Doctorate in *Sciences and Technologies of Chemistry and Materials*  
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**Part 1 - Istituto Italiano di Tecnologia (IIT, Genova – IT)**

Design and synthesis of novel Cystic Fibrosis (CF) modulators

**Part 2 - Helmholtz Institute for Pharmaceutical Research Saarland (HIPS, Saarbrücken - DE)**

Development of novel inhibitors of the anti-infective target DXS using  
Dynamic Combinatorial Chemistry (DCC)

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Preface

I hereby declare that the subjects presented in this thesis dissertation are the results of the research work carried out throughout my Ph.D. course.

The research outlined in this Ph.D. thesis concerns two main topics: the first three Chapters (Chap. 1-3) report the relevant outcomes of a medicinal chemistry project, which deals with the identification of novel Cystic Fibrosis (CF) modulators. It was developed over a three years period at the D3-Pharmachemistry department of the Istituto Italiano di Tecnologia (IIT) in Genova (Italy), under the supervision of Dr. Tiziano Bandiera and Dr. Fabio Bertozzi and the co-supervision of Prof. Paola Fossa. Biological tests of all the synthesized compounds were conducted at Istituto Giannina Gaslini (IGG, Genova), while kinetic solubility and metabolic stability analyses were carried out at the Analytical Chemistry Lab - IIT (Genova).

The last Chapter (Chap. 4) describes a bio- and organic chemistry project, which was carried out during my 6 months of research experience abroad in the lab of Prof. Dr. Anna K. H. Hirsch, at the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), in Saarbrücken (Germany). IC₅₀ and MIC tests were performed by the Department Drug Design and Optimization (DDOP) at HIPS (Saarbrücken).
Abstract

Cystic Fibrosis (CF) is a lethal, autosomal recessive genetic disease characterized by an accumulation of viscous mucus at epithelia surface of multiple organs, including lungs, pancreas, gut and testis, which results in obstruction, infection, inflammation and ultimately organ failure. The primary cause of CF is the mutation of a gene, the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), which leads to a decrease in CFTR chloride channel function and ultimately to a reduced ionic and water homeostasis at epithelial surfaces. Historically conventional CF treatments focused on symptomatic therapy, until recently when the growing understanding of the molecular basis of CF pathologies stimulated the development of small-molecule drugs, called CFTR modulators, which address the primary cause of CF with the hope to repair the defects in mutated CFTR.

Aiming to expand the portfolio of novel modulators available to CF patients, also considering the relevant but limited pharmacological efficacy elicited by some of the current treatments, there is still the need to develop more CFTR small molecule modulators, primarily correctors, which may address the primary cause of CF by rescuing the activity of defective CFTR (Chapter 1).

The present PhD thesis describes the design, synthesis and biological characterization of novel CFTR correctors.

Starting from primary hits ARN9364 and ARN5562, selected for their promising initial biological activity after a High-Throughput Screening (HTS) campaign, new analogs were designed and synthesized to elucidate the Structure-Activity Relationship (SAR) patterns around these chemotypes (Chapter 2).

The biological test of these novel compounds in a phenotypic cell-based assay (HS-YFP assay) using CFBE41o- cells, allowed to get clear information about the most suited structural modifications needed to improve rescuing activity of defective F508del-CFTR. An iterative process of design, synthesis and biological testing led to the identification of slightly or even more potent CFTR correctors (Chapter 3).

In Chapter 4 the development of selective and potent inhibitors of the anti-infective target 1-deoxy-d-xylulose-5-phosphate synthase (DXS), using target-directed Dynamic Combinatorial Chemistry (tdDCC), as hit-identification strategy, was reported. Biochemical evaluation of several hit compounds amplified in the tdDCC experiment against M.
*tuberculosis* DXS and *D. radiodurans* DXS afforded inhibitors with IC$_{50}$ in the range of 30 μM – 190 μM.
Notes

All compounds synthesized during my Ph.D. work at IIT (Chapter 2–3) are reported with increasing numbers (1–97).

All compounds synthesized during my Ph.D. period at HIPS (Chapter 4) are reported preceded by the letter “G” (G1–G54).

List of abbreviations used

glacial acetic acid (AcOH), acetonitrile (MeCN), ammonium acetate (NH₄OAc), ammonium chloride (NH₄Cl), N-bromosuccinimide (NBS), n-butyllithium (n-BuLi), cesium acetate (CsOAc), cesium carbonate (Cs₂CO₃) cesium fluoride (CsF), chloroform (CHCl₃), cyclohexane (Cy), diatomaceous earth (Celite), bis(dibenzylideneacetone)palladium(0) ([Pd₂(dba)₃]), 1,2-dichloroethane (DCE), dichloromethane (DCM), 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (XPHOS), diethyl ether (Et₂O), 4,5-bis(diphenylphosphino)-9,9-dimethylxanthen (Xantphos), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxid hexafluorophosphate (HATU), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI), N,N-diisopropylethylamine (DIPEA), dimethylsulfoxide (DMSO), N,N-dimethylformamide dimethyl acetal (DMF*DMA), 4-(dimethylamino)-pyridine (DMAP), [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) ([Pd(dppf)Cl₂]), ethanol (EtOH), ethyl acetate (EtOAc), equivalent (eq.), 1-hydroxybenzotriazole (HOBt), hydrochloric acid (HCl), iodomethane (CH₃I), lithium bis(trimethylsilyl)amide (Me₃SiNH-Li), lithium diisopropylamide (LDA), lithium hydroxide (LiOH), methanol (MeOH), methylmagnesium bromide (MeMgBr), palladium(II) acetate ([Pd(OAc)₂]), palladium on carbon ([Pd/C]), potassium acetate (KOA), potassium carbonate (K₂CO₃), potassium phosphate tribasic (K₃PO₄), retention time (Rₜ), room temperature (rt), silica gel (SiO₂), sodium acetate (NaOAc), sodium bicarbonate (NaHCO₃), sodium carbonate (Na₂CO₃), sodium hydride (NaH), sodium hydroxide (NaOH), sodium sulfate (Na₂SO₄), sodium triacetoxyborohydride (NaBH₄ACO₃), tetrakis(triphenylphosphine)nickel(0) ([Ni(PPh₃)₄]) tert-butyl methyl ether (TBME), tetrabutylammonium iodide (TBAI), tetrahydropyran (THP),
tetrahydrofuran (THF), triethylsilane (Et$_3$SiH), trifluoroacetic acid (TFA), tetrakis(triphenylphosphine)palladium(0) ([Pd(PPh$_3$)$_4$]), triethylamine (Et$_3$N), 2-(trimethylsilyl)ethoxymethyl chloride (SEM-Cl), di-tert-butyl dicarbonate (Boc$_2$O), water (H$_2$O).
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Summary
Part 1: Design and synthesis of novel Cystic Fibrosis (CF) modulators

1. Introduction

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is a lethal, autosomal recessive genetic disease among Caucasian, identified by Dorothy Andersen in 1938, with an estimated worldwide patient population of around 85,000. In the past the life expectancy for CF patients ranged from months to a few years; nowadays thanks to early diagnosis and improved treatments, the median survival age has increased progressively till more than 40 years.

CF is caused by mutations in the gene coding for the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), a chloride channel generally found in the membrane of epithelial cells. This protein plays a critical role in fluid and electrolyte transport in epithelial cells (in particular in the transport of chloride and bicarbonate ions from cytoplasm to the extracellular environment) and contributes to regulate also mucociliary clearance in the airways.

The clinical manifestations of this disorder are characterized by chronic obstruction of ducts in multiple organs leading to an array of pathological conditions. In lungs, the defect in CFTR function results in the formation of thick, sticky mucus clogging the lungs and causing difficulties in breathing. In addition to that, the mucus becomes the substrate where various pathogens can grow leading to severe infections and chronic inflammation.

The major cause of morbidity and mortality are the respiratory failure and lung disease. The pathophysiological cascade, which characterizes these respiratory disorders, is caused by the insufficient or absence of CFTR function, leading to an impairment of chloride or bicarbonate anion permeability in airway cells (Figure 1.1). The defective CFTR also causes a dysregulation of the conductance of other ions (such as the excess sodium mediated by the epithelial sodium channel, negatively regulated by CFTR), which reduces the water content and the pH of the airway surface liquid. In addition to that, the increase in mucin polymer
cross-linking and in the viscosity and the amount of the mucus, severely impair the removal of mucus and bacteria by ciliary beating.

Considering the importance of the mucociliary clearance as defense mechanism against pathogens, the impairment of this physiological function leads to an increase of chronic infections in particular by Pseudomonas Aeruginosa, Staphylococcus Aureus and Haemophilus. Furthermore, accumulation of this sticky mucus leads to obstruction of the airways, inflammation, bronchiectasis, lung damage and in the last stage of the disease, respiratory failure.5,8

![Figure 1.1: The Cystic Fibrosis (CF) pathogenesis cascade in the lung. The mechanism behind lung dysfunction starts with the primary CFTR gene defect and ultimately leads to severe lung deficiency. Picture taken from Lopes-Pacheco M., Front. Pharmacol., 2016, 7, 275.](image_url)

Other common pathological findings in CF patients are pancreatic and digestive insufficiency, often associated to malnutrition, bowel obstruction, diabetes, hepatic damage and male sterility.1

Moreover, another clinical manifestation of the disease is the salt loss due to the faulty chloride reabsorption thought CFTR in the sweat glands.9 Therefore, sweat chloride measurement is commonly used as biomarker of CFTR activity and to monitor the response to treatment with disease-modifying agents.10
1.2 The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) chloride channel

CFTR belongs to the ATP-binding cassette (ABC) transporter family of membrane proteins, which is widely distributed throughout all the kingdoms of life. ABC transporters utilize the chemical energy derived from the hydrolysis of ATP to pump substrates across cellular membranes against a concentration gradient, and can be importers or exporters. This family of proteins transport small ions, amino acids, sugars, drugs and proteins, and are required for metabolite transport, signal transduction, protein secretion and antigen presentation. Considering their important roles, mutations in this family give rise to several human diseases such as CF, Stargardt and Tangier disease.

CFTR is the only known member of the ABC family that acts as an anion channel, not as a transporter, allowing ions (primarily Cl\(^-\) and HCO\(_3\)^-) diffusion through their electrochemical gradient. In particular, CFTR acts as chloride and bicarbonate channel, both of which are important for the extracellular fluid properties.

In addition to chloride, many monovalent anions such as fluoride, iodide, bromide, bicarbonate, thiocyanate, formate and nitrate are able to permeate through this channel; their relative permeability decrease as a function of anion size.

The CFTR gene is located on chromosome 7q31.2, and was cloned in 1989 through positional cloning approaches. A major breakthrough in the structure of wt-CFTR was recently achieved with the publication of medium-to-high-resolution 3D structures of the full-length wild-type protein, first from zebrafish and then human determined by the Cryo Electron-Microscopy (cryo-EM) technique. These structures provided insights about a non-phosphorylated, apo-form of the channel, representing a closed and inactive channel state.

CFTR is a 1480-residue long membrane protein consisting of two transmembrane domains (TMDs), two nucleotide binding domains (NBDs), and a unique structural motif termed R-domain (R) (Figure 1.2). All these domains are arranged from N- to C-terminus in the order: TMD1-NBD1-R-TMD2-NBD2.
The TMDs consist of six membrane-spanning α-helices, form the channel pore and allow transport of chloride ions across the membrane. A large number of lysines and arginines are distributed on the TMDs domains and their positive charge attracts anions into the pore through electrostatic interaction. TMDs are connected to their extracellular ends by short extracellular loops (ECLs) and to the cytoplasmic ends by long intracellular loops (ICLs). These ICLs are predicted to interact and transduce information between TMDs and NBDs.

NBDs control the gating of the channel participating in ATP binding and hydrolysis. They have similar folds to that of other ABC proteins, but present an additional ~35-residue insertion in NBD1 and ~80-residue extension in NBD2. As for ABC-NBDs, CFTR seems to be associated in a head-to-tail arrangement to form two nucleotide-binding sites at their interface. In CFTR, ATP hydrolysis occurs only at one of the two ATP-binding sites, whereas the other one is defective in ATP hydrolysis and is stably occupied by a nucleotide.

The R domain is a unique residue comprising 241 amino acids. It is located in the linker region, connects the two parts of the protein, and regulates channel activity controlling the channel opening and closing by its phosphorylation. This region is highly charged and contains nine sequences for protein kinase A (PKA) phosphorylation, and target sites for other kinases, such as protein kinase C (PKC) and AMP-activated protein kinase (AMPK). The R domain is a disordered region, with a α-helical secondary structure in the non-phosphorylated state, and interacts with other CFTR domains. In this context, phosphorylation of R domain seems to alter different interactions with other protein regions,
and is required for the molecular mechanism of CFTR. In particular, phosphorylation occurs in at least nine sites in the R domain and one in NBD1. A single phospho-residue is not enough for channel activation, and the activity increases with phosphorylation in an additive manner. Phosphorylation also appears to render the CFTR protein more compact, increasing R-region interactions with the C-terminus but decreasing NBDs interactions, according to a role in gating mediation by NBD dimerization. Moreover, ATP binding and ATP-dependent channel opening are modulated by PKA-dependent phosphorylation.

