (1C, s br, C11), 126.2 (C3), 125.9 (C8), 124.6 (1C, q, $J = 270.9$ Hz, C14), 119.4 (1C, q, $J = 32.1$ Hz, C12), 116.1 (C10), 51.8 (C20), 50.8 (C16), 48.6 (C26), 38.7 (C24), 35.90 (1C, C17/C27), 35.85 (1C, C17/C27), 30.7 (C21), 26.6 (C23), 21.9 (C22); **HRMS** (ESI) calcd for [C$_{28}$H$_{30}$N$_4$O$_{10}$F$_3$ + H]^+: 675.1681, found: 675.1697.
Characterisation data for peptides of 4 amino acids or longer:

<table>
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<tr>
<th>Number</th>
<th>Sequence</th>
<th>Yield (%)</th>
<th>HPLC t. (min) (5-95% B)</th>
<th>HPLC t. (min) (20-60% B)</th>
<th>Calculated MW [M+H]^+ (Da)</th>
<th>Found MW [M+H]^+ (Da)</th>
<th>Fmoc-SPPS method</th>
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<tr>
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<td>78-AADD-OH</td>
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<td>689.1452</td>
<td>Microwave</td>
</tr>
<tr>
<td>96</td>
<td>78-AADD-NH₂</td>
<td>12</td>
<td>9.22</td>
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<td>688.1633</td>
<td>688.1635</td>
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<tr>
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<td>8.70</td>
<td>8.49</td>
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<td>1280.2712</td>
<td>Automated</td>
</tr>
<tr>
<td>111</td>
<td>74-dADDDSDDDD-OH</td>
<td>15</td>
<td>9.01</td>
<td>8.89</td>
<td>1312.2229^2</td>
<td>1312.2288^2</td>
<td>Automated</td>
</tr>
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</table>

All peptides were purified by semi-preparative HPLC with a gradient of 30-50% over 20 min and reported HPLC t. refers to the retention time reported on the analytical HPLC.

^1 HPLC t. (min) (30-80% B)

^2 Calculated MW [M-H]^- (Da)

^3 Found MW [M-H]^- (Da)
4.6 Computational Docking Procedure

All the docking studies reported herein were performed using Glide from the Schrödinger package, set to default parameters.\(^{254-257}\)

The protein structures were prepared from the PDB files of the X-ray crystal structures generated by Dr. Paul Brear (Hyvönen Group, Department of Biochemistry, University of Cambridge). The protein structure was prepared for docking using the Protein Preparation Wizard application. Bond orders were assigned, hydrogen atoms added and water molecules beyond 5 Å from hetero groups were deleted. The structure was refined by sampling of hydrogen bonds and water molecule orientations at pH 7 \(\pm 2\). To keep the conformation of the protein as close as possible to the X-ray crystal structures, no modifications such as ‘cap termini’, ‘filling loops’ or ‘side chain’ were performed. The energy of the structure was then minimised.

The parent ligands of the X-ray crystal structures were used as the templates for Glide grid generation. No positional constraints were given and all parameters were left as default. An OPLS_2005 force field was used.

Chemical structures were imported from SDF files. Ligands were then prepared with the Ligand Preparation wizard, using OPLS_2005 as the force field. All other parameters were left as default. Conformations of the output structures were generated by the Conformation Generation wizard using fast mode, default setting and OPLS_2005 force field.

Virtual screening mode was then used to rigidly dock molecules into the receptor grid. The docking score consisted of Van Der Waals, lipophilic, hydrogen bonding and metal interaction components, along with non-hydrogen bonding polar interactions in hydrophobic sites.

Subsequent protein-ligand visualisation and image rendering was performed using Pymol from the Schrödinger package.\(^{258}\)

The following X-ray crystal structure PDB files were used for computational docking. The chemical structures of the original αD pocket ligands are provided and the figure numbers reporting the results:

<table>
<thead>
<tr>
<th>PDB File</th>
<th>Ligand Chemical Structure</th>
<th>Docking Results</th>
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</thead>
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<td><img src="image" alt="Chemical Structure" /></td>
<td>Figure 28</td>
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<tr>
<td>58</td>
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<td>Figure 38</td>
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<tr>
<td>61a</td>
<td><img src="image3" alt="Chemical Structure 61a" /></td>
<td>Figure 41</td>
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<tr>
<td>78</td>
<td><img src="image4" alt="Chemical Structure 78" /></td>
<td>Figure 55, 57, 58, 59, 61, 62 and 63</td>
</tr>
</tbody>
</table>
4.7 Crystallisation and Screening Conditions

Crystallisation and screening experiments were conducted by Dr. Paul Brear, Department of Biochemistry, University of Cambridge. Cell-based assays were conducted by Dr Maxim Rossman, Department of Biochemistry, University of Cambridge.

Protein Expression and Purification

Two constructs of CK2α were used in this project. For ITC and kinase activity assays CK2α_WT was used (residues 2-329). For crystallization purposes CK2α_KA was used. CK2α_KA (residues 2-329) contained four mutations designed to aid crystallization by reducing the overall charge of the protein; R21S, K74A, K75A and K76A. CK2α_KA was cloned into pHAT2 vector to give constructs with cleavable His6-tags. Recombinant plasmids containing one of the two constructs (CK2α_WT/CK2α_KA) were introduced into Escherichia coli BL21(DE3) for protein production. Single colonies of the cells were grown in 6×1L of 2×TY with 100 μg/mL ampicillin at 37°C. Isopropylthio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression when the optical density at 600 nm reached 0.6. The cells were incubated overnight at 25°C then harvested by centrifugation at 4,000 g for 20 minutes. The same extraction and purification procedure was used for both constructs, with the exception that CK2α_KA used 350 mM NaCl in the buffer, whereas, CK2α_WT required 500 mM NaCl. The cell pellets were suspended in 20 mM Tris, 350/500 mM NaCl, pH 8.0 and lysed using a high pressure homogenizer. Protease inhibitor cocktail tablets (one tablet per 50 mL extract; Roche Diagnostics) and DNase I were then added. The crude cell extract was then centrifuged at 10,000 g for 45 minutes, the supernatant was filtered with a 0.22 μm filter. The soluble supernatant was applied on a Ni Sepharose Fast Flow 6 column at pH 8.0, washed and eluted in 20 mM Tris pH 8.0, 350/500 mM NaCl, 200 mM imidazole. After overnight dialysis into 20 mM Tris, pH 8.0, 350/500 mM NaCl the N-terminal His6-Tag was cleaved overnight by TEV protease and passed through a second metal affinity column to remove uncleaved protein and the protease. The cleaved protein was further purified on a Sepharose Q HP anion-exchange column and the main peak fraction from this column was further purified by gel filtration on a Superdex 75 16/60 HiPrep column equilibrated with Tris 20 mM, pH 8.0, 350/500 mM NaCl. Pure protein was concentrated to 15 mg/mL and flash frozen in liquid nitrogen.
**X-ray Crystallography**

CK2α_KA at 5 mg/mL in 20 mM Tris, pH 8.0, 350 mM NaCl, 1 mM DTT, and 25 mM ATP was crystallised with 112.5 mM MES pH 6.5, 35% glycerol ethoxylate and 180 mM ammonium acetate in a 1:1 ratio with a total volume of 2 μL by the hanging drop vapour-diffusion method. The fragments were soaked as singletons at 2-100 mM into these crystals for 15–20 h in 107 mM MES pH 6.5, 35% glycerol ethoxylate and 1.04 M ammonium acetate after which the crystals were cryo-cooled in liquid nitrogen for data collection. 12 X-ray diffraction data was collected at the Diamond synchrotron radiation source, then processed using the pipedream package by Global Phasing Ltd. Structures were solved by using programs from the CCP4 package. Models were iteratively refined and rebuilt by using AutoBuster and Coot programs. Ligand coordinates and restraints were generated from their SMILES strings using the Grade software package. All crystal structures reported in this thesis are of the resolution 1.4 – 2.5 Å.