The CFTR activity is characterized by three states for the channel: open, closed and open ready (which does not allow anions flux but could rapidly change its conformation into the open state). PKA phosphorylation of the R region is required as well as the presence of intracellular ATP and Mg$^{2+}$. The CFTR molecular mechanism is reported in Figure 1.3.

![Figure 1.3: Schematic CFTR mechanism.](Image)

**Figure 1.3: Schematic CFTR mechanism.** Picture taken from Gout T., Ann. Thorac. Med., 2012, 7, 115-121. Phosphorylation of the R domain disrupts an interaction between the R region and NBD1 that allows ATP to bind at the NBDs. ATP-binding induces the formation of a closed NBD head-to-tail sandwich dimer, leaving the channel in an open-ready transition state conformation (the channel is still closed but could be open). The ATP-induced dimerization of NBDs causes a conformational change in TMD region, the channel opens and chloride flux occurs across the plasma membrane. Hydrolysis of ATP at the active site in the NBDs caused destabilization of the NBD dimer and the released of Pi and ADP causes the closure of the channel gate.
Despite the excellent resolution for the MSD regions observed in the cryo-EM 3D structure,\textsuperscript{11a,b} the resolution obtained for the cytosolic domains (NBDs and intracellular loops, ICLs) has resulted to be quite low, giving very little information for the critical regulatory R region. Moreover, these first 3D structures correspond to inactive forms of the channel, and information about further conformational states, representing relevant stages in the channel-gating cycle, is still missing.\textsuperscript{11c}

To address these limitations, 3D models of the assembly of CFTR-MSDs/NBDs, in different forms relevant to the gating cycle, were built by comparative modeling, based on crystal structures of similar ABC transporters. Short MD simulations or other approaches enabled development of models of the open form of the channel, consistent with experimental data. Taking into account that these models were built from sequences with low levels of identity to CFTR, particularly for the MSDs, further studies are needed to improve the accuracy of the CFTR models by local superimposition of the experimental and theoretical data.

### 1.2.1 CFTR biogenesis

CFTR biogenesis is a cellular process by which new synthesized CFTR chains attain their fully mature conformation. It is characterized by different steps: post-transcriptional splicing, protein translation, folding at the endoplasmic reticulum (ER), glycosylation in the Golgi compartment, trafficking to the apical membrane, endosomal recycling and retrieval.\textsuperscript{5} Only 20-40% of all CFTR nascent chains reach the plasma membrane in a mature state \textsuperscript{22} and the process is regulated by multiple quality control systems. The latter systems are important to prevent accumulation of misfolded or incorrectly processed CFTR that could be toxic to the cell.\textsuperscript{23} Among these quality control systems, the ubiquitin-proteasome destroy the major fraction of misfolded proteins, proteostasis control CFTR maturation pathway, and lysosomes eliminate non-native protein that escape from endoplasmic reticulum associated degradation (ERAD).\textsuperscript{5}

The first steps of the protein folding process are controlled by the ER. In particular, chaperones and co-chaperones such as HSP40/70/90 and calnexin control the folded state of CFTR and target the misfolded protein for degradation.\textsuperscript{24,25} Moreover, histone deacetylase (HDAC) and vasolin-containing protein (VCP) translocate misfolded CFTR to proteasomes and aggresomes. Surface CFTR expression is modulated by the CFTR-associate ligand (CAL), located at Golgi apparatus, that promotes protein degradation in lysosomes. Finally,
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a system of peripheral proteins quality control (PPQC) and NHERF-1, a CFTR binding protein, regulate distribution and function of CFTR at the plasma membrane, removing improperly folded protein.\(^5\)

### 1.2.2 CFTR mutations

More than 2000 mutations of the gene coding for CFTR are reported in literature and are classified in six groups according to the different mechanism by which they are known to affect CFTR production and/or function (Figure 1.4).\(^1\)

![Figure 1.4: Classes of CFTR mutations](Picture taken from Elborn J. S. The Lancet, 2016, 388, 2519-2531.)

**Class I** mutations result in no protein synthesis due to the insertion of a premature terminal signal caused by splice site abnormalities, insertions or deletions that generate frameshifts and nonsense mutations. Normally, stop codons arising from nonsense mutations serve as signal for translation termination and generate premature stop codons (PTCs). The transcribed mRNA is truncated and unstable resulting in no protein synthesis.\(^26\)

**Class II** mutations lead to a misfolded protein, which has an increased the turnover resulting in reduced amounts of protein at the cell surface. This class includes the most common CFTR mutation, the deletion of phenylalanine at position 508 (i.e., F508del).\(^6\)
which results in retention of the misfolded protein at the endoplasmic reticulum, with a subsequent degradation of mutated CFTR in the proteasome. Nevertheless, few mutant channels manage to be eventually incorporated into the plasma membrane. However, as they are not correctly or completely folded, the conductance and the open probability of the channel is low.

Class III gene mutations affect channel regulation, impairing channel opening and for this reason are often referred to as gating mutations. The G551D mutation, resulting from a glycine-to-aspartate substitution at position 551, has been characterized as one of the most common class III mutations. In this case, although the resulting mutant protein can traffic normally to the apical membrane and is normally phosphorylated, once in the membrane, the ion channel is characterised by a drastically reduced channel activity due to an extremely low open probability.27

Similarly to Class III mutations, Class IV mutants show normal gating but reduced conduction and decreased flow of ions. R334W, a mutation in the CFTR channel pore, is an example of this class.6

Class V and VI mutations are reported to cause substantial reduction of CFTR quantity, and increased turnover of the protein and substantial membrane protein instability, respectively. In particular, class V includes splicing mutants that allow the synthesis of normal CFTR mRNA and protein, although at low level, and mutations that reduce transcription and amino acid modifications, causing inefficient protein maturation.28 Instead, class VI mutations result in reduced stability of CFTR at the cell surface, leading to increased turnover. C.120del23 is an example of this class and is characterized by the absence of the cytoskeleton-anchoring N-terminus of CFTR.29

Several mutations lead to a protein showing features of more than one class, and this fact represents a limitation of the above reported classification. As an example, Class II F508del mutants could be also included in classes III and VI because the protein exhibits defective gating and a reduced cell surface half-life due to its intrinsic instability.26,30,31

Although more than 2000 different mutations are known to be present in CFTR, most of them are present on NBD1, including F508del and G551D.6

All CF patients presents CFTR mutations in both alleles. In particular, people with a loss of function CFTR mutation in both alleles (such as class I to III) have no or low levels of CFTR protein, or protein with reduced activity. This leads to a more severe phenotype of the disease. On the contrary, people having at least one residual function CFTR mutation in one of the alleles show a less severe disease phenotype and better prognosis.5
1.3 Therapies for Cystic Fibrosis treatment

Current CF treatments are based on a combination of therapies trying to address different pathophysiological targets for both the clinical manifestations and underlying genetic defects. Historically, conventional CF treatments have been focused on the symptomatic therapy, including antibiotics (to prevent and treat lung infections), anti-inflammatory drugs, mucus thinning drugs, bronchodilators (to help relax muscles in bronchial tubes), pancreatic enzymes (to improve absorption of nutrients), oxygen therapy, bowel surgery, and ultimately lung transplant.32

Nowadays, aided by the growing understanding of the molecular basis of CF pathology, a pharmacological approach based on small-molecule drugs that address the primary causes of CF is being pursued.

In particular, drug-discovery efforts have demonstrated that CFTR proteins carrying class II and III mutations are a possible target for pharmacotherapy using small molecule compounds known as CFTR modulators.33

1.3.1 Symptomatic therapies

Lung disease treatment in CF is central to clinical management of CF patients and targets airways clearance. Inflammation and repeated respiratory tract infections sustained by viruses and bacteria, such as Haemophylus Influenzae, Staphylococcus Aureus and Pseudomonas Aeruginosa, are the principal targets. Oral and inhaled antibiotics, such as ciprofloxacin, colestin and tobramycin, are used to both prevent and eradicate infections and reduce exacerbations of the diseases.1

In the management of inflammation, only one non-steroidal anti-inflammatory drug, i.e. ibuprofen, has demonstrated efficacy. A1-antitrypsine is the main inhibitor of neutrophil elastase in the lung and dry power inhalation of it is in clinical trials and seems to reduce neutrophil elastase activity. 34 N-acetylcysteine, an antioxidant medication, and sildenafil are currently studied to evaluate the effect on inflammation.35 Similarly, macrolide azithromycin shows a substantial impact on inflammation in vitro and in clinical trials.35, 36

Moreover, therapies have been developed to restore the airway surface liquid. This can be achieve by mucolytic agents, inhibitor of sodium absorption, osmotic therapy (such as hypertonic saline and mannitol) and stimulation of chloride secretion. Indeed, fluid and
electrolyte transport defects cause by CFTR mutations play a key role in the cycle of airway obstruction, inflammation and infection. Agents that alter rheologic proprieties of the mucus and restore the airway surface liquid could improve mucociliary clearance, relieve airway obstruction and facilitating removal of toxic pro-inflammatory materials.\textsuperscript{32} CFTR dysfunction affects also epithelial cells in the pancreatic and biliary ducts, which cause chronic obstructive pancreatitis and nutritional consequences, and pancreatic insufficiency. These problems can be treated with a pancreatic enzyme replacement therapy and with a specific dietetic support.\textsuperscript{37} The pancreatic disease, which destroys islet cell function, leads to insulin deficiency and diabetes mellitus, occurring in up to 40% of adults. This disorder is treated with insulin replacement and maintaining a high energy diet.\textsuperscript{38} An optimum management of nutrition is required also for the increased metabolic rate caused by several factors such as increase work of breathing, metabolic consequences of chronic infection and underlying gene mutation.\textsuperscript{1}

Osteopenia, and consequently osteoporosis, is common in CF due to the lower bone mineral density of CF patients. Therefore, calcium, vitamin D and bisphosphonate therapies are generally recommended.\textsuperscript{39}

Progressive effects of infection and inflammation lead to extensive bronchiectasis and respiratory failure that could be resolved only with lung transplantation.\textsuperscript{40}

Moreover, the psychosocial effects of chronic disorders, such as depression and anxiety, should also be taken into consideration. These conditions are commonly treated with appropriately cognitive therapies and/or pharmacological support.

### 1.3.2 CFTR modulators

CFTR modulators are small molecules that target specific defects caused by mutations in the CFTR gene and can be divided into five main therapeutic categories: \textit{read-through agents}, \textit{potentiators}, \textit{correctors}, \textit{stabilizers} and \textit{amplifiers}.\textsuperscript{32}

**Read-through agents**

Compounds that promote ribosomal read-through of nonsense mutations are envisaged to allow the synthesis of full-length CFTR proteins from genes with class I mutations. These agents permit the translation to the normal end of transcript by ribosomal over-reading of a premature stop codon.\textsuperscript{41} These read-through effects have been observed for aminoglycosides...
antibiotic class (gentamicin and tobramycin) that are used for the treatment of P. Aeruginosa infections. These compounds are able to inhibit ribosomal proof-reading by binding to the decoding site of rRNA and consequently, reducing the codon anticodon pairing adhesion, allowing the translation to continue to the end of the gene. However, these agents are not specific, having the ability to read-through stop codon in different genes, so possibly generating toxic aggregates that can cause nephrotoxicity and ototoxicity.

In this class of compounds, Ataluren (also known as PTC124, Figure 1.5), a drug initially developed for the treatment of Duchenne muscular dystrophy, has been reported to restore in vitro and in vivo functional CFTR production and reached phase III clinical trials in CF patients carrying class I mutations. In early 2017, the development of Ataluren for CF was terminated because the drug failed to achieve the efficacy endpoint of the clinical study. Only a slight improvement in respiratory performance was observed in a subgroup of patients.

![Figure 1.5: Chemical structure of Ataluren.](image)

Other read-through agents have shown promising results in preclinical studies when combined with other CFTR modulators. Among them, the FDA-approved natural herbal agent, Escin (a mixture of saponins found in Aesculus hippocastanum), already used for its anti-inflammatory, anti-edematous, and vasoprotective properties, showed high levels of read-through and consequent cell surface CFTR expression.

**CFTR potentiators**

Potentiators are compounds that increase the activity of CFTR proteins carrying class III or IV mutations. They increase the flow of chloride ions through mutated CFTR channels that are already on the epithelial cell surface but exhibit no or reduced gating activity. Moreover, CFTR potentiators could also be used in CF patients with class I or II mutations, when the synthesis or the trafficking of the mutated protein are rescued since those proteins usually have a defective channel activity.
In 2012, the first small-molecule drug, Ivacaftor® (VX-770, Kalydeco®, Figure 1.6), developed by Vertex Pharmaceuticals, was approved to restore the function of G551D-CFTR, the most common class III mutant, which is found in circa 5% CF patient population.\textsuperscript{47} Later on, its efficacy was also demonstrated for other less common mutations (such as G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N and S549R),\textsuperscript{48} which allowed this drug to be further approved for the treatment of CF patients with CFTR harbouring those mutations.