**Isothermal Titration Calorimetry (ITC)**

All ITC experiments were performed at 25 °C using a MicroCal itc200 instrument (GE Healthcare). CK2α_WT (20 mg/mL, 20 mM Tris pH 8.0, 500 mM NaCl) was diluted in Tris buffer (200 mM Tris, 300 mM NaCl, 10% DMSO) and concentrated to 20–50 μM. Compounds in 100x stock solutions were diluted into the buffer ensuring that the DMSO concentrations were carefully matched. In a typical experiment CK2α_WT (40 μM) was loaded into the sample cell and 0.4–2.0 mM of the ligand was titrated in nineteen 2 μL injections of 2 s duration at 150 s intervals, with injector speed of 750 rpm. Heats of dilution were determined in identical experiments, but without protein in the cell. The data fitting was performed with a single site binding model using the Origin software package. Experiments were performed only once.

** Kinase Assays**

The kinase assays were performed using the ADP-Glo™ kinase assay kit (Promega). 50 nM CK2α_WT was incubated in the kinase reaction buffer (40 mM Tris pH7.5, 200 mM NaCl, 20 mM MgCl₂, 0.1 mg/mL BSA, 25 μM ATP, 50 μM substrate peptide (RRRADSDDDDD, Enzo Life Sciences Inc.), 5% (v/v) DMSO) in the presence of different concentrations of the inhibitor at 25 °C for 40 min. 5 μL aliquots of the kinase reaction were quenched with 5 μL of ADP-glo™ solution. After another 40 min the kinase detection reagent was added and maintained at 25 °C for 30 minutes. The luminescence was recorded using a PHERAstar FS plate reader (BMG LABTECH) with an integration time of 1 s. Percentage
inhibition was calculated relative to a DMSO control and a baseline measurement without ATP. The IC\textsubscript{50} curves were fitted using Sigma plot 11.0. Percentage inhibition experiments were carried out in triplicate and IC\textsubscript{50} experiments were carried out in duplicate.

**Cell Culture**

All cell lines used were obtained from ATCC and were supplied as mycoplasma free. HCT116 colon carcinoma cells were maintained in McCoy’s 5A (1x) + Glutamax-I growth medium (Gibco, 36600-021) supplemented with fetal bovine serum (FBS, Gibco Life Technologies, 10270-106) at a final concentration of 10%. All cells were grown at 37°C / 5% CO\textsubscript{2} in a humidified environment and all the assays were performed using these culturing conditions.

**Growth Inhibition Assays**

Adherent cell lines (HCT116) were seeded into flat-bottomed tissue culture 96-well plates in a volume of 150 μL of growth medium. HCT116 cells were seeded at 750 cells per well. After 24 hours, compounds dissolved in DMSO were diluted in growth medium and were added to cells such that the final DMSO concentration was 1% (v/v) and the final volume in the well was 200 μL. Cells were then incubated in the presence compound for 72 hours before fixation. Without removing supernatant 100 13 μL of cold 10% (v/v) trichloroacetic acid was added to each well and the plates were incubated for 30 minutes at 4 °C. After that the plates were washed three times in tap water and left to dry at room temperature. The fixed cells were stained in a 0.057% sulforhodamine B/1% acetic acid solution (w/v) and incubated at room temperature with agitation for 30 minutes after which the dye was removed and the plates washed in 1% (v/v) acetic acid and left to dry. The dye was then solubilised in 200 μL 10 mM Tris solution (pH 10.5) and incubated for 30 minutes under agitation. The 510 nm absorbance was then measured using a PHERAstar plus plate reader (BMG Labtech). Percentage of growth inhibition was calculated relative to DMSO controls and GI\textsubscript{50} values were calculated using Graphpad Prism. All experiments were carried out in duplicate.
References


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Appendix

Appendix 1: A Fragment-Based Drug Discovery Approach for the Development of Selective Inhibitors of Protein Kinase CK2α

Appendix 1A: $^1$H and $^{13}$C NMR Spectra

Appendix 1B: Analytical HPLC Traces for Selected Peptides

Appendix 1C: Mass Spectra for Selected Peptides

Appendix 2: Publications

Appendix 2A: Second-Generation CK2α Inhibitors Targeting the αD Pocket

Appendix 2B: Partially Saturated Bicyclic Heteroaromatics as an sp³-Enriched Fragment Collection
Appendix 1: A Fragment-Based Drug Discovery Approach for the Development of Selective Inhibitors of Protein Kinase CK2α