![Figure 1.6: Chemical structure of Ivacaftor® (VX-770, Kalydeco®)](image)

Clinical trials in CF patients with the above-reported mutations showed that Ivacaftor® reduced sweat chloride concentration and improved FEV\textsubscript{1} (i.e., Forced Expiratory Volume in one second, that is the volume of air that can forcibly be blown out in first one second after full inspiration). The drug was reported to be generally well tolerated and significantly efficacious in decreasing the risk of exacerbations.\textsuperscript{49} Despite its proven therapeutic effect in treating CF patients carrying a G551D mutation, the efficacy of the monotherapy with Ivacaftor® in improving FEV\textsubscript{1} of F508del homozygous patients resulted to be quite limited. Nevertheless, its combination, using a co-administration approach with the corrector Lumacaftor® (Orkambi®, see below), led to a more efficient rescue of the mutated protein reduction of pulmonary exacerbations, slight decrease of sweat chloride concentration and significant, although modest, improvement in FEV\textsubscript{1}.\textsuperscript{50}

Along with the commercially approved Ivacaftor®, other classes of potentiators have been studied and tested to possibly treat the gating defect of mutated CFTR in CF patients.

In particular, GLPG1837 (Figure 1.7) developed by Galapagos is beneficial in class III mutations, e.g., G551D, G178R, and S549N.\textsuperscript{51} Clinical studies to evaluate safety and efficacy showed significant decrease in patients’ sweat chloride concentration from 97.7 mmol/L (baseline) to 68.7 mmol/L (end of GLPG1837 treatment) and a percent predicted forced expiratory volume in 1 s (FEV\textsubscript{1}) declined from 73.3% at screening to 68.5%. Compound was reported to be generally well tolerated and adverse events were more frequently reported in the high dose treatment period with respiratory adverse events most common.\textsuperscript{52}
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Among other compounds, which have been reported to show some efficacy in improving the gating function of mutated CFTR, the chemical class of flavonoids comprises few molecules. In particular, Genistein and Curcumin have shown synergistic effects in rescuing CFTR activity when combined with the corrector Lumacaftor®\textsuperscript{53}.

**CFTR correctors**

This class of modulators has been shown to modulate the quantity of CFTR present at the level of plasma membrane. CF patients with class II mutations (such as F508del) are the primary target for this kind of compounds, considering that the protein is not correctly folded and is retained within the endoplasmic reticulum and targeted for degradation.\textsuperscript{26}

Correctors act either by enhancing the conformational stability of CFTR, interacting directly with the protein to facilitate the correct folding (i.e., behaving as pharmacological chaperones), thus rescuing its trafficking to the plasma membrane, or by indirectly modulating components of the cellular quality control machinery (i.e., behaving as proteostasis regulators) (Figure 1.8).\textsuperscript{54}

Considering the mechanism of action, pharmacological chaperone correctors could be divided into three classes: compounds that stabilize the interaction between NBD1 and intracellular loops 1 and 4 (referred to as type C-1 correctors), molecules that restore NBD2 stability (referred to as type C-2 correctors), and compounds that directly stabilize NBD1 (referred to as type C-3 correctors).\textsuperscript{5} Proteostasis regulators could act both on peripheral quality control and on ER quality control systems. They have been evaluated for their ability to restore CFTR expression at plasma membrane, considering that wild type CFTR and F508del-CFTR present different interactomes during processing and trafficking.\textsuperscript{54}
The amount of correction needed to provide significant clinical benefit is unknown, but recent findings suggest that 25% of correction in airways epithelial cells could be required to be therapeutically useful. To reach these levels of correction it may be necessary to have more than one corrector with different mechanisms of action, giving an additive or synergistic effect.

Many clinical trials have been conducted in the last few years to determine safety and efficacy parameters for newly identified modulators (Table 1.1). A breakthrough was reached in late 2015, when the first small molecule CFTR corrector Lumacaftor® (VX-809, Figure 1.9), developed by Vertex Pharmaceuticals, was approved by FDA as a combination therapy with Ivacaftor®, with the trade name of Orkambi®, for the treatment of homozygous CF patients, carrying two copies of the F508del CFTR gene (circa 50% CF cases).
Table 1.1: Modulators developed for CF treatment. Picture taken from Chaudary N. Therapeutics and Clinical Risk Management, 2018, 14, 2375-2383.

<table>
<thead>
<tr>
<th>Name</th>
<th>Clinical stage</th>
<th>Target</th>
<th>Mode of action</th>
<th>Company</th>
</tr>
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<tr>
<td>Ivacaftor</td>
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<td>CFTR</td>
<td>Potentiator</td>
<td>Vertex Pharmaceuticals</td>
</tr>
<tr>
<td>Lumacaftor + ivacaftor</td>
<td>Approved</td>
<td>CFTR</td>
<td>Corrector + potentiator</td>
<td>Vertex Pharmaceuticals</td>
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<td>Approved</td>
<td>CFTR</td>
<td>Corrector + potentiator</td>
<td>Vertex Pharmaceuticals</td>
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<tr>
<td>VX-445 + tezacaftor + ivacaftor</td>
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<td>CFTR</td>
<td>VX-445 – new corrector</td>
<td>Vertex Pharmaceuticals</td>
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<tr>
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<td>Phase II</td>
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<td>Potentiator</td>
<td>Novartis Pharmaceuticals</td>
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<tr>
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<td>Phase II</td>
<td>CFTR</td>
<td>Corrector</td>
<td>Flatley Discovery Lab</td>
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<tr>
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<td>CFTR</td>
<td>VX-152 – new corrector</td>
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<tr>
<td>VX-440 + tezacaftor + ivacaftor</td>
<td>Phase III</td>
<td>CFTR</td>
<td>VX-440 – new corrector</td>
<td>Vertex Pharmaceuticals</td>
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<tr>
<td>VX-561 (CTF-656)</td>
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<td>Potentiator (deuterated ivacaftor)</td>
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<td>Potentiator</td>
<td>Galapagos NV/AbbVie</td>
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<td>GLPG2222</td>
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<td>Potentiator</td>
<td>Galapagos NV/AbbVie</td>
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<tr>
<td>GLPG3067 + GLPG2222 + GLPG2737</td>
<td>Phase I</td>
<td>CFTR</td>
<td>Potentiator + C1 corrector + C2 corrector</td>
<td>Galapagos NV/AbbVie</td>
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<tr>
<td>PTI-428</td>
<td>Phase I</td>
<td>CFTR</td>
<td>Amplifier that increases amount of CFTR protein</td>
<td>Proteostasis Therapeutics</td>
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<tr>
<td>QR-010</td>
<td>Phase I</td>
<td>CFTR</td>
<td>Oligonucleotide that repairs CFTR mRNA</td>
<td>ProQR Therapeutics</td>
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<td>CFTR</td>
<td>Delivers CFTR mRNA</td>
<td>Translate Bio</td>
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<td>PTI-801</td>
<td>Phase I</td>
<td>CFTR</td>
<td>Corrector</td>
<td>Proteostasis Therapeutics</td>
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<td>Phase I</td>
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<td>Potentiator</td>
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<td>ENaC</td>
<td>Inhibits ENaC activity</td>
<td>Novartis Pharmaceuticals</td>
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<tr>
<td>SPC-101</td>
<td>Phase II</td>
<td>ENaC</td>
<td>Peptide that induces ENaC internalization</td>
<td>Spyxx Biosciences</td>
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<tr>
<td>AZD6344</td>
<td>Phase I</td>
<td>ENaC</td>
<td>Inhibits ENaC activity</td>
<td>AstraZeneca</td>
</tr>
<tr>
<td>BI 43651</td>
<td>Phase I</td>
<td>ENaC</td>
<td>Inhibits ENaC activity</td>
<td>Boehringer Ingelheim</td>
</tr>
</tbody>
</table>

Notes: Summary of ion channel-targeting therapeutics according to their target, mode of action, and stage of clinical testing or approval. Adapted from Chest, 154(1), Gentzsch M. Mail MA, Ion Channel Modulators in Cystic Fibrosis, 383–393, Copyright (2018), with permission from Elsevier.

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na channel.

VX-809 was shown to restore F508del-CFTR expression and function in human bronchial epithelial (HBE) cells and seems to act on the early steps of CFTR synthesis. This notwithstanding, Lumacaftor® treatment alone showed a marked decrease only in sweat chloride levels, with minimal improvements in forced expiratory volume in one second (FEV₁) in phase II trials with F508del homozygous patients.

![Figure 1.9: Chemical structure of Lumacaftor® (VX-809).](image)

Although CFTR modulators, particularly correctors, provided new perspectives and advances in the treatment of patients with common and rare mutations, in vitro and clinical
studies showed that monotherapy with correctors alone was not sufficiently efficacious to provide clinical benefit in patients, in particular those with F508del mutations. Therefore, combination therapies of a corrector and a potentiator were evaluated and showed to enhance the rescue of CFTR bearing F508del compared to monotherapies. These findings led to the approval of Orkambi® (VX-809+VX-770) as the first treatment for CF patients, carrying a homozygous F508del/F508del mutation.\textsuperscript{5}

The rationale supporting the use of these combination therapies was based on their complementary mechanism of action considering that correction of protein folding and channel opening are both required to treat CF patients with type II CFTR mutations. Orkambi\textsuperscript{®} proved to be safe and clinically efficacious with remarkable reduction of sweat chloride values and a small but significant improvement (around 3\%) of FEV\textsubscript{1}.\textsuperscript{58}

Furthermore, CF patients treated with this combined therapy showed also a decrease in pulmonary exacerbations, reduction in the rate of lung function decline, and improved nutritional status. Intravenous antibiotics and hospitalization were also reduced.\textsuperscript{59} Therapeutic treatment with Orkambi\textsuperscript{®} had and still has an overall acceptable side-effect profile, although the incidence of some respiratory adverse events was higher with Lumacaftor\textsuperscript{®}/Ivacaftor\textsuperscript{®} compared to the placebo group.\textsuperscript{48,60}

The drug discovery efforts to identify novel and better modulators led to the market authorization of a new combination therapy. In 2018, FDA approved Symdeco\textsuperscript{®,} a combination of Tezacaftor\textsuperscript{®} (VX-661, Figure 1.10) and Ivacaftor\textsuperscript{®} (Figure 1.6) for the treatment of CF patient aged 12 years or older with either two copies of the F508del mutation or with one F508del mutation and one additional mutation that results in residual CFTR function.\textsuperscript{32}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{tezacaftor.png}
\caption{Chemical structure of Tezacaftor\textsuperscript{®} (VX-661).}
\end{figure}

A 24-week phase III study showed that this new combination improved lung function (around 4\%), decreased sweat chloride levels and most importantly reduced pulmonary exacerbations in subjects homozygous for F508del. Another study in CF individuals compound heterozygous for F508del and G551D proved that Symdeko\textsuperscript{®} could decrease sweat chloride and increase in FEV\textsubscript{1} levels (ca. 5\%) to a slightly higher extent compared to
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homozygous patients. Interestingly, data from clinical trials reported Symdeko® to show less side effects and drug-drug interactions compared to Orkambi®, overall proving more favorable pharmacokinetic and drug-like profiles.

New generations of correctors, featuring a different mechanism of action to the ones previously developed, have been also evaluated in combination regimens trying to further stabilize/rescue mutated CFTR and improve its function. Pharmaceutical companies are actively trying to identify and test novel modulators to be used in combination therapies, and addressing several mutations with better results than the current treatments.

The newest and most promising research involves the use of first-generation potentiators and correctors in combination with a so-called next-generation drug. This therapeutic approach, called triple combination, has shown promising results in the partial restoration of the activity of the most common CFTR mutant. In particular, Vertex Pharmaceuticals moved aggressively in this field, trying to verify the hypothesis that a triple combination therapy could provide greater efficacy in CF patients bearing F508del and other less abundant mutations. A few second-generation correctors were identified (e.g. VX-440, VX-445, VX-152 and VX-659) and the most promising ones tested in clinical trials in combination with first generation correctors (i.e., Lumacaftor® or Tezacaftor®) and the potentiator Ivacaftor®. Some of these studies reported much better improvements in efficacy (measured as FEV₁ and sweat chloride values) than previously approved treatments.

From these latest studies, one compound showed very promising therapeutic results and in October 2019 FDA approved a novel triple combination with the trade name of Trikafta®. This CF therapy combines the action of two correctors Elexacaftor® (VX-445, Figure 1.11) and Tezacaftor® (VX-661), having different mechanisms of action, plus the potentiator Ivacaftor® (VX-770).