Appendix 1A: $^1$H and $^{13}$C NMR Spectra

2'-Chloro-[1,1'-biphenyl]-3,4'-dicarbonitrile (44a)
(2'-Chloro-[1,1'-biphenyl]-3,4'-diyl)dimethanamine TFA salt (44)
2-Chloro-3'-methyl-[1,1'-biphenyl]-4-carbonitrile (45a)
(2-Chloro-3'-methyl-[1,1'-biphenyl]-4-yl)methanamine TFA salt (45)
2-Chloro-3'-vinyl-[1,1'-biphenyl]-4-carbonitrile (46a)
(2-Chloro-3'-vinyl-[1,1'-biphenyl]-4-yl)methanamine TFA salt (46)
2-Chloro-4-cyanophenyl trifluoromethanesulfonate (48)
2-Chloro-3'-hydroxy-[1,1'-biphenyl]-4-carbonitrile (51a)
4′-(Aminomethyl)-2′-chloro-[1,1′-biphenyl]-3-ol TFA salt (51)
2-Chloro-3’-(hydroxymethyl)-[1,1’-biphenyl]-4-carbonitrile (54a)
(4'-Aminomethyl)-2'-chloro-[1,1'-biphenyl]-3-yl)methanol TFA salt (54)
2-Chloro-3'-ethyl-[1,1'-biphenyl]-4-carbonitrile (55a)
2-Chloro-3'-fluoro-[1,1'-biphenyl]-4-carbonitrile (56a)
(2-Chloro-3'-fluoro-[1,1'-biphenyl]-4-yl)methanamine TFA salt (56)
2-Chloro-3'-ethynyl-[1,1'-biphenyl]-4-carbonitrile (57a)
(2-Chloro-3’-ethynyl-[1,1’-biphenyl]-4-yl)methanamine TFA salt (57)
2-Chloro-3'-{(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (58a)
(2-Chloro-3’-(trifluoromethyl)-[1,1’-biphenyl]-4-yl)methanamine TFA salt (58)
3-Chloro-4-(thiophen-2-yl)benzonitrile (59a)
(3-Chloro-4-(thiophen-2-yl)phenyl)methanamine HCl salt (59)
3-Chloro-4-(thiophen-3-yl)benzonitrile (60a)
(3-Chloro-4-(thiophen-3-yl)phenyl)methanamine HCl salt (60)
2-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-4-(trifluoromethyl)phenol (61b)
2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (61a)
4'-{aminomethyl}-2'-chboro-5-{trifluoromethyl}-[1,1'-biphenyl]-2-ol TFA salt (61)
2-Chloro-2'-hydroxy-[1,1'-biphenyl]-4-carbonitrile (62a)
4'-{aminomethyl}-2'-chloro-[1,1'-biphenyl]-2-ol HCl salt (66)
2-chloro-2'-hydroxy-5'-{(trifluoromethyl)-[1,1'-biphenyl]-4-carbaldehyde (63)
2'-chloro-4'-(hydroxymethyl)-5-(trifluoromethyl)-[1,1'-biphenyl]-2-ol (64)
2'-hydroxy-2-methyl-5'-(trifluoromethyl)[1,1'-biphenyl]-4-carbonitrile (65)
3-amino-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (66)
4-bromo-3,5-dichlorobenzonitrile (67a)
2,6-Dichloro-4-cyanophenyl trifluoromethanesulfonate (67c)
2,6-dichloro-2'-hydroxy-5'-[trifluoromethyl]-[1,1'-biphenyl]-4-carbonitrile (67)
2'-hydroxy-2,5'-bis(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (68)
2-amino-2'-hydroxy-5'-{ trifluoromethyl}-[1,1'-biphenyl]-4-carbonitrile (69)
2',3-dihydroxy-5'-{trifluoromethyl}-[1,1'-biphenyl]-4-carbonitrile (70)
2'-hydroxy-3-(methylsulfonyl)-5'-trifluoromethyl-[1,1'-biphenyl]-4-carbonitrile (71)
2'-hydroxy-2,6-dimethyl-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (72)
4-ethoxybenzoic acid (73)
Ethyl 4-amino-3,5-dichlorobenzoate (74b)
Ethyl 4-bromo-3,5-dichlorobenzoate (74a)
Ethyl 4-iodo-3,5-dichlorobenzoate (74e)
Ethyl 3,5-dichloro-4-(((trifluoromethyl)sulfonyl)oxy)benzoate (74g)
Ethyl 2,6-dichloro-2’-hydroxy-5’-(trifluoromethyl)-[1,1’-biphenyl]-4-carboxylate (74d)
Lithium 2,6-dichloro-2'-hydroxy-5'-{(trifluoromethyl)-1,1'-biphenyl}-4-carboxylate (74)
Ethyl 4-bromo-3-chlorobenzoate (76b)
Ethyl 2-chloro-2'-hydroxy-5'-([trifluoromethyl]-[1,1'-biphenyl]-4-carboxylate (76c)
2-Chloro-2'-hydroxy-5'-{(trifluoromethyl)}-[1,1'-biphenyl]-4-carboxylic acid (76)
2'-Chloro-6-hydroxy-[1,1'-biphenyl]-3,4'-dicarboxylic acid (77)
Lithium 2-chloro-2'-hydroxy-5'-((trifluoromethyl)-[1,1'-biphenyl]-4-carboxylate (78)
Lithium 2-chloro-2'-hydroxy-5'-(methoxycarbonyl)-[1,1'-biphenyl]-4-carboxylate (79)
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-alanine (92)
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-alanyl-L-alanine (93)
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-alanyl-L-alanyl-L-aspartic acid (94)
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-L-aspartyl-L-alanyl-L-aspartic acid (97)
(S)-4-Amino-3-((S)-6-amino-2-((S)-3-carboxy-2-(2-chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carboxamido)propanamido)hexanamido)-4-oxobutanoic acid TFA salt (98)
(2-Chloro-2'-hydroxy-5'-{trifluoromethyl}-{1,1'-biphenyl}-4-carbonyl)-L-aspartyl-L-lysyl-L-glutamic acid TFA salt (99)
(2-Chloro-2′-(2,2,2-trifluoroacetoxy)-5′-(trifluoromethyl)-[1,1′-biphenyl]-4-carbonyl)-L-aspartyl-L-lysyl-L-glutamic acid TFA salt (99a)
(2-Chloro-2'-hydroxy-5'-{trifluoromethyl} [1,1'-biphenyl]-4-carbonyl)-L-aspartyl-L-glutaminyl-L-aspartic acid (100)
(2-Chloro-2'-{2,2,2-trifluoroacetoxy}-5'-(trifluoromethyl)-{1,1'-biphenyl}-4-carbonyl)-L-aspartyl-L-glutaminyl-L-aspartic acid (100a)
(2-Chloro-2'-hydroxy-5'-{trifluoromethyl}-1,1'-biphenyl-4-carbonyl)-L-aspartyl-L-lysyl-L-asparagine TFA salt (101)
(2-Chloro-2'-[(2-chloro-2'-hydroxy-5'-[trifluoromethyl]-[1,1'-'biphenyl]-4-carbonyl)oxy]-5'-
(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl-L-aspartyl-L-lysyl-L-asparagine TFA salt (101a)
(2-Chloro-2'-hydroxy-5'-{(trifluoromethyl)}-[1,1'-biphenyl]-4-carbonyl)-L-aspartyl-L-tryptophyl-L-aspartic acid (102)
((2-Chloro-2'-hydroxy-5'-{trifluoromethyl}-[1,1'-biphenyl]-4-yl)methyl)-L-aspartyl-L-alanyl-L-aspartic acid TFA salt (107)
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)-D-aspartyl-L-alanyl-L-aspartic acid (108)
(2-Chloro-2'-hydroxy-5'-(trifluoromethyl)-1,1'-biphenyl-4-carbonyl)-D-aspartyl-L-lysyl-L-aspartic acid TFA salt (109)
Appendix 1B: Analytical HPLC Traces for Selected Peptides

HPLC traces refer to the analytical HPLC trace of purified peptide samples. The percentage in brackets above each trace refers to the linear gradient of solvent B (0.05% (v/v) TFA in acetonitrile) where solvent A is 0.05% (v/v) TFA in water. Data collected over 15 min at a flow rate of 1 mL min\(^{-1}\) and UV detection (\(\lambda_{\text{max}} = 220\) nm and 254 nm). Retention times (t\(_r\)) are reported in the peptide experimental section.

95 (78-AADD-OH)

(5-95% B)

(30-80% B)
96 (78-AADD-NH₂)

(5-95% B)

(30-80% B)
105 (78-PGGD-OH)

(5-95% B)

(20-60% B)
106 (78-PGGN-OH)

(5-95% B)

(20-60% B)
110 (78-dADDSDDD-OH)

(5-95% B)

(20-60% B)
111 (74-dADDDSDDD-OH)

(5-95% B)

(20-60% B)
Appendix 1C: Mass Spectra for Selected Peptides

**95 (78-AADD-OH)**
Calculated MW [M+H]^+ (Da): 689.1468  
Found MW [M+H]^+ (Da): 689.1452

**96 (78-AADD-NH₂)**
Calculated MW [M+H]^+ (Da): 688.1633  
Found MW [M+H]^+ (Da): 688.1635

106 (78-PGGN-OH)  Calculated MW [M+H]$^+$ (Da): 642.1573  Found MW [M+H]$^+$ (Da): 642.1557
110 (78-dADDDSDDD-OH)  
Calc. MW [M+H]$^+$ (Da): 1280.2764  
Found MW [M+H]$^+$ (Da): 1280.2712

111 (74-dADDDSDDD-OH)  
Calc. MW [M-H]$^-$ (Da): 1312.2229  
Found MW [M-H]$^-$ (Da): 1312.2288
Appendix 2: Publications

Appendix 2A: Second-Generation CK2α Inhibitors Targeting the αD Pocket

Chem. Sci. 2018, 9, 3041-3049

Appendix 2B: Partially Saturated Bicyclic Heteroaromatics as an sp3-Enriched Fragment Collection

Angew. Chem. Int. Ed. 2016, 55, 12479-12483
Second-generation CK2α inhibitors targeting the αD pocket†

Jessica legre,‡a Paul Brear,‡b Claudia De Fusco,‡b,c Masao Yoshida,‡d
Sophie L. Mitchell,a Maxim Rossmann,‡b Laura Carro,‡a Hannah F. Sore,a
Marko Hyvönen‡kb and David R. Spring‡ka

CK2 is a serine/threonine kinase that is a key regulator of many cellular processes and is involved in cellular proliferation and anti-apoptotic mechanisms.1 In vivo it exists mainly as a holoenzyme composed of two catalytic (α and/or α′) and a dimer of regulatory (β) subunits, but it can also be found as the isolated subunits.2 Unlike most other kinases it is constitutively active and more than 300 proteins have been identified as CK2 substrates, making it probably one of the most pleiotropic proteins in eukaryotic systems.3 Elevated levels of CK2 have been found in a variety of cancers, including leukaemia, breast, lung, prostate, colorectal, renal and glioblastoma brain tumours.4,5 It has been shown that cancer cells are particularly susceptible to CK2 inhibition because they rely on high levels of the kinase to survive.6 CK2 overexpression has been associated with multi-drug resistance phenotypes and it has been demonstrated that CK2α inhibition leads to an increased uptake of known drugs in multidrug resistant cells.7,8 It has been shown that CK2 inhibitors are able to limit the growth of a range of cancer cell lines.9,10 Hence, CK2 has been recognised as a highly promising target for anti-cancer therapies.

Like the majority of kinase inhibitors, most of the known CK2 inhibitors target the ATP binding site, presenting the issue of poor selectivity over other kinases.11–13 This is the case for CX4945 (known as silmitasertib), the first in class CK2 inhibitor currently in phase II clinical trials.14,15 The IC50 of CX4945 against CK2 is 1 nM but it also inhibits 12 other kinases with nanomolar affinity and it is more potent against Clk2 than against CK2.16,17

Previous work from our groups led to the discovery of a new binding pocket on CK2α, which is located adjacent to the ATP binding site. This pocket was revealed in a X-ray crystallographic screen, in which several weakly binding fragments where found to occupy this novel site formed through a movement of the αD helix, hence the name of αD pocket.18,19 Through fragment growing and linking, we generated a novel selective CK2 inhibitor: CAM4066 (Fig. 1).

CAM4066 was a valuable tool for validating the concept of using the αD site to develop selective inhibitors of CK2α; however, it has several structural features that are undesirable in a lead molecule or chemical tool. These features, shown in Fig. 1, include a long flexible linker (circled in orange), a zwitterionic nature (the amine and the carboxylate are highlighted in green), amide bonds (coloured blue) and a high MW, which is often associated with poor oral bioavailability (Fig. 1).

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††Electronic supplementary information (ESI) available: All experimental details, crystallographic data collection and refinement statistics, details of chemical synthesis, additional figures and tables. See DOI: 10.1039/c7sc05122k
‡‡These authors contributed equally to the work presented here.

Introduction

CK2 is a critical cell cycle regulator that also promotes various anti-apoptotic mechanisms. Development of ATP-non-competitive inhibitors of CK2 is a very attractive strategy considering that the ATP binding site is highly conserved among other kinases. We have previously utilised a pocket outside the active site to develop a novel CK2 inhibitor, CAM4066. Whilst CAM4066 bound to this new pocket it was also interacting with the ATP site: herein, we describe an example of a CK2α inhibitor that binds completely outside the active site. This second generation αD-site binding inhibitor, compound CAM4712 (IC50 = 7 μM, GI50 = 10.0 ± 3.6 μM), has numerous advantages over the previously reported CAM4066, including a reduction in the number of rotatable bonds, the absence of amide groups susceptible to the action of proteases and improved cellular permeability. Unlike with CAM4066, there was no need to facilitate cellular uptake by making a prodrug. Moreover, CAM4712 displayed no drop off between its ability to inhibit the kinase in vitro (IC50) and the ability to inhibit cell proliferation (GI50).
Moreover, the carboxylate forms a salt bridge with the conserved Lys68 in the ATP binding site. As expected due to its physicochemical properties, \textit{CAM4066} suffers from poor cellular permeability and therefore the methyl ester derivative, \textit{pro-CAM4066}, was used as a pro-drug to improve cellular activity and target engagement.\textsuperscript{18} The aim of this work was to develop enhanced CK2\textsubscript{\alpha} inhibitors that have improved physicochemical properties and bind to the \textit{\alpha}D pocket without reaching deep into the ATP pocket. Our ideal lead-like candidate would have a smaller number of rotatable bonds (<10), not be susceptible to protease action (absence of amide groups), and be cell permeable without resorting to the use of a pro-drug. In addition, we aimed to develop inhibitors that do not rely on any of the conserved interactions within the ATP binding site.

The strategy (shown in Fig. 2) involved a fragment optimisation and a fragment-growing stage, followed by merging of the best compounds. Firstly, we would optimise the \textit{\alpha}D site fragment further to gain higher affinity and secondly, we would grow the fragment into the upper part of the \textit{\alpha}D pocket in order to gain inhibition.

Finally, the compounds with the most promising substitution patterns would be combined to provide the final inhibitor that would show inhibition of the kinase activity, good cell permeability and efficacy in cellular assays.

**Results and discussion**

Previously we have reported our preliminary studies on the exploration of the \textit{\alpha}D pocket, based on the development of primary hits from the crystallographic screen, which led to the identification of compound 1 (\(K_d = 267\ \mu\text{M}\)), shown in Fig. 2. Our strategy to optimise the \textit{\alpha}D site fragment was to concentrate on ring A of the biaryl structure; however, a brief investigation of ring B was also performed. In parallel, optimisation of the amine substituent was carried out growing in the channel that connects the \textit{\alpha}D site and the ATP binding pocket.

**Optimization of the \textit{\alpha}D site fragment**

\textbf{Ring A}. Ring A effectively fills the bottom of the hydrophobic pocket of the \textit{\alpha}D site (Fig. 3). However, on closer examination we uncovered a side channel off the main pocket filled by several well-defined water molecules that could be targeted to improve
the affinity. Comparison of the co-crystal structure of CK2α and 1 (PDB: 5CSH, Fig. 3a) with the closed apo structure (PDB: 5CSP, Fig. 3b) shows that the side channel in the closed structure was occupied by Tyr125. This indicates that this channel can be targeted as it has sufficient volume to accommodate sizable groups, i.e. the phenolic ring of the Tyr125, and that the waters can be displaced as this happens in the closed form of the zD loop. Modelling studies indicated that 1 contains the right vectors to grow into the Tyr channel by substitutions on the 2-position of ring A.