![Figure 1.11: Chemical structure of Elexacaftor® (VX-445).](image-url)
In randomized phase III clinical studies, this triple combination showed increased therapeutic efficacy with respect to Orkambi® or Symdeko® in CF patients aged 12 years or older, being homozygous with a F508del/F508del mutation or heterozygous with a single F508del and a mutation with a residual function. This new medication showed significantly improved F508del-CFTR protein processing and trafficking, resulting in a FEV₁ increase of up to 13.8% and markedly reduced sweat chloride concentration (in heterozygous patients) compared to Symdeco®. Moreover, the treatment with Trikafta® led also to an increase of body weight and body-mass index, and resulted in a 63% lower annualized rate of pulmonary exacerbations. The triple combination regimen was generally safe and well tolerated and the most adverse events were mild or moderate.

Different classes of correctors have been also developed by Galapagos and Abbvie. Among them, ABBV/GLPG-2222 and ABBV/GLPG-3221 (Figure 1.12) were advanced to clinical trials. ABBV/GLPG-2222 was well tolerated and showed sweat chloride decreased at the 50, 100, and 200mg doses. In particular, the decrease was dependent on the specific dose, with the biggest decrease at the 200mg dose (-15.8 mmol/L p< 0.000). ABBV/GLPG-3221 is being studied as part of a triple combination therapy, along with another corrector and a potentiator molecule. The Phase 1 trial of the triple combination resulted in an increase in chloride transport and in the restoration of protein levels on the surface of human bronchial epithelial (HBE) cells from patients with the F508del mutation.

Figure 1.12: Chemical structure of ABBV/GLPG-2222 and ABBV/GLPG-3221.

Among the proteostasis regulators, Cysteamine (approved by FDA for nephropatic cystinosis) has shown activity for the treatment of CF. In particular, its association with epigallocatechin gallate has been shown to restore the function and stability of CFTR at plasma membrane by correcting autophagy flux and rescuing CFTR trafficking. This
combination has been reported to decrease both sweat chloride concentration and inflammatory biomarkers levels and to improve FEV₁ in F508del-homozygous and heterozygous patients in a phase II clinical trial.⁷¹

Other proteostasis regulators such as phosphodiesterase inhibitors (Sildenafil and its analogs), histone deacetylase inhibitors, ouabain and ADP-ribosome polymerase (PARP) inhibitors (latonduine analogs) are currently studied to evaluate their effects on trafficking of CFTR.⁵⁴

**Stabilizers**
This is a class of compounds that corrects the instability of class IV mutations by anchoring CFTR channel at the plasma membrane, leading to a decrease in its degradation. Cavosonstat (Figure 1.13) is the first stabilizer in phase II clinical trials in F508del homozygous patients treated with the combination therapy of Lumacaftor®/Ivacaftor®. This compound has been described to increase S-nitrosoglutathione levels, which enhance CFTR maturation and stability.⁵

![Figure 1.13: Chemical structure of Cavosonstat.](image)

**Amplifiers and correctors of the splicing**
These types of CFTR modulators act primarily on mRNA. **CFTR amplifiers** are novel mutation-independent modulators that work by increasing immature CFTR protein expression by stabilising CFTR mRNA. These amplifiers, acting synergistically with correctors and potentiators to increase therapeutic benefits, can find application to the treatment of CF patients independently of the CFTR mutations they carry.⁷² At the time being, there are only two types of amplifiers, which have shown promising results in pre-clinical and clinical trials, PTI-428 (Figure 1.14) and PTI-CH, both developed by Proteostasis Therapeutics.⁷³
In recent \textit{in vitro} studies, PTI-428 has been shown to nearly double CFTR protein activity when used in combination with other therapies, increasing CFTR function across multiple gene mutations.\textsuperscript{74} PTI-428 is currently being studied both alone as well as in combination with two other CFTR modulators. A clinical trial sponsored by Proteostasis Therapeutics evaluated PTI-428’s safety, tolerability, and pharmacokinetics in CF patients with any mutations alone and in combination with other modulators. In particular, initial results showed that patients who received PTI-428, as an add-on to their existing Orkambi\textsuperscript{®} treatment, had improved lung function, compared with those taking Orkambi\textsuperscript{®} alone. Treatment with PTI-428 appeared well-tolerated, with no serious side effects. However, changes in sweat chloride did not correlate with lung function improvements. A phase II study of PTI-428 in combination with other modulators in people with two copies of the F508del CFTR mutation is underway.\textsuperscript{75}

Antisense oligonucleotide-based therapy is a new approach that aims to correct \textit{class V} mutations (caused by aberrant mRNA generated by an alternative splicing) by modulating the splicing defect and restoring the normal full-length CFTR transcript, therefore rescuing a functional protein. Actually, an RNA oligonucleotide (QR-010) is in phase I clinical trials to evaluate its effects both on F508del homozygous and heterozygous patients.\textsuperscript{76}

\section*{1.4 Aim of the project}

So far, Orkambi\textsuperscript{®}, Symdeco\textsuperscript{®} and Trikafta\textsuperscript{®} are the combinations of small molecule drugs approved for the treatment of CF patients carrying at least one copy of F508del mutation. The compounds have been developed, studied and are currently commercialized by Vertex Pharmaceuticals.

Aiming to expand the portfolio of novel modulators available to CF patients, considering the promising, but limited pharmacological efficacy elicited in particular by treatments with first generation’s correctors (e.g. Lumacaftor\textsuperscript{®} and Tezacaftor\textsuperscript{®}), and the reported liabilities (i.e., side effects) highlighted for some of these drugs, there is still the
need to develop other CFTR small molecule modulators, primarily correctors, which may address the primary cause of CF by rescuing the activity of defective CFTR proteins.

The aim of my PhD project was focused on the design, synthesis and biological characterization of novel small-molecules as CFTR correctors.

The first part of my research work was mainly dedicated to the synthesis of the primary hit ARN9364 and close analogs. ARN9364 was identified as a hit in a high-throughput screening (HTS) of the IIT compound collection, consisting of 11,334 commercial small molecules, conducted at the Istituto Giannina Gaslini (IGG) in Genoa, using a phenotypic cell assay on two different cell lines (FRT and CFBE41o- cells stably transfected with F508del-CFTR).

Unexpectedly, the careful UPLC/MS and NMR characterizations first, and biological data afterwards (Chapter 2, paragraph 2.1 and Chapter 3, paragraph 3.2), proved that the chemical structures of the library hit and of the in-house re-synthesized compound were not in agreement. After an accurate analysis of NMR data (both $^1$H and $^{13}$C mono- and bi-dimensional studies), a new structure was assigned to the library hit compound, which was then re-synthesized following a suitable protocol (Chapter 2, paragraph 2.1).

Through the insertion of small modifications on the chemical structure of the actual hit, new analogs were designed and synthetized, aiming to possibly elucidate the Structure-Activity Relationship (SAR) patterns around this novel chemo-type (Chapter 2, paragraph 2.2 and Chapter 3, paragraph 3.2). In particular, our attention was focused on the modifications on the pyrrolidine, pyrazole, and amino-pyridine moieties present in the molecule.

The biological test of these compounds in a phenotypic assay in the CFBE41o- cell line (Chapter 3, paragraph 3.1) allowed getting additional information about the most suited structural modifications, thus starting an iterative process which led to the identification of slightly more potent CFTR modulators.

Along with the synthesis of new ARN9364 analogs, a side-project of this PhD work was dedicated to the design and synthesis of new derivatives of another hit compound, ARN5562 (Chapter 2, paragraph 2.3). I took an active part in the synthesis of a few analogs trying to expand the SAR and improve the drug-like properties of a novel chemo-type derived from the hit ARN5562.
1.5 Bibliography

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2. Chemistry

2.1 Synthesis of ARN9364

The hit compound ARN9364 (CAS: 1794126-00-3, Figure 2.1) was identified by a high-throughput screening (HTS) of the IIT compound library consisting of 11,334 commercial small molecules. The screening was conducted at the Istituto Giannina Gaslini (IGG, Genoa), using a phenotypic cell assay, on two different cell lines, Fischer Rat Thyroid [FRT] and Cystic Fibrosis Bronchial Epitelial-41o- [CFBE41o-] cells, both stably expressing the F508del-CFTR protein and transfected with the Halide Sensitive Yellow Fluorescent Protein (HS-YFP). Arn9364 was selected as a starting point for this PhD project due to its initial promising biological activity.

![Reported structure of ARN9364](image)

The confirmation of the chemical structure of a hit is of pivotal importance in any screening of commercial compounds. ARN9364 is characterized by a pyrazole core connected with a pyrrolidine motif in position 3 and a substituted pyridine moiety in position 4. Leaving out the functionalization of pyrrolidine and pyridine, we envisioned two reasonable retrosynthetic disconnections (Scheme 2.1).

*Retrosynthetic path 1* required the formation of the pyrazole core through a late stage cyclization of a β-ketoaldehyde B. Intermediate B could be obtained by α-formylation of ketone derivative C, which in turn can be prepared by acylation of the 4-methylpyridine E with properly substituted derivatives of the carboxylic acid D. In the *retrosynthetic path 2*, we envisioned the sequential functionalization of a pre-formed pyrazole scaffold. As already reported, 3-substituted pyrazoles such as compound G, could be easily halogenated in position 4 to furnish intermediates F, which could be exploited in cross-coupling reactions.
to insert the pyridine moiety. In a similar manner, compound G could be obtained from commercially available building blocks, such as H and J.

Scheme 2.1: Proposed retro-synthetic disconnections to ARN9364.

Based on the proposed retro-synthetic disconnections, two different synthetic routes were outlined and planned. The first synthesis (Scheme 2.2) involved as the first step the formation of the triflate 3, starting from commercially available building blocks 1 and 2. Unfortunately, after few unsuccessful attempts to obtain compound 3 leading to only trace amounts of desired product along with many unidentified impurities, this route was abandoned in favor of the other protocol.
Scheme 2.2: First proposed synthetic route to the synthesis of ARN9364.

The re-synthesis of ARN9364 was then planned following the synthetic approach reported in Scheme 2.3.
This protocol is based on the possibility to functionalize the 4-alkyl position of a 2-halopyridine with an acylating agent via a metalation process. The first synthetic step was the formation of the Weinreb amide 14, from the corresponding acid 12 and N,O-dimethylhydroxylamine hydrochloride (13), using EDC as coupling reagent in presence of HOBT and DIPEA in DCM in 92% yield. The formation of ketone 16 from Weinreb amide 14 and 2-bromo-4-methyl pyridine (15) required a careful optimization of the reaction conditions (Table 2.1). This reaction turned out to be the limiting step in this route, due to a problematic purification and modest yield. In the first attempt (entry 1, Table 2.1), LDA
(1.1 eq.) was added to a solution of 15 at 0°C, followed by addition of the amide 14 at -78°C, affording the desired product 16 in 28% yield. Increasing the equivalents of LDA (1.3 eq.), with the same temperature control, led to a disappointing result (entry 2). However, using the same amount of LDA (1.3 eq.), but performing both the addition of the reagents and the reaction at -78°C (entry 4), compound 16 was obtained in 75% yield. In general, after repeating this reaction step few times, we noticed a clear effect of the temperature and the quality of LDA on the overall outcome.

Table 2.1: Optimization of the formation of ketone 16.

<table>
<thead>
<tr>
<th>ENTRY</th>
<th>15 (eq.)</th>
<th>14 (eq.)</th>
<th>LDA (eq.)</th>
<th>Temperature (LDA addition)</th>
<th>Temperature (amide addition)</th>
<th>Yield (%)</th>
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</thead>
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<tr>
<td>1</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
<td>0°C</td>
<td>-78°C</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3</td>
<td>0°C</td>
<td>-78°C</td>
<td>No reaction</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3</td>
<td>-78°C to 0°C for 1h</td>
<td>-78°C</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3</td>
<td>-78°C</td>
<td>-78°C</td>
<td>75</td>
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</table>

The next reaction step consisted in the formation of an intermediate suitable for the pyrazole preparation, such as a 1,3-dicarbonyl derivative or a masked β-ketoaldehyde. Since in literature several cyclization protocols to pyrazoles have been reported starting from β-enaminones, we decided to follow this approach. The reaction of compound 16 with dimethylformamide dimethylacetal (DMF-DMA) in toluene at 120°C for 1 h afforded the enamine 17, which was submitted to the cyclization step in the presence of hydrazine without purification. This process afforded pyrazole 18 in 63% yield over two steps. The subsequent deprotection of Boc-pyrrolidine was performed with HCl 3 M in methanol at room temperature for one hour, obtaining compound 19 in quantitative yield. The following cyclic amine acylation was carried out with EDC, as coupling reagent, in presence of HOBT and DIPEA in DCM to afford compound 20 in high yield (69%). At this stage of the synthetic
process, a pyrazole protection step was necessary to improve the outcome of the following cross-coupling Buckwald-Hartwing reaction. To do so, we decided to install a suited tetrahydro-pyryl (THP) group. Treatment of 20 with 3,4-dihydro-2H-pyran in the presence of trifluoroacetic acid at 60 °C gave the protected pyrazole 22 in high yield (70%).