A robust crystallographic system for CK2α enabled us to use X-ray crystallography as our primary screening technique. Therefore, co-crystal structures of all the compounds were attempted. The majority of the structures showed the ligand bound to CK2 and for all the compounds showing a clear electron density $K_d$ was determined via ITC (overview of the results of the ITC experiments can be found in Table S1†).

Several structures that did not show clear electron density for the ligand were also investigated using ITC to provide SAR data and controls. A number of mono- and di-substituted rings and bicyclic systems were investigated (Table 1 and 2, Fig. 4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ring A</th>
<th>$K_d$ (μM)</th>
<th>LE</th>
<th>PDB</th>
<th>Compound</th>
<th>Ring A</th>
<th>$K_d$ (μM)</th>
<th>LE</th>
<th>PDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>267</td>
<td>0.33</td>
<td>5CSH</td>
<td>7</td>
<td>OH</td>
<td>375</td>
<td>0.30</td>
<td>5OSL</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>41</td>
<td>0.38</td>
<td>5ORH</td>
<td>8</td>
<td>&gt;500</td>
<td>—</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>17</td>
<td>0.39</td>
<td>5ORJ</td>
<td>9</td>
<td>&gt;500</td>
<td>—</td>
<td>5OUL</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>205</td>
<td>0.29</td>
<td>5OS7</td>
<td>10</td>
<td>&gt;500</td>
<td>—</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>O</td>
<td>244</td>
<td>0.30</td>
<td>5OQU</td>
<td>11</td>
<td>105</td>
<td>0.33</td>
<td>5OS8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>234</td>
<td>0.32</td>
<td>5ORK</td>
<td>12</td>
<td>250</td>
<td>0.27</td>
<td>n.a</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Measured by ITC. $^b$ LE = ligand efficiency. $^c$Ligand efficiency is defined as the ratio of the Gibbs free energy of binding of a ligand divided by the number of heavy (non-hydrogen) atoms in the molecule ($LE = \Delta G / [\text{number of heavy atoms}]$).20
10) appeared not to be tolerated as only the fluoro-derivative 9 yielded a co-crystal structures. The disubstituted 2-Me-4-F derivative 11 was found to have a $K_d$ of 105 $\mu$M, marginally higher than the 2-methyl compound 2, but with a lower ligand efficiency ($LE = 0.33$). Expanding ring A to a naphthyl group 12 improved the affinity only slightly, while the indolyl derivative 13 did not show any significant binding and we were unable obtain structures of these in complex with CK2z. Therefore, compound 3 represented the fragment with the best LE and it was chosen as the best optimized fragment as far as the ring A is concerned.

**Ring B.** In many of the crystal structures of the compounds with different ring A substituents two alternative binding modes were observed for ring B, in which the chlorine atom could be on either side of the pocket. Therefore, the dichloro derivative of 1 (14) was synthesized to fulfill the interactions that both of these chlorine positions made. 14 showed an improved $K_d$ of 12 $\mu$M with the highest LE of 0.43 (Table 2). The co-crystal structure is shown in Fig. S1b† (PDB: 5OTR) where 14 (in blue) is overlapped with the two binding poses of 1 (green). The final compound 15, merging both the dichloro functionality on ring B and the 2-ethyl group on ring A, was synthesized and showed an improved $K_d$ of 7 $\mu$M ($LE = 0.41$) with its binding mode overlapping well with that of the merged fragments (Fig. 4e, PDB: 5OTZ). Although compounds 3 and 15 showed a comparable LE (0.39 vs. 0.41 respectively), compound 15 was chosen as

### Table 2  
SAR studies on the bottom ring (ring A) of dichloro derivatives 14 and 15

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ring A</th>
<th>$K_d$ [$\mu$M]</th>
<th>LE$^b$</th>
<th>PDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td></td>
<td>12</td>
<td>0.43</td>
<td>5OTR</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>6.5</td>
<td>0.41</td>
<td>5OTZ</td>
</tr>
</tbody>
</table>

$^a$ Measured by ITC. $^b$ LE = ligand efficiency$^a$. $^*$Ligand efficiency is defined as the ratio of the Gibbs free energy of binding of a ligand divided by the number of heavy (non-hydrogen) atoms in the molecule ($LE = \Delta G/\text{[number of heavy atoms]}$).$^{20}$
hit to bring forward due to its ability to occupy a larger part of the zD pocket (Fig. 4e).

**Growing towards the entrance to the ATP site.** The compounds tested so far were not able to effectively inhibit the protein activity as ATP was not displaced. Indeed, most of the co-crystal structures featured a molecule of ATP/ADP in the ATP binding site as well as the ligands bound in the zD pocket (Fig. 4 and 5).

During the development of CAM4066 a series of flexible linkers were designed and tested to join the zD site fragment to an ATP site fragment. These compounds revealed that it was possible to induce the opening of a small channel between the zD and the ATP sites. Our aim was to induce the opening of the channel with shorter, more rigid compounds than CAM4066. The flipping of the side chain of Met163 allows the formation of the channel and results in blocking the ATP site – Met163 is located just underneath the adenine base of ATP. Therefore, these compounds would not need to grow deep into the ATP site to achieve inhibition. The channel from the zD site is lined by Met163 and His160 and we envisioned that compounds with aromatic groups that stacked between these amino acid residues would improve the affinity and cause the conformational change that would lead to inhibition. Toward this end, the effect of several aromatic groups on the amine were investigated using a kinase activity assay (Table 3).

Whilst the reference compound 1, tested at 500 μM, inhibited only 21% of the protein activity, the inhibitory activity of the aromatic derivatives 16–19, were found to be considerably improved with compounds 16 and 19 being the most promising (54 and 52% respectively). In order to pick up additional H-bond interactions the pyrrole derivative 19 was chosen over 16 and we hypothesized that the heterocycle should go further up into the channel. Whilst the imidazole derivative 20 did not show significant improvement, the benzimidazole derivative 22 was found to be the most potent compound with an IC50 of 58 μM. Fig. 5d and f show the co-crystal structure of 21 and 22 with CK2α, respectively. As expected, Met163 flips upon binding of the more extended compounds compared to the co-crystal structure of 1 (Fig. 5b). This explains the displacement of ATP and therefore activity inhibition even without fragments binding directly in the ATP pocket.

As these compounds showed increased activity, the concentration for the inhibition assay was decreased to 10 μM and cellular activity was investigated (Table 4). Although 15 had the highest affinity of the zD binders (Table 2), 3 was chosen for further studies for synthetic reasons, with the idea of retrieving the substitution pattern of 15 in the final compound. Merging 3 with 22 provided compound 23, which featured higher potency than the original fragments in the inhibition assay and a promising GI50 of 10 μM in HCT116 cells. Therefore, SAR

![Table 3](image)

**Table 3.** N-substituents on the benzylamine^a^

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Inhibition of the kinase activity @ 500 μM (%)</th>
<th>PDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>21</td>
<td>5CSH</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td>n.a.</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td>n.a.</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td>6EI</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td>5OT6</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>5OU</td>
</tr>
<tr>
<td>21</td>
<td>n.d.</td>
<td></td>
<td>SOUM</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>98 [IC50 58]</td>
<td>SOUU</td>
</tr>
</tbody>
</table>

^a n.d. = interference with the assay by the test compound.
studies around the benzimidazole ring were then performed (compounds 24–30), with both electron withdrawing and donating groups in position α and β of the benzimidazole improving the inhibition activity in respect to the derivative 23. The α and β methoxy derivatives 26 and 29 were found to be the most promising in the respective α and β substituted series with IC₅₀ of 8 and 7 μM, respectively (Table 4, IC₅₀ and GI₅₀ curves are shown in Fig. S4 and S5†).

**Further modifications of ring B.** Alternative substitutions around the middle ring were also investigated and compared to compound 23. Firstly, the dichloro derivative was synthesized as it gave promising results as a fragment (compound 15, Table 2). With an IC₅₀ of 7 μM, CAM4712 was the most potent compound compared to the methyl, trifluoromethyl, methoxy and trifluoromethoxy derivatives (compounds 31, 32, 33 and 34, respectively) (Table 5). Substitution of the chlorine atom in compound 23 (compounds 31–34) was investigated in order to improve the moderate solubility of the related CAM4712 in water. Unfortunately, compounds 31–34 resulted in loss of activity and were therefore not pursued further.

With the optimisation around each aromatic ring in hand, compounds 35 and 36 were designed via a merging strategy so that they contained the most promising substitution patterns. The methoxy derivatives 35 and 36 were synthesized and tested but, disappointingly, gave worse results than CAM4712 (Fig. 6).

**Validation of CAM4712.** As CAM4712 was the most advanced compound in this series a more detailed investigation was performed. Firstly, CAM4712 showed an improved IC₅₀ of 7 μM compared to the compound 22 (58 μM). This data validated our fragment merging strategy to improve upon both α and inhibition. Unfortunately, as with the other compounds in the series, once the benzimidazole group had been added CAM4712 was not soluble enough for ITC. Therefore, an ITC competition study was performed to confirm the binding mode and to estimate the affinity of CAM4712 for the αD site (an overview of the results of the ITC experiments can be found in Table S1†). In order to achieve this, several probe molecules that have well characterised binding modes and affinities were titrated into CK2α in the presence of CAM4712. From these experiments, it was possible to determine not only the affinity for the αD site but also which part of the ATP site the benzimidazole group blocks.

Four probes were used for this study in four separate competition experiments, each of them titrated into CK2α in the presence of and absence 20 μM CAM4712 giving the following results:

1. Inhibition of binding in the αD or ATP site by CAM4712:
   - The binding of CAM4066 to CK2α was inhibited by 20 μM CAM4712. From this the affinity of CAM4712 was estimated to be 4.0 μM (Fig. S2†). This experiment confirms that CAM4712 binds to CK2α but the binding could partially occur in the ATP site.

2. Inhibition of binding in the αD site:
   - 15 was titrated into CK2α in the presence of 20 μM CAM4712 (Fig. S3†). This showed that CAM4712 was also able to inhibit the binding of 15 to CK2α and from this the affinity was estimated to be 5.0 μM. As 15 binds in the αD site this experiment confirms that the binding site of CAM4712 is the αD pocket as well as confirming the affinity.
(3) Inhibition of binding to Lys68:

2-Hydroxyl, 5-methyl benzoic acid (37) binds to the conserved Lys68 in the ATP site and occupies the right-hand side of the pocket (PDB: 5CSP). The binding of compound 37 was not inhibited by CAM4712 (Fig. S6f) confirming that the benzimidazole ring does not interact with the right-hand side of the ATP pocket and validates the binding mode derived from the crystal structure (PDB: 5OTZ). This result predicted that it would be possible to generate a crystal structure of CAM4712 and 37 bound simultaneously to CK2α and this was confirmed by a crystal structure showing both compounds binding simultaneously to CK2α (Fig. S6f, PDB: 6EHK).

(4) Inhibition of binding in the ATP site/hinge region:

CX4945, which is from the analyses of crystal structures would clash with CAM4712 in the hinge region, was titrated into CK2α in the presence of CAM4712 (Fig. S7†). CAM4712 was shown to inhibit the binding of CX4945 to CK2α. The affinity of CAM4712 for CK2α was estimated to be 3.0 μM. This confirms that the benzimidazole ring binds in the Met163 channel and blocks access to the ATP site as this would inhibit the binding of CX4945.

In summary, these competition experiments suggest firstly that the $K_d$ of CAM4712 towards CK2α is approximately 4 μM. Secondly, they confirm that the binding mode of CAM4712 in the zD pocket and mouth of the ATP site corresponds to that seen in the crystal structure.

The validation experiments of CAM4712 and the crystal structures allowed us to rationalise the difference in in vitro potency between CAM4066 and CAM4712 (IC₅₀ 7 μM and 0.37 μM respectively). Whilst the binding of both compounds to CK2 resulted in the flipping of the Met163, CAM4712 did not H-bond the conserved Lys68 in the ATP binding site. Instead, a low-energy hydrophobic π–π interaction between the His160 and the benzimidazolone was introduced (as shown for the related compound 22 in Fig. 5f) resulting in loss of binding affinity and potency compared to CAM4066.

The efficacy of CAM4712 in cellular assays was tested in HCT116 cell line, which is known to overexpress CK2α. A cell growth inhibition assay yielded a GI₅₀ for CAM4712 of 10.0 ± 3.6 μM, which is similar to that of the clinical trials candidate CX4945 (11.3 ± 1.2 μM). It is also similar to pro-CAM4066 (GI₅₀ 9.1 ± 1.4 μM, IC₅₀ 0.37 μM), but importantly, no drop-off in potency was observed from the functional to the cellular assay. This represents a large step forward compared to CAM4066, which had to be administered as the prodrug pro-CAM4066. The target engagement by CAM4712 was analysed by following the CK2α dependent phosphorylation of Ser129 of Akt1. This showed good inhibition of the phosphorylation of Ser129 by CAM4712 as well as by its close analogues 23 and 26 which confirms that these compounds inhibit CK2α in the cellular environment (Fig. 7).

CAM4712 showed a 10-fold decrease in potency compared to CAM4066 and therefore the selectivity of CAM4712 was screened against a panel of 140 kinases at a concentration of 30 μM (4 × IC₅₀). CAM4712 showed good selectivity against the 20 closely related CMGC kinases in the panel (Fig. S6a†). However, 4 kinases (CAMK1, SmMLCK, EF2K and SGK1) were inhibited by

![Fig. 7](image)

CAM4712 for more than 50% (Fig. S8b†) and hence, CAM4712 showed a reduced overall selectivity compared to CAM4066 (which was screened at 2 μM). This is not surprising considering the high concentration used in the selectivity screen due to CAM4712 being less potent than CAM4066. Nevertheless, CAM4712 showed a more selective profile than other CK2α inhibitors (Fig. S8b†) and represents, therefore, a good starting point for further development of selective CK2α inhibitors. Our aim in this work has not been to gain specificity but rather to demonstrate mechanistically that an inhibitor that is not making significant contacts with the conserved active site is able to inhibit the kinase effectively. To fully exploit the
selectivity that zD binding offers, further optimization of CAM4712 is needed to increase its affinity towards CK2α.