With compound 22 in hand, our attention moved to the insertion of an amino group in position 2 of the pyridine ring to give the intermediate for the preparation of the target n-propylamino derivative. We envisaged installing a Boc-protected amino group via a Buchwald-Hartwing coupling, starting from 2-bromo pyridine 22.

Table 2.2: Buckwald-Hartwing reaction optimization.

<table>
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<tr>
<th>ENTRY</th>
<th>Pd-CATALYST</th>
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<th>BASE</th>
<th>SOLVENT</th>
<th>YIELD (Temp.)</th>
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<tr>
<td>1</td>
<td>[Pd2(dba)3]</td>
<td>X-Phos</td>
<td>Cs2CO3</td>
<td>Toluene (120°C)</td>
<td>No reaction</td>
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<tr>
<td>2</td>
<td>[Pd(OAc)2]</td>
<td>X-Phos</td>
<td>Cs2CO3</td>
<td>Dioxane (90°C)</td>
<td>No reaction</td>
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<tr>
<td>3</td>
<td>[Pd2(dba)3]</td>
<td>Xantphos</td>
<td>Cs2CO3</td>
<td>Dioxane (110°C)</td>
<td>55%</td>
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</table>

A careful optimization of the reaction parameters (Table 2.2.) allowed obtaining the desired product in reasonable yield. The combination of [Pd2(dba)3], xantphos as ligand, and Cs2CO3 as the base, in dioxane at 110°C (entry 3, Table 2.2) led to full conversion of the starting material and afforded the desired product 24 in 55% yields.

The next step of the synthesis was the alkylation of intermediate 24. In the first attempts using cesium carbonate in DMF and 1-bromopropane, we observed the formation of intermediate 25, though with low conversion both at room temperature and at 70°C. However, good results were obtained using tetrabutylammonium iodide (TBAI), as phase
transfer catalyst, cesium carbonate in DMF at room temperature, giving compound 25 in 60% yield.

Finally, the deprotection of intermediate 25 with HCl 3.0 M in MeOH led to the formation of 26 in 76% yield (7% yield over 10 steps).

Once synthesized, compound 26 was submitted to internal structural identity quality control. Unexpectedly, the careful UPLC/MS and NMR characterizations first (see below), and biological data afterwards (see Chapter 3, Paragraph 3.2), proved that the chemical structures of the library hit and of the in-house re-synthesized compound were not in agreement (Figure 2.2).

*Synthesized compound*

![UPLC/MS chromatograms of synthesized compound](image1)

*Purchased library compound*

![UPLC/MS chromatograms of purchased library compound](image2)

Figure 2.2: UPLC/MS chromatograms of synthesized compound (top) and purchased library compound (bottom).
Although the UPLC/MS of the synthesized compound showed the same mass (MW) of the purchased compound, both the retention time and UV profile of the two samples were not overlapping.

Similarly, when comparing the $^1$H NMR spectra in deuterated DMSO major differences in the proton spectrum were identified (Figure 2.3). Concerning the aromatic portion of the spectrum, discrepancies were evident both in terms of chemical shift and multiplicity for the pyridine signals (from 6.5 to ca. 8.5 ppm Figure 2.3, box A’ and A’’). In addition, the presence of a singlet at 10.25 ppm (Figure 2.3, box C’’) in the purchased sample was not shown in the synthesized hit (Figure 2.3, box C’).

*Synthesized compound*

*Purchased library compound*

Figure 2.3: NMR spectra of synthesized compound (top) and purchased library compound (bottom).
Major differences were also present in the aliphatic portion of the proton spectra. In fact, although the first signal (ca. 1.0 ppm, Figure 2.3, box B’ and B”’) matched in both chemical shift and multiplicity, the other set of signals (range from 1.50 ppm to 3.75 ppm, Figure 2.3, box B’ and B”’) was not consistent for the two compounds. Lastly, a different multiplicity was also noticed in the signals around the water signal (3.3 ppm).

Based on the careful, in-depth UPLC/MS and NMR analyses, we were reasonably confident that the reported structure of purchased ARN9364 was not consistent with experimental data.

In order to unambiguously determine the correct structure of ARN9364, further NMR experiments (both 1H and 13C mono- and bi-dimensional studies) were performed on the purchased sample. Some of these spectra (e.g. 1H-13C-HSQC, 1H-15N-HMOC, 1H-15N-HMBC, 1H-13C-HMBC) used for the correct assignments of the chemical structure are reported below in Figures 2.4a-d.

Figure 2.4a: 1H-13C-HSQC spectra.
Figure 2.4b: $^1$H-$^{15}$N-HMQC spectra.

Figure 2.4c: $^1$H-$^{15}$N-HMBC spectra.
Some of the key-assignments for ARN9364 structure determination are outlined below and reported in Table 2.3.

In particular, from the NMR spectra we could observe:

- Strong long range 1,2-interaction between NH in position 20 and the $^{13}$C in position 21 at 173.8 ppm typical of amide group.
- Strong long range 1,3-interaction between CH in position 23 and the $^{13}$C in position 21 at 173.8 ppm.
- Strong long range 1,4-interaction between CH (axial) in position 26+24 and the $^{13}$C in position 21 at 173.8.
- Strong long range 1,3-interaction between CH in position 12 and the $^{13}$C in position 4 at 116.8 ppm.
- Strong long range 1,3 interaction between CH in position 12 and the $^{13}$C in position 11 at 143.1.
- Weak long range 1,4-interaction between NH in position 20 and the $^{13}$C in position 11 at 143.1.

Taken together, this information allowed us to reasonably propose a new structure of our hit compound. In particular, the interactions between NH in position 20 with either the carbon in position 11, showing a signal at 143.1 ppm, or the carbon in position 21, showing
a signal at 173.8 ppm (see corresponding arrows in Figure 2.5), clearly indicated that the
cyclobutanecarbonyl group was on the amino pyrimidine portion of the molecule and not on
the pyrrolidine (i.e. the amide moiety and the alkyl chain are swapped, Table 2.3, Figure
2.5).

Supported by this accurate NMR analysis, a new structure was assigned to the
purchased compound, as depicted in Figure 2.5. To confirm our hypothesis, a synthetic
procedure to prepare the compound was designed.

Figure 2.5: Correct chemical structure of ARN9364.

Table 2.3: $^1$H, $^{13}$C and $^{15}$N NMR assignments (DMSO-$d_6$ 600 MHz) for the correct structure of ARN9364.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>$^1$H</th>
<th>J$_{C-H}$(Hz)</th>
<th>$^{13}$C</th>
<th>$^{15}$N</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>278.5</td>
<td>N</td>
</tr>
<tr>
<td>1</td>
<td>12.87</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(NH) br s, 1H</td>
</tr>
<tr>
<td>20</td>
<td>10.26</td>
<td>-</td>
<td>-</td>
<td>140.5</td>
<td>s 1H</td>
</tr>
<tr>
<td>12</td>
<td>8.23</td>
<td>-</td>
<td>110.9</td>
<td>-</td>
<td>s, 1H</td>
</tr>
<tr>
<td>15</td>
<td>8.229</td>
<td>5.25</td>
<td>148.1</td>
<td>-</td>
<td>ps d, 1H</td>
</tr>
<tr>
<td>5</td>
<td>7.98</td>
<td>-</td>
<td>116.7</td>
<td>-</td>
<td>br s, 1H</td>
</tr>
<tr>
<td>16</td>
<td>7.14</td>
<td>5.25</td>
<td>117.6</td>
<td>-</td>
<td>ps d, 1H</td>
</tr>
<tr>
<td>6</td>
<td>3.64</td>
<td>-</td>
<td>34.6</td>
<td>-</td>
<td>m, 1H</td>
</tr>
<tr>
<td>23</td>
<td>3.37</td>
<td>8.52</td>
<td>39.1</td>
<td>-</td>
<td>quint, 1H</td>
</tr>
<tr>
<td>7</td>
<td>3.11 and 2.62</td>
<td>-</td>
<td>59.4</td>
<td>m, 2H</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2.81 and 2.60</td>
<td>-</td>
<td>53.4</td>
<td>m, 2H</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2.45</td>
<td>-</td>
<td>57.4</td>
<td>-</td>
<td>br s, 2H</td>
</tr>
<tr>
<td>10</td>
<td>2.29 and 1.95</td>
<td>-</td>
<td>30.6</td>
<td>m, 2H</td>
<td></td>
</tr>
<tr>
<td>26+24</td>
<td>2.22 and 2.09</td>
<td>-</td>
<td>24.5</td>
<td>m, 4H</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1.93 and 1.80</td>
<td>-</td>
<td>17.7</td>
<td>m, 2H</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.48</td>
<td>7.44</td>
<td>21.3</td>
<td>-</td>
<td>sext, 2H</td>
</tr>
<tr>
<td>19</td>
<td>0.88</td>
<td>7.43</td>
<td>11.9</td>
<td>-</td>
<td>t, 3H</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>-</td>
<td>173.8</td>
<td>-</td>
<td>CO</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>152.6</td>
<td>-</td>
<td>C</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>143.1</td>
<td>-</td>
<td>q C</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>116.8</td>
<td>-</td>
<td>q C</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>Not detected</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
For the synthesis of the correct structure of **ARN9364**, changes were made to the previously described protocol. Based on a reasonable retrosynthetic disconnection (Scheme 2.4), this approach implied the sequential functionalization of the pre-formed pyrazole scaffold C. As already reported, 3-substituted pyrazoles, such as C,² can be easily halogenated in 4 position (compound B), giving the possibility to exploit cross-coupling reactions to insert aromatic moieties (e.g. a pyridine ring) thus affording 3,4-disubstituted analogs A.

![Scheme 2.4: Proposed retro-synthetic disconnections to ARN9364.](image)

The formation of the pyrazole scaffold could be obtained through the cyclization of a β-ketoaldehyde D, which could be prepared by α-formylation of ketone derivative E, synthesized via a Grignard reaction from commercially available carboxylic acids F.

The synthetic route used to obtain **ARN9364** with the correct structure is reported in Scheme 2.5.

This protocol required the initial formation of the pyrazole by a cyclization reaction of a suitable pyrrolidine derivative.¹¹ Therefore, the first synthetic step entailed the formation of Weinreb amide 14 from the corresponding carboxylic acid 12 and N,O-dimethylhydroxylamine hydrochloride (13), using EDC in presence of HOBT and DIPEA in DCM.
Next, a Grignard reaction with methylmagnesium bromide on amide 14 gave ketone 27 in quantitative yield. The reaction was initially performed at -78°C, showing only partial conversion of 14 into 27. For this reason, after MeMgBr addition, we decided to increase gradually the temperature up to 0°C, allowing obtaining the ketone 27 in quantitative yield and acceptable purity, which was used in the following step without further purification.

The next key-step consisted in the preparation of the 3-(3-pyrazolyl)-1-tert-butoxycarbonylpiperidilnine, also in this case via a suited β-enaminone as precursor. The reaction of methyl ketone 27 with dimethylformamide-dimethylacetal (DMF-DMA) in DMF
at 140°C for 24 h afforded the enamine 28, which was submitted, without purification, to a cyclization step in the presence of hydrazine in EtOH at 85°C. This process afforded the corresponding pyrazole 29 in 80% overall yield, over three steps. With compound 29 in hand, our attention moved to the regioselective functionalization of 4-position of the pyrazole with a halogen, as the intermediate for the insertion of the pyridine moiety through a cross-coupling reaction. Following a regioselective reaction, using N-bromosuccinimide in DMF, a bromine atom was installed at position 4 of the pyrazole, affording compound 30 in quantitative yield.

At this stage of the synthetic process, the N-1-pyrazole protection step was necessary to improve the outcome of the following Suzuki cross-coupling reaction. As reported in the literature, we inserted a 2-(trimethylsilyl)ethoxymethyl (SEM) protecting group on N-1 position of compound 30 under basic conditions, affording the desired protected pyrazole 31 in high yield (94%). Next, the Suzuki reaction between bromo-derivative 31 and the commercially available boronic ester 32 was performed to obtain compound 33 (Table 2.4). This synthetic step required the optimization of the reaction conditions in order to increase the initially modest yield, due to the incomplete conversion of compound 31 and to the formation of the corresponding de-halogenated byproduct (33-byprod). After several attempts modifying the reaction parameters, such as solvent, base, Pd-catalyst and temperature (entry 1-7), we were able to find the optimal conditions to perform the Suzuki reaction (entry 8). In fact, using a combination of 0.2 equivalents of [Pd(dppf)Cl]2*DCM and Cs2CO3, as the base, in dioxane/H2O (3:1, conc.: 0.03 M) at 110°C, full conversion of the starting material was achieved affording the desired product 33 in acceptable yields (65%).
Table 2.4. Optimization of Suzuki reaction to compound 33.

<table>
<thead>
<tr>
<th>Entry</th>
<th>32 (eq.)</th>
<th>Base</th>
<th>Pd-Catalyst</th>
<th>Solvent (concentration)</th>
<th>Temperature</th>
<th>Yield/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>Cs₂CO₃</td>
<td>[Pd(dppf)Cl₂]*DCM (0.07 eq)</td>
<td>Dioxane/H₂O 3:1 (M=0.03)</td>
<td>110°C</td>
<td>37% mix of 33-byprod and 31.</td>
</tr>
<tr>
<td>2</td>
<td>1.3</td>
<td>Cs₂CO₃</td>
<td>[Pd-Tetrakis] (0.07 eq)</td>
<td>Dioxane/H₂O 3:1 (M=0.03)</td>
<td>90°C</td>
<td>Trace amount of 33; mix of 33-byprod and 31.</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>Cs₂CO₃</td>
<td>[Pd(dppf)Cl₂]*DCM (0.07 eq)</td>
<td>DME/H₂O 3:1 (M=0.03)</td>
<td>80°C</td>
<td>Trace amount of 33; mix of 33-byprod and 31.</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>Na₂CO₃</td>
<td>[Pd(dppf)Cl₂]*DCM (0.07 eq)</td>
<td>DME/H₂O 3:1 (M=0.03)</td>
<td>80°C</td>
<td>Trace amount of 33; mix of 33-byprod and 31.</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>Na₂CO₃</td>
<td>[Pd(dppf)Cl₂]*DCM (0.07 eq)</td>
<td>Dioxane/H₂O 3:1 (M=0.03)</td>
<td>80°C</td>
<td>Trace amount of 33; mix of 33-byprod and 31.</td>
</tr>
<tr>
<td>6¹⁶</td>
<td>1.5</td>
<td>CsOAc</td>
<td>[Pd(dppf)Cl₂]*DCM (0.07 eq)</td>
<td>THF (M=0.1)</td>
<td>80°C</td>
<td>Trace amount of 33; mix of 33-byprod and 31.</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>CsF</td>
<td>[Pd(dppf)Cl₂]*DCM (0.07 eq)</td>
<td>THF (M=0.1)</td>
<td>80°C</td>
<td>Trace amount of 33; mix of 33-byprod and 31.</td>
</tr>
<tr>
<td>8</td>
<td>1.5</td>
<td>Cs₂CO₃</td>
<td>[Pd(dppf)Cl₂]*DCM (0.2 eq)</td>
<td>Dioxane/H₂O 3:1 (M=0.03)</td>
<td>110°C</td>
<td>65% (full conversion of 31) trace amount of 33-byprod</td>
</tr>
</tbody>
</table>

The acylation step of the amino group on the pyridine ring was at first performed under different reaction conditions, using either the corresponding carboxylic acid or the acyl chloride (Table 2.5). The best results, in terms of conversion and yield, were
achieved performing the reaction with cyclobutane-carboxylic acid chloride 34 and Et₃N, in DCM at room temperature. Although a sub-stoichiometric amount of acyl chloride 34 was used, we generally observed the formation of significant amount of the di-acylated derivative 35, along with the desired compound 36, accompanied by unreacted starting material 33. For this reason, we decided to use 2 equivalents of acyl chloride 34 to fully convert 33 into di-acylated 35, followed by treatment with NaOH 2 M in methanol to afford the corresponding mono-acylated compound 36 in 84% yield over two steps.

Table 2.5: Amino-pyridine acylation optimization.

<table>
<thead>
<tr>
<th>Entry</th>
<th>34 (R)</th>
<th>Base</th>
<th>Coupling reagent</th>
<th>Solvent/ Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OH</td>
<td>DIPEA</td>
<td>EDCI / HOBT</td>
<td>DCM, 24 h</td>
<td>16% (and unreacted 33)</td>
</tr>
<tr>
<td>2</td>
<td>OH</td>
<td>DIPEA</td>
<td>HATU</td>
<td>DCM, 24 h</td>
<td>68% (full conversion of 33)</td>
</tr>
<tr>
<td>3</td>
<td>Cl</td>
<td>Et₃N</td>
<td>-</td>
<td>DCM, 2 h</td>
<td>84% over two steps.</td>
</tr>
</tbody>
</table>

The subsequent simultaneous removal of Boc- and SEM-protecting groups on compound 36 was achieved in a straightforward manner using 10% TFA in DCM at room temperature to give 37, which was directly used in the following step of the synthesis. The final reductive amination step was carried out with propionaldehyde, NaBH(OAc)₃ in MeOH to afford compound 38 (correct structure of ARN9364) in 34% yield (11% yield over 11 steps).

Once synthesized, compound 38 (ARN9364) was characterized by UPLC/MS (Figure 2.6) and ¹H NMR (Figure 2.7) analysis. A complete overlap of the data with those of the library sample, tested initially in the HTS, was observed.
Figure 2.6: UPLC/MS chromatograms of synthesized compound (top) and purchased library compound (bottom)
2.2 Synthesis of ARN9364 analogs

At this point, in order to investigate the structure-activity relationships (SARs) for this class of compounds, we decided to explore the impact on the biological activity of different $N$-substitutions on the pyrrolidine and pyridine moieties, and the effect of an $N$-methyl substitution on the pyrazole.

All the new racemic ARN9364 analogs featuring a different $N$-substitution on the pyrrolidine ring were synthesized starting from intermediate 37. Compounds 39a-f were prepared in a similar manner, in low/moderate yields (24-37%), via a reductive amination using the appropriate aldehyde in the presence of NaBH(OAc)$_3$ in MeOH (Table 2.6).
Table 2.6: Synthesized ARN9364 analogs 39a-f.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R^I</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>39a</td>
<td></td>
<td>24%</td>
</tr>
<tr>
<td>39b</td>
<td></td>
<td>31%</td>
</tr>
<tr>
<td>39c</td>
<td></td>
<td>31%</td>
</tr>
<tr>
<td>39d</td>
<td></td>
<td>29%</td>
</tr>
<tr>
<td>39e</td>
<td></td>
<td>37%</td>
</tr>
<tr>
<td>39f</td>
<td></td>
<td>27%</td>
</tr>
</tbody>
</table>

Next, the di-amide 40 was prepared by N-acylation of the pyrrolidine ring with propionic acid using EDC in presence of HOBT and DIPEA in DCM (44% yield, Scheme 2.6).

The corresponding methyl-carbamate derivative 41 was obtained in 28% yield by reaction of 37 with methylchloroformate and Et$_3$N, in DCM at room temperature (Scheme 2.6).
To evaluate whether a different substituent on the amino group of the pyridine moiety could increase the overall activity in rescuing mutated CFTR, few acyl groups, with different sterical and electronical properties, were investigated.

For the synthesis of these new ARN9364 analogs, a few changes were made to the previously described protocol (Scheme 2.5). In particular, willing to insert this diversification (i.e., acylation of the 2-amino pyridine) in the last step of the synthetic route, we envisaged to install orthogonal protecting groups onto the core structure, which could be removed in a chemo-selective manner to obtain a versatile starting point, such as compound 55 (Scheme 2.7). According to this approach, the orthogonal protection of the pyrrolidine and the pyrazole rings with a Cbz- and a SEM-group, respectively, could help obtaining the key-intermediate 51.

The synthetic approach followed to obtain the common building block (55) to be used for the synthesis of these novel analogs is reported in Scheme 2.7.
Intermediate 51 was obtained following the same synthetic route described previously (Scheme 2.5), starting this time from carboxylic acid 42. With compound 51 in hand, our attention moved to the Boc-protection of the amino group on the pyridine ring. This reaction was performed in a straightforward manner with Boc\(_2\)O in DCM giving compound 52 in 64\% yield.

At this point, compound 52 was subjected to a chemo-selective deprotection to remove the Cbz group on the pyrrolidine moiety. As the first attempt, the reaction was carried out with 1,4-cyclohexadiene and 10\% Pd/C in a mixture of THF/MeOH; this approach failed to produce the desired compound 53 (condition A, Scheme 2.8). The reaction was then performed in an H-Cube hydrogenation flow reactor, using 10\% Pd/C as catalyst at 1 atm and at 30\^\circ\mathrm{C} in EtOH. Under these conditions, a mixture of compound 53 and the unexpected byproduct 56 (condition B, Scheme 2.8) was obtained. A careful investigation of this outcome (i.e., formation of the N-ethyl pyrrolidine derivative 56 by Boc-deprotection, followed by reductive insertion of an ethyl moiety) showed how this type of side-reaction
could occur in Pd-catalyzed alkylations of amines using alcohols.\textsuperscript{21, 22} Trying to take advantage of this synthetic drawback and willing to insert a \textit{n}-propyl group on the pyrrolidine nitrogen, we decided to perform the same reaction in the presence of \textit{n}-propanol as the solvent (condition C, Scheme 2.8). The reaction led to a mixture of Cbz-deprotected compound 53 and \textit{n}-propyl alkylated intermediate 54 in 37% yield. Due to the long reaction time (72 h, many hydrogenation cycles), partial conversion and low overall yield, we decided to abandon this approach and focus on an alternative deprotection procedure. Eventually, performing the reaction using 10\% Pd/C in the presence of an excess of triethylsilane\textsuperscript{23} (3 eq.) in EtOH, compound 53 was obtained in high yield (82\%) (condition D, Scheme 2.8).

The next reductive amination step was carried out with propionaldehyde, using NaBH(OAc)$_3$ in MeOH to afford compound 54 in 79\% yield (Scheme 2.7). The subsequent simultaneous removal of Boc- and SEM-protecting groups on compound 54 was achieved using HCl 3.0 M in MeOH at room temperature to give the final intermediate 55 in quantitative yield.

At this point, few racemic analogs of hit ARN9364 were synthesized starting from intermediate 55.

A common approach was initially envisaged to obtain the new compounds via an acylation reaction with the appropriate acyl chloride under basic conditions (Scheme 2.9).
In general, although a sub-stoichiometric amount of the corresponding acyl chloride was used, the formation of significant amounts of the di- (57) and tri-acylated (58) derivatives was generally observed, along with the desired final compound 59 and some unreacted starting material 55 (Scheme 2.9).

![Scheme 2.9: Initially attempted synthesis of novel racemic ARN9364 analogs.](image)

For this reason, to fully convert 55, an excess of acyl chloride (ca. 4 eq.) was employed leading to a mixture of both di- (57) and tri-acylated (58) derivatives, which by treatment with NaOH 2.0M in methanol led to the corresponding desired mono-acylated compounds 59a-d in low/moderate yields (Table 2.7).

Table 2.7: Synthesis of ARN9364 racemic analogs 59a-d.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>59a</td>
<td></td>
<td>39%</td>
</tr>
<tr>
<td>59b</td>
<td></td>
<td>33%</td>
</tr>
<tr>
<td>59c</td>
<td></td>
<td>14%</td>
</tr>
<tr>
<td>59d</td>
<td></td>
<td>17%</td>
</tr>
</tbody>
</table>

Following a similar protocol as for the synthesis of ARN9364 (Scheme 2.5), compound 60 was obtained starting from Boc-protected intermediate 36 via a benzylation
step with benzyl bromide (61), giving intermediate 62, which was then Boc-deprotected (compound 63) and subjected to reductive amination to afford the final analog 60 in 46% yield (Scheme 2.10).

Scheme 2.10: Synthesis of analog 60.

Following the same synthetic route described for the synthesis of compound 55 (Scheme 2.7), the N-methyl pyrazole derivative 64 was synthesized starting from Cbz-protected intermediate 48 (Scheme 2.11). The N-methylation of the pyrazole was performed with iodomethane and Cs₂CO₃ in DMF, giving, as expected, a mixture of two N-methyl regioisomers (65 and 66). After flash chromatography purification, the desired regioisomer 66 was isolated (71% yield) and characterized by NMR analyses. Then, a Suzuki coupling with 2-amino-pyridine boronic ester 32 followed by acylation of the corresponding amino group with cyclobutanecarboxyl chloride (34) afforded intermediate 68 in good yield (87%, over 3 steps). The Cbz-deprotection of the pyrrolidine was carried out with 1,4-cyclohexadiene and 10% Pd/C to give compound 69, which was then converted into the desired final analog 64 by a reductive amination step using of n-propanaldehyde (33% yield).
From the high-throughput screening which led to the discovery of ARN9364, another close hit-analog, 78 (Figure 2.8), was identified as a compound showing a preliminary promising biological activity. To confirm its chemical structure and verify its activity, compound 78 was synthesized following the synthetic approach reported in Scheme 2.12.

Figure 2.8: Structure of compound 78.
the desired product 75. The benzylation step of intermediate 75 was performed using 4-fluorobenzyl bromide (77) with cesium carbonate, and tetrabutylammonium iodide (TBAI), as phase transfer catalyst,\(^\text{10}\) in DMF at room temperature, to give 77 in 30% yield over two steps.

Finally, the deprotection of intermediate 77 with HCl 3.0M in MeOH led to the formation of 78, as hydrochloride salt, in 71% yield.