One of the aims of this work was to generate an improved inhibitor compared to the previous compound CAM4066. We managed to design a compound with the physico-chemical properties falling into the range for bioavailable compounds according to Lipinski’s rules: the number of rotatable bonds was reduced, the amide groups were removed and the compound entered the cells and showed activity without the use of a prodrug. Moreover, the number of H-bond donors and acceptors was reduced to 2 and the molecular weight was kept below 500 Da (Table 6). This was all achieved without interacting with the deep and conserved ATP binding site.

Conclusions

In conclusion, we have developed a series of second-generation CK2α inhibitors that target the zD site. This was achieved by first optimising the fragments that bound in the zD site, followed by identification of groups that grow towards the mouth of the ATP site to provide potent inhibitors of CK2α.

In our previous work we demonstrated that selectivity could be achieved anchoring the inhibitors in the zD pocket and with this work we achieve inhibition with ligands that do not target the active site. CAM4712 showed high cellular activity (10.0 ± 3.6 µM) and target engagement was demonstrated. This second generation of zD pocket inhibitor overcomes the limitations of our first inhibitor, including the fact that it does not need to be administered as a pro-drug to exert anti-proliferative activity. We have also shown that it is not necessary to interact with the ATP site directly, but effective inhibition of the kinase and displacement of ATP can be achieved by blocking the mouth of the ATP site with no need to interact with conserved features of the ATP binding site. These results demonstrate an entirely new approach to CK2α inhibition and will allow the future development of drug-like molecules, lead compounds and chemical tools that utilise the novel properties of the zD site.

Conflicts of interest

There are no conflicts to declare.

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Partially Saturated Bicyclic Heteroaromatics as an sp\textsuperscript{3}-Enriched Fragment Collection

David G. Twigg, Noriyasu Kondo, Sophie L. Mitchell, Warren R. J. D. Galloway, Hannah F. Sore, Andrew Madin, and David R. Spring*

Dedicated to Professor Stuart L. Schreiber on the occasion of his 60th birthday

Abstract: Fragment-based lead generation has proven to be an effective means of identifying high-quality lead compounds for drug discovery programs. However, the fragment screening sets often used are principally comprised of sp\textsuperscript{2}-rich aromatic compounds, which limits the structural (and hence biological) diversity of the library. Herein, we describe strategies for the synthesis of a series of partially saturated bicyclic heteroaromatic scaffolds with enhanced sp\textsuperscript{3} character. Subsequent derivatization led to a fragment collection featuring regio- and stereo-controlled introduction of substituents on the saturated ring system, often with formation of new stereocenters.

Fragment-based drug discovery (FBDD) is a well-established method for generating high-quality hits and leads.\textsuperscript{[1]} The approval of the B-Raf kinase inhibitor vemurafenib (Zelboraf) in 2011\textsuperscript{[2]} and the Bel-2 inhibitor venetoclax (Venclexta) in 2016,\textsuperscript{[3]} coupled with the ongoing evaluation of over 20 candidates in clinical trials,\textsuperscript{[4]} validates this approach as a complementary strategy to other hit-discovery techniques such as high-throughput screening.\textsuperscript{[5]} While the growing prevalence of fragment-based approaches is encouraging, evaluation of many existing fragment libraries shows a predominance of (hetero)aromatic, “flat” compounds, with a deficiency of chiral, sp\textsuperscript{3}-rich examples.\textsuperscript{[6,7]}

Studies by Ritchie et al.\textsuperscript{[8]} and Lovering et al.\textsuperscript{[9]} demonstrate improvements in project progression by, for example, increasing the fraction of sp\textsuperscript{3} centers within molecules or restricting the number of aromatic rings. Furthermore, computational analysis demonstrates that greater 3D conformational character is observed in compounds that have been clinically evaluated in humans, compared to those found in commercial libraries.\textsuperscript{[10]} This indicates the importance of sp\textsuperscript{3}-richness in both the design of screening collections and the subsequent development of hits to leads.

Examples of previous studies aiming to synthesize collections of sp\textsuperscript{3}-enriched fragments have been limited. Diversity-oriented synthesis\textsuperscript{[7,11]} and natural product derivatives\textsuperscript{[12]} have been used to generate 3D fragment collections, but there remains an unmet need to access new scaffold types. Recently there have been calls\textsuperscript{[13]} for new approaches and methodologies for designing fragments with multiple synthetically accessible growth vectors in three dimensions, to allow rapid and efficient elaboration of hits to leads after initial screening, with some early success.\textsuperscript{[14]}

With these points in mind, the study described herein was aimed at developing efficient synthetic routes to a series of partially saturated bicyclic heteroaromatic (PSBH) fragments with enhanced sp\textsuperscript{3} content relative to existing fragment libraries. Compounds featuring PSBHs have been shown to display bioactivity against a range of targets (Figure 1),\textsuperscript{[15]} and so a series of related fragments might be expected to serve effectively as a screening collection for FBDD applications.

![Figure 1. Selected examples of bioactive compounds containing functionalized partially saturated bicyclic heteroaromatics (highlighted in red).](image-url)
The targets of this study featured a variable aromatic heterocycle fused to a partially saturated carbocycle. The heterocycle could bear either a polar (e.g., amino) group, which should greatly enhance aqueous solubility, a property necessary for fragment screening at higher concentrations, or alternatively a hydrophobic (e.g., chloro) group, which can forge key interactions with protein targets. The synthetic route (Scheme 1) employed a modular and divergent approach, using simple cross-coupling and alkylation reactions to install a pair of terminal olefins that could be reliably cyclized through ring-closing metathesis (RCM). This allowed excellent control of the carbocycle ring size and the position and orientation of the resultant endocyclic olefin growth vector, which could undergo subsequent functionalization to produce a range of fragments suitable for screening and/or further elaboration.

We selected pyrazole and pyridine as representative aromatic heterocycles. Whilst previous studies have shown the synthesis of related structures, they have incorporated less control over the position of the olefin and do not feature the amino group found in many of our compounds. Furthermore, there are only very few examples where the olefin is used as a branch point and further functionalized beyond simple reduction.

Starting from readily available 3-nitro-1H-pyrazole (4), 2-(trimethylsilyl)ethoxymethyl (SEM) protection, selective iodonation, and subsequent Suzuki coupling with potassium vinyltrifluoroborate gave vinyl derivative 7a (Table 1). Deprotection followed by N-alkylation with an alkyl bromide of varying C-chain length provided metathesis precursors 9a–c. Upon treatment with either Grubbs’ or Hoveyda-Grubbs’ 2nd generation catalysts yielded the desired scaffolds 10a–c. Inclusion of further heteroatoms in the formation of medium-sized partially saturated rings was achieved through treatment of vinyl intermediate 8a with either tosylate 11 (leading, after RCM, to O-containing fragment 10d), or 3-Boc-1,2,3-oxathiazolidine 2,2-dioxide 12, which gave access to the N-containing scaffold 10e after allylation and metathesis.

Use of a different Suzuki coupling partner gave methyl-substituted product 7f, which could be elaborated to PSBH fragment 10f. Alternatively, direct allylation at the C-5 position of SEM-protected intermediate 5 could be achieved upon treatment with lithium disopropylamide (LDA) and alkyl bromide. This led, in an analogous way, to scaffolds 10g–h with non-conjugated olefins.