Once synthesized, compound 78 was characterized by UPLC/MS and NMR analysis. A complete overlap of its analytical data with those of the library sample, tested initially in the HTS, was observed.

![Scheme 2.12: Synthesis of compound 78.](image)

2.3 Synthesis of ARN5562 analogs

The second part of this project, aimed at the discovery of novel potent CFTR correctors, was focused on the design and synthesis of close analogs of a corrector deriving from another hit compound identified in the same HTS. The initial hit, ARN5562 (Figure 2.9), belonged to a chemical class different from that of ARN9364. Therefore, a different synthetic procedure was outlined for the evolution of this chemo-type (*data not reported*).
Rounds of chemical modifications of the structure of ARN5562 provided the information to build Structure-Activity Relationships (SAR) and led to the identification of two structural (sub)-classes of correctors. The chemical expansion of one of the chemo-types allowed identifying ARN21750 (Figure 2.10), a compound showing a better biological profile than ARN5562 (see Chapter 3, paragraph 3.3).

SAR studies on the tetrahydro-pyridyl ring of the pyrazolo[4,3-c]pyridine moiety led to the discovery of different biologically active analogs (*data not reported*). Among them, compounds featuring a bridged aza-bicyclic group carrying a 3,5-dimethylisoxazole sulfonamide (Figure 2.11), showed a promising activity profile (*data not shown*).

To efficiently synthesize a few new analogs within this chemo-type, to investigate the importance of the heterocyclic moiety on the right-end side of the molecule (R, Figure 2.11), we envisioned retrosynthetic disconnections requiring the formation of the tricyclic scaffold.

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*a A full SAR evolution/expansion starting from hit ARN5562 leading to potent analogs of identified lead compound ARN21750 will be carefully described and reported in a manuscript to be submitted for publication to a peer-reviewed journal.*
B via a cyclization reaction between a di-ketone of type C and 3-hydrazino-benzoic acid D (Scheme 2.13). Compound C could be obtained from trifluoromethyl-acetylation of commercially available ketone E.

Scheme 2.13: Proposed retro-synthetic disconnections for tricyclic scaffold.

The synthetic approach to obtain the building block 83, as a common intermediate for the synthesis of these novel analogs, is reported in Scheme 2.14.

Scheme 2.14: Synthesis of intermediate 84.

The first step of this synthetic route consisted in the formation of a diketone suitable for the pyrazole preparation. Starting from the commercially available Boc-protected tropinone 79 and ethyl 2,2,2-trifluoroacetate (80), treatment with LDA in THF at -78°C, afforded the di-ketone 81 in 21% yield. The following cyclization step was performed using 3-hydrazinobenzoic acid (82) in AcOH, leading to the formation of the tricyclic compound 83 (90% yield), which was then used for the synthesis of new heteroaryl-amides on the right-end side of the molecule.

The synthetic route followed for the synthesis of compound 89 and 90 is reported in Scheme 2.15.
HATU-mediated amide coupling between tricyclic benzoic acid 83 and substituted N-methyl aniline 84 led to the formation of intermediate 85 (54%), which was then subjected to a Boc-deprotection with TFA 20% in MeOH. The hydrochloride 86 was used without any purification in the following reaction for the synthesis of the two final compounds, 89 and 90. Compound 89 was synthetized in a straightforward manner by reductive amination of the tetrahydropyridine nitrogen with paraformaldehyde (87), whereas sulfonamide 90 was smoothly obtained by a coupling reaction between sulfonyl chloride 88 and amine 86.

Concerning the synthesis of other derivatives (Scheme 2.16, Table 2.8), characterized by different amidic right-end side, the protection of the benzoic acid derivative 83 as the ethyl ester 91 was first required to efficiently obtain analogs bearing a 3,5-dimethylisoxazole sulfonamide moiety on the nortropane ring.

Boc-deprotection of ethyl ester 91 gave the secondary amine 92, which was coupled with sulfonyl chloride 88 to furnish sulfonamide 93. The common intermediate 94 for the synthesis of these new derivatives was obtained through the hydrolysis of the ethyl ester 93 with LiOH in a THF/H$_2$O mixture.

For the synthesis of three analogs within this series, bearing a heteroaryl group on the right-end side of the molecule, other two steps were required (Scheme 2.16). The amide
coupling of the carboxylic acid intermediate **94** with the mono- and bicyclic amino-substituted heteroaryls **95a-c** with HATU, as coupling reagent, and DIPEA in DMF led to the formation of the corresponding secondary amides **96a-c** in moderate yields. The following methylation step with methyl iodide in DMF gave the final racemic tertiary amides **97a-c** in moderate yields (45-60%, Table 2.8).

Scheme 2.16: Synthesis of heteroaryl substituted tertiary amides **97a-c**.

Table 2.8: Yields of the syntheses of intermediates **96a-c** and final racemic compounds **97a-c**.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sup&gt;i&lt;/sup&gt;</th>
<th>96 (yield)</th>
<th>97 (yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td><img src="image" alt="Image of a" /></td>
<td>59% (over three steps)</td>
<td>46%</td>
</tr>
<tr>
<td>b</td>
<td><img src="image" alt="Image of b" /></td>
<td>38% (over three steps)</td>
<td>60%</td>
</tr>
<tr>
<td>c</td>
<td><img src="image" alt="Image of c" /></td>
<td>31% (over three steps)</td>
<td>45%</td>
</tr>
</tbody>
</table>
2.4 Experimental Part

2.4.1 Chemicals, materials and methods

All the commercial available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (THF, Et₂O, DCM, DMF, DMSO, MeOH) were purchased from Sigma-Aldrich. Automated column chromatography purifications were performed on Teledyne ISCO apparatus (CombiFlash® Rf) with pre-packed silica gel columns of different sizes (from 4 g up to 120 g) (Redisep). Mixtures of increasing polarity of cyclohexane (CyHex) and ethyl acetate (EtOAc) or cyclohexane and methyl tert-butyl ether (TBME) or dichloromethane (DCM) and methanol (MeOH) were used as eluents. NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for ¹H, and 100.62 MHz for ¹³C), equipped with a BBI probe and Z-gradients and Bruker FT NMR Avance III 600 MHz spectrometer equipped with a 5 mm CryoProbeTM QCI ¹H/¹⁹F–¹³C/¹⁵N–D quadruple resonance, a shielded z-gradient coil and the automatic sample changer SampleJet™ NMR system (600 MHz for ¹H, 151 MHz for ¹³C and 565 MHz for ¹⁹F). Chemical shifts for ¹H and ¹³C spectra were recorded in parts per million using the residual non-deuterated solvent as the internal standard (for CDCl₃: 7.26 ppm, ¹H and 77.16 ppm, ¹³C; for DMSO-d₆: 2.50 ppm, ¹H; 39.52 ppm, ¹³C, for D₂O: TSP as internal standard 0.00 ppm). The analyses by UPLC/MS were run on a Waters ACQUITY UPLC/MS system consisting of a SQD (Single Quadrupole Detector) Mass Spectrometer equipped with an Electrospray Ionization interface and a Photodiode Array Detector. The PDA range was 210-400nm. The analyses were performed on either an ACQUITY UPLC HSS T3 C18 column (50x2.1mmID, particle size 1.8µm) with a VanGuard HSS T3 C18 pre-column (5x2.1mmID, particle size 1.8µm) (LogD<1) or an ACQUITY UPLC BEH C₁₈ column (50x2.1mmID, particle size 1.7µm) with a VanGuard BEH C₁₈ pre-column (5x2.1mmID, particle size 1.7µm) (LogD>1).

The mobile phase was 10mM NH₄OAc in H₂O at pH 5 adjusted with AcOH (A) and 10mM NH₄OAc in MeCN-H₂O (95:5) at pH 5 (B). Electrospray ionization in positive and negative mode was applied in the mass scan range 100-650Da or 150-750Da. Analyses were performed either with “Polar method”, “Generic method” and “Apolar Method” herein reported:
**Polar method:**
Column: Waters ACQUITY UPLC HSS T3 C18, 1.8μm, 50x2.1mmID  
Pre-column: VanGuard HSS T3 C18, 1.8μm, 5x2.1mmID  
Linear gradient: 0-0.2min: 0%B, 0.2-2.7min: 0-50%B, 2.7-2.8min: 50-100%B, 2.8-3.0min: 100%B  
Flow rate: 0.5mL/min

**Generic method:**
Column: Waters ACQUITY UPLC BEH C18, 1.7 μm, 50x2.1 mmID  
Pre-column: VanGuard BEH C18, 1.7 μm, 5x2.1 mmID  
Linear gradient: 0-0.2 min: 5%B, 0.2-2.7 min: 5-95%B, 2.7-2.8 min: 95-100%B, 2.8-3.0 min: 100%B  
Flow rate: 0.5 mL/min

**Apolar method:**
Column: Waters ACQUITY UPLC BEH C18, 1.7 μm, 50x2.1 mmID  
Pre-column: VanGuard BEH C18, 1.7μm, 5x2.1 mmID  
Gradient: 0-0.2 min: 50%B, 0.2-2.7 min: 50-100%B, 2.7-3.0 min: 100%B  
Flow rate: 0.5 mL/min

Hydrogenation reactions were performed using H-Cube continuous hydrogenation flow reactor (SS-reaction line version), employing disposable catalyst cartridges (CatCart) preloaded with the required heterogeneous catalyst.

Microwave heating was performed using Explorer®-48 positions instrument (CEM). 
All tested compounds showed ≥ 95% purity by NMR and UPLC/MS analysis.
2.4.2 Characterization of compounds (14–97)

**[(rac)-tert-Butyl 3-[methoxy(methyl)carbamoyl]pyrrolidine-1-carboxylate (14)](image)**: In a round-bottomed flask, under N\(_2\), compound 12 (3.00 g, 13.95 mmol) was dissolved in DCM (25 mL). To this resulting solution, 13 (1.80 g, 18.42 mmol), DIPEA (6.22 mL, 35.71 mmol), HOBT (2.14 g, 13.95 mmol) and EDC (2.94 g, 15.34 mmol) were added. After 18 h, the reaction was diluted with DCM (20 mL) and the organic layer was washed with water and brine and dried over Na\(_2\)SO\(_4\) and filtered. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/MTBE 70:30) to give the pure title compound, as a yellow solid (3.32 g, 92%).

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 3.68 (s, 3H), 3.51 – 3.41 (m, 1H), 3.42 – 3.29 (m, 2H), 3.29 – 3.18 (m, 2H), 3.11 (s, 3H), 2.09 – 1.97 (m, 1H), 1.97 – 1.80 (m, 1H), 1.39 (s, 9H).

UPLC-MS: \(t_R = 1.79\) min (generic method); MS (ESI) m/z calcd for C\(_{12}\)H\(_{23}\)N\(_2\)O\(_4\): (M+H)\(^+\) : 259.3.; found: 259.5.

**[(rac)-tert-Butyl 3-[2-(2-bromo-4-pyridyl)acetyl]pyrrolidine-1-carboxylate (16)](image)**: In a three neck flask, at -78°C under N\(_2\), compound 15 (0.30 g, 1.74 mmol) was dissolved in THF (15 mL) and a solution of LDA (2.0 M in THF, 1.13 ml, 2.26 mmol) was added dropwise and then stirred for 1 hour at -78 °C. 14 (0.49 g, 1.92 mmol) was added, the reaction was stirred at the same temperature. After 2 h, the reaction was poured into a NH\(_4\)Cl saturated solution, the aqueous phase was extracted with EtOAc (x3) and the combined organic layers were washed with brine and dried over Na\(_2\)SO\(_4\) and filtered. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (CyHex/EtOAc 70:30) to give the pure title compound, as a light brown solid (0.48 g, 75%).

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.31 (d, \(J = 5.0\) Hz, 1H), 7.51 (s, 1H), 7.27 (dd, \(J = 5.0, 1.4\) Hz, 1H), 4.07 – 3.94 (m, 2H), 3.48 – 3.33 (m, 3H), 3.31 – 3.19 (m, 6H), 2.20 – 2.05 (m, 1H), 2.06 – 1.91 (m, 1H), 1.39 (s, 9H). UPLC-MS: \(t_R = 2.15\) min (generic method); MS (ESI) m/z calcd for C\(_{16}\)H\(_{22}\)BrN\(_2\)O\(_3\): (M+H)\(^+\) : 369.2.; found: 369.3.
(rac)-tert-Butyl-3-[(E)-2-(2-bromo-4-pyridyl)-3-(dimethylamino)prop-2-enoyl]pyrrolidine-1-carboxylate (17): In a round-bottomed flask, under N₂, compound 16 (0.582 g, 1.58 mmol) was dissolved in Toluene (15 mL). To this resulting solution, DMF-DMA (293 µL, 2.21 mmol) was added and the reaction was heated to 120 °C. After 1 h, water was added and the aqueous phase was extracted with EtOAc (x3). The combined organic layers were washed with brine and dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky yellow oil (0.689 g), which was not subjected to any purification and directly used in the next step of reaction.