A similar approach was used to generate PSBH scaffolds from pyridine 13 (Table 2). Attempts to mask the 2-amino group as a nitro group proved ineffective since, despite successful cross-coupling reactions, the 2-nitropyridines were unstable to strong base and did not undergo the desired alkylations at the 4-methyl position. Mono-Boc protection was also unsuitable due to poor yields in the cross-coupling step, possibly due to catalyst chelation. The 2-amino group could be rendered synthetically tractable, however, either through bis-Boc protection or through substitution with a 2-chloro group, which itself can serve as a synthetic handle.

Bis-Boc substrate 14 (prepared in one step from 13) was functionalized at the 5-position using either Suzuki coupling (for vinyl substituents) or Stille coupling (for allyl substituents) to produce intermediates 15a,d,f. Treatment with LDA and trapping of the resultant anion with a variable alkyl bromide electrophile gave a range of metathesis substrates (16a–f), which under standard ring-closing metathesis conditions yielded PSBH scaffolds 17a–f. The 2-chloro substrate 18 could be allylated in the 5-position by using an excess of i-PrMgCl-LiCl and trapping the resultant organometallic intermediate with alkyl bromide. Allylation at the 4-methyl position and RCM gave scaffold 17g in superior yields.

Following PSBH synthesis, a series of simple one-, two-, or three-step functionalizations were performed on selected pyrazole and pyridine scaffolds to demonstrate the synthetic potential of this approach.
utility of the olefin π-bond as a growth vector and to generate a variety of new stereocenters (Scheme 2).

Catalytic hydrogenation of nitropyrazoles 10a–d,f served to reduce both the olefin π-bond and the nitro group in moderate to good yields, revealing the latent amino functionality and, in the case of 19f, creating a new stereocenter. Other one-step reactions include aziridination, dibromination, allylic oxidation dihydroxylation, difluorocyclopropanation, hydroxybromination, epoxidation, and hydroboration (20–28) to introduce functionalities at the 4-, 5- and 6-positions of the fused pyrazole systems. Demonstrating that these initial products can serve as intermediates to other fragments, the products of hydroboration can react further to incorporate Br, F, and N substituents (30–33), whilst epoxide 26 can be opened by nucleophiles such as fluoride and hydride to form fluorohydrin 35 and alcohol 36. Whilst the yields of some reactions were modest, sufficient material was obtained for full characterization and future screening campaigns.

The pyridine-based scaffolds 17a–g can undergo a similar range of transformations. Catalytic hydrogenation and subsequent acid-mediated deprotection of bis-Boc compounds 17a–f generated novel fragments, many of which include new stereocenters (37a–f). Aziridination, dibromination, dihydroxylation, hydroboration, α-bromoketone formation, and hydroxybromination were also carried out (38–43). Further reactions included amino-alcohol (44) and epoxide (45).

### Table 2: Synthesis of pyridine-based PSBH scaffolds.

<table>
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<th>Step</th>
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<th>n</th>
<th>R1</th>
<th>R2</th>
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<th>B (16) [%]</th>
<th>C (17) [%]</th>
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<td></td>
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<td>H</td>
<td>H</td>
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<td></td>
<td>b</td>
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<td>0</td>
<td>H</td>
<td>Me</td>
<td>–</td>
<td>69</td>
</tr>
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<td></td>
<td>c</td>
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<td>H</td>
<td>CF3</td>
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<td>1</td>
<td>H</td>
<td>H</td>
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<td>79</td>
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Reaction conditions: [a] Boc2O (2.5 equiv), DMAP (0.1 equiv), THF, 70 °C. [b] R’-BF3 K or R’-B(MIDA) (1.5 equiv), Pd(dppf)Cl2·CH2Cl2 (10 mol%), K2CO3 (3.0 equiv), THF/H2O, 70 °C. [c] Allyltributyltin (1.1 equiv), Pd(PPh3)4 (10 mol%), KOtBu (2 equiv), toluene, 110 °C. [d] i-PrMgCl·LiCl (1.5 equiv), allyl bromide (1.2 equiv), THF, –15 °C to RT. [e] LDA (1.2 equiv), alkyl bromide (1.5 equiv), THF, –78 °C to RT. [f] Grubbs II (5 mol%), CH2Cl2, 40 °C. DMAP = 4-dimethylaminopyridine.

Scheme 2. Functionalization of PSBH scaffolds. For reaction conditions, see the Supporting Information.
formulation, both from bromohydrin 43. 3-Chloro fragments 46–51 could also be readily accessed using similar conditions.

Calculation of a range of physicochemical properties was carried out on all of the PSBH products. Almost all fragments were shown to conform to the so-called “Rule of Three”, a set of criteria commonly associated with greater hit rates in fragment screening collections.[21] Of particular note are the low mean values for molecular weight (190), SlogP (1.45), and “fraction aromatic” (0.43) and the high mean number of chiral centres (0.88), especially when compared to existing commercial libraries (Table 3).

In conclusion, we have developed simple, scalable routes to a series of partially saturated pyrazole- and pyridine-based scaffolds that can readily undergo a range of synthetic transformations to generate a collection of sp²-rich fragments, which are suitable for use either as screening members in a library or as intermediates to “higher-content fragments”. The compounds adhere to recognized guidelines for fragment physicochemical properties, whilst displaying enhanced sp³ character and greater chirality, and providing a range of three-dimensional growth vectors for synthetic development. It is envisioned that the strategy could be applied to a vast range of analogous scaffolds with varied heterocycles and substituents and that several of the functionalization reactions detailed in Scheme 2 could be rendered asymmetric based on related precedents.[19,22]

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### Keywords

drug design - drug discovery - fused-ring systems - nitrogen heterocycles - synthetic methods

### Table 3: Mean physicochemical properties of fragment collections.

<table>
<thead>
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<th>Property[4]</th>
<th>Ideal Range[5]</th>
<th>This work</th>
<th>Chembridge</th>
<th>Maybridge</th>
</tr>
</thead>
<tbody>
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<td>SlogP</td>
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<td>1.45 1.31</td>
<td>2.55</td>
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<tr>
<td>MW</td>
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<td>190 222</td>
<td>265</td>
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</tr>
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<td>50.0 53.9</td>
<td>57.5</td>
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<tr>
<td>HBA</td>
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<td>1.35 1.81</td>
<td>2.12</td>
<td></td>
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<tr>
<td>HBD</td>
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<td>0.55 1.04</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>HAC</td>
<td>10-16</td>
<td>12.8 15.5</td>
<td>18.0</td>
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<td>0.6 3.2</td>
<td>2.8</td>
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<tr>
<td>Chiral centres</td>
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<td>0.88 0.27</td>
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<tr>
<td>Fraction Aromatic</td>
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<td>0.43 0.42</td>
<td>0.52</td>
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</tr>
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</table>

[a] MW = molecular weight, PSA = polar surface area, HBA = number of hydrogen-bond acceptors, HBD = number of hydrogen-bond donors, HAC = heavy atom count, RBC = rotatable bond count. [b] Based on the guidelines used by Astex Pharmaceuticals.[14,21] Green: within ideal range, orange: at extreme of ideal range, red: outside ideal range. See the Supporting Information for further details.

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