¹H NMR (400 MHz, DMSO-d₆) δ 8.25 (d, J = 5.0 Hz, 1H), 7.76 (s, 1H), 7.37 – 7.32 (m, 1H), 7.27 – 7.16 (m, 1H), 7.13 (dd, J = 5.0, 1.4 Hz, 1H), 3.66 – 3.54 (m, 1H), 3.20 (q, J = 9.0 Hz, 2H), 2.77 (s, 4H), 1.98 – 1.83 (m, 2H), 1.39 (s, 9H). UPLC-MS: tᵣ = 2.09 min (generic method); MS (ESI) m/z calcd for C₁₉H₂₇BrN₃O₃: (M+H)⁺: 426.4; found: 426.4.

(rac)-tert-Butyl 3-[4-(2-bromo-4-pyridyl)-1H-pyrazol-3-yl]pyrrolidine-1-carboxylate (18): In a round-bottomed flask, compound 17 (0.689 g, 1.62 mmol) was dissolved in EtOH (18 mL). To this resulting solution, hydrazine monohydrate 65% (120 µL, 2.43 mmol) was added and the reaction was stirred at room temperature. After 1 h, solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/EtOAc 60:40) to give the pure title compound, as a light brown solid (0.48 g, 55% over two steps).

¹H NMR (400 MHz, DMSO-d₆) δ 13.2 – 13.0 (bs, 1H), 8.3 (d, J = 5.2 Hz, 1H), 8.3 (s, 1H), 7.7 – 7.7 (m, 1H), 7.6 – 7.5 (m, 1H), 4.2 – 4.0 (m, 1H), 3.8 – 3.7 (m, 1H), 3.7 – 3.6 (m, 1H), 3.5 – 3.4 (m, 2H), 2.3 – 2.1 (m, 1H), 2.1 – 1.9 (m, 1H), 1.4 (s, 9H). UPLC-MS: tᵣ = 2.08 min (generic method); MS (ESI) m/z calcd for C₁₇H₂₂BrN₄O₂: (M+H)⁺: 393.1.; found: 393.1.
(rac)-2-Bromo-4-(3-pyrrolidin-3-yl-1H-pyrazol-4-yl)pyridine;hydrochloride \textbf{(19)}:  

Compound \textbf{18} (0.322 g, 0.82 mmol) was dissolved in a solution of HCl 3.0 M in MeOH (2.4 mL, 8.2 mmol) at room temperature. The solution was stirred for 1 h. The solvent was removed under vacuum and compound was precipitated with Et\textsubscript{2}O as dichlorohydrate salt, to give the compound, as a white solid (0.282 g, 94%).

\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}) \(\delta\) 9.6 – 9.3 (\textit{app}-m, 2H), 8.4 (d, \(J = 5.2\) Hz, 1H), 8.3 (s, 1H), 7.7 (d, \(J = 1.4\) Hz, 1H), 7.5 (dd, \(J = 5.2, 1.6\) Hz, 1H), 3.8 (p, 1H), 3.6 – 3.5 (m, 1H), 3.4 – 3.2 (m, 3H), 2.4 – 2.2 (m, 1H), 2.1 – 1.9 (m, 1H). UPLC-MS: \(t_R = 1.19\) min (generic method); MS (ESI) \textit{m/z} calcd for C\textsubscript{12}H\textsubscript{14}BrN\textsubscript{4}: (M+H)\textsuperscript{+} : 293.0; found: 293.1.

\textbf{(rac)-[3-[4-(2-Bromo-4-pyridyl)-1H-pyrazol-3-yl]pyrrolidin-1-yl]-cyclobutylmethanone} \textbf{(20)}: In a round-bottom flask, at 0 °C under N\textsubscript{2}, compound \textbf{19} (0.280 g, 0.77 mmol) was dissolved in DCM (40 mL). The resulting solution was treated with DIPEA (415 \textmu L, 3.08 mmol), HOBT (0.114 g, 0.85 mmol) and EDC (0.191 g, 1.00 mmol). Then cyclobutanecarboxylic acid (74 \textmu L, 0.77 mmol) was added and the temperature was raised up to room temperature. After 2 h, the reaction was poured into a NH\textsubscript{4}Cl saturated solution, the aqueous phase was extracted with EtOAc and the combined organic layers were washed with brine and dried over Na\textsubscript{2}SO\textsubscript{4} and filtered. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/MeOH 96:4) to give the pure title compound, as an oil (0.200 g, 69%).

\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}) \(\delta\) 13.1 (bs, 1H), 8.3 (d, \(J = 5.2\) Hz, 1H), 8.3 (s, 1H), 7.7 – 7.7 (m, 1H), 7.6 – 7.5 (m, 1H), 3.9 – 3.6 (m, 2H), 3.6 – 3.4 (m, 3H), 3.3 – 3.1 (m, 1H), 2.3 – 1.9 (m, 6H), 1.9 – 1.8 (m, 1H), 1.8 – 1.7 (m, 1H). UPLC-MS: \(t_R = 1.72\) min (generic method); MS (ESI) \textit{m/z} calcd for C\textsubscript{17}H\textsubscript{20}BrN\textsubscript{4}O: (M+H)\textsuperscript{+} : 375.0; found: 375.1.
(rac)- [3-[4-(2-Bromo-4-pyridyl)-1-tetrahydropyran-2-yl-pyrazol-3-yl]pyrrolidin-1-yl]-cyclobutyl-methanone (22): In a round-bottomed flask, under N$_2$, compound 20 (0.200 g, 0.53 mmol) was dissolved in 2,3-Dihydropyran (21) (2 mL). To the resulting solution TFA (4 µL, 0.53 µmol) was added and the reaction was heated to 60 °C. After 3 h, the reaction was diluted with EtOAc, washed with NaHCO$_3$, brine and the organic layer was dried over Na$_2$SO$_4$ and filtered. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/MeOH 98:2) to give the pure title compound as two regioisomers, as a oil (0.171 g, 70%).

Major isomer: $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.3 (d, $J = 5.1$ Hz, 1H), 7.8 (s, 1H), 7.5 – 7.4 (m, 1H), 7.2 (dd, $J = 5.2$, 1.5 Hz, 1H), 5.4 – 5.3 (m, 1H), 4.2 – 4.0 (m, 1H), 3.8 – 3.4 (m, 6H), 3.3 – 3.1 (m, 1H), 2.5 – 1.8 (m, 1H). UPLC-MS: $t_R$ = 2.21 min (generic method); MS (ESI) m/z calcd for C$_{22}$H$_{28}$BrN$_4$O$_2$: (M+H)$^+$ : 459.1.; found: 459.1.

(rac)-tert-Butyl N-[3-[1-(cyclobutanecarbonyl)pyrrolidin-3-yl]-1-tetrahydropyran-2-yl-pyrazol-4-yl]-2-pyridyl]carbamate (22): In a round-bottomed flask, under N$_2$, compound 22 (0.100 g, 0.22 mmol) was dissolved in dioxane (7 mL). To the resulting solution 23 (0.038 g, 0.32 mmol), Cs$_2$CO$_3$ (0.142 g, 0.44 mmol), [Pd$_2$(dba)$_3$] (0.004 g, 0.0043 mmol) and xantphos (0.007 g, 0.01 mmol) were added and the reaction was heated to 110 °C. After 18 h, the reaction was diluted with EtOAc and filtered through a plug of celite. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (CyHex/EtOAc 15:85) to give the pure title compound, as an oil (0.055 g, 55%).

Major isomer: $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 9.8 (d, $J = 9.2$ Hz, 1H), 8.3 – 8.2 (m, 1H), 8.2 (d, $J = 5.2$ Hz, 1H), 7.9 – 7.8 (m, 1H), 7.2 – 7.1 (m, 1H), 5.4 (dd, $J = 10.0$, 2.3 Hz, 1H), 4.0 – 3.9 (m, 1H), 3.9 – 3.8 (m, 1H), 3.8 – 3.6 (m, 3H), 3.6 – 3.4 (m, 3H), 3.3 – 3.2 (m, 1H), 2.2 – 2.0 (m, 7H), 2.0 – 1.8 (m, 3H), 1.8 – 1.6 (m, 2H), 1.6 – 1.5 (m, 2H), 1.5 (s, 9H). UPLC-MS: $t_R$ = 2.40 min (generic method); MS (ESI) m/z calcd for C$_{27}$H$_{38}$N$_5$O$_4$: (M+H)$^+$ : 496.3.; found: 496.3.
(rac)-**tert-Butyl N-[4-[3-[1-(cyclobutane carbonyl)pyrrolidin-3-yl]1-tetrahydropyran-2-yl-pyrazol-4-yl]-2-pyridyl]-N-propyl-carbamate** (25): In a round-bottomed flask, under \( \text{N}_2 \), compound 24 (0.052 g, 0.11 mmol) was dissolved in DMF (2 mL). To the resulting solution Cs\(_2\)CO\(_3\) (0.110 g, 0.34 mmol) and TBAI (0.125 g, 0.34 mmol) were added and the reaction was stirred at room temperature. After 30 min, 1-bromopropane (0.042 g, 0.34 mmol) was added and the reaction was stirred for 18 h. The mixture was then poured into water and extracted with EtOAc. The combined organic layers were washed with brine and dried over Na\(_2\)SO\(_4\) and filtered. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/MeOH 98:2) to give the pure title compound, as a colorless oil (0.036 g, 60%).

Major isomer: \(^1\text{H} \text{NMR} (400 \text{ MHz, DMSO-}d_6) \delta 8.4 \ (d, J = 5.2 \text{ Hz, 1H}), 8.3 – 8.3 \ (m, 1H), 7.7 – 7.6 \ (m, 1H), 7.3 – 7.2 \ (m, 1H), 5.4 \ (dd, J = 10.0, 2.3 \text{ Hz, 1H}), 4.0 – 3.9 \ (m, 1H), 3.9 – 3.6 \ (m, 6H), 3.6 – 3.3 \ (m, 3H), 3.3 – 3.2 \ (m, 1H), 3.2 \ (d, J = 5.0 \text{ Hz, 1H}), 2.3 – 2.0 \ (m, 7H), 2.0 – 1.8 \ (m, 4H), 1.8 – 1.5 \ (m, 7H), 1.4 \ (app-d, 9H), 0.8 \ (t, J = 7.4 \text{ Hz, 3H}). UPLC-MS: \( t_R = 1.87 \text{ min (apolar method)}; MS (ESI) m/z \text{ calcd for } C_{30}H_{44}N_5O_4: (M+H)^+ : 538.3; \text{ found: 538.3}. \)

\( \text{rac-Cyclobutyl-[3-[4-[2-(propylamino)-4-pyridyl]-1H-pyrazol-3-yl]pyrrolidin-1-yl]methane} \) (26): Compound 25 (0.036 g, 0.067 mmol) was dissolved in a solution of HCl 3.0 M in MeOH (0.22 mL, 0.67 mmol) at room temperature. The solution was stirred for 5 h. The solvent was removed under vacuum and the residue obtained was subjected to flash chromatography (DCM/DCM*MeOH NH\(_3\) 1N 90:10) to give the pure title compound, as a colorless oil (0.036 g, 60%).

\(^1\text{H} \text{NMR} (400 \text{ MHz, DMSO-}d_6) \delta 12.9 \ (bs, 1H), 8.0 – 7.8 \ (m, 2H), 6.6 – 6.5 \ (m, 1H), 6.5 – 6.4 \ (m, 2H), 3.8 – 3.5 \ (m, 3H), 3.5 – 3.4 \ (m, 2H), 3.3 – 3.1 \ (m, 3H), 2.3 – 2.0 \ (m, 6H), 2.0 – 1.8 \ (m, 1H), 1.8 – 1.6 \ (m, 1H), 1.5 \ (h, J = 7.4 \text{ Hz, 2H}), 0.9 \ (t, J = 7.4 \text{ Hz, 3H}). UPLC-MS: \( t_R = 1.54 \text{ min (apolar method)}; MS (ESI) m/z \text{ calcd for } C_{20}H_{26}N_5O: (M+H)^+ : 354.2; \text{ found: 354.2}. \text{ MS (ESI) m/z \text{ calcd for } C_{20}H_{26}N_5O: (M–H)^- : 352.2; \text{ found: 352.2}. \)
(rac)-tert-Butyl 3-acetylpyrrolidine-1-carboxylate (27): In a three-neck flask, at -78 °C under N₂, compound 14 (0.30 g, 1.74 mmol) was dissolved in THF (3 mL) and a solution of MeMgBr (3 M in Et₂O, 0.87 mL, 2.6 mmol) was added dropwise and then the temperature raised up to room temperature. After 1 h, the reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with EtOAc (x3) and the combined organic layers were washed with brine and dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil (0.243 g), which was not subjected to any purification and directly used in the next step of reaction.

¹H NMR (400 MHz, DMSO-ｄ₆) δ 3.4 – 3.3 (m, 2H), 3.3 – 3.1 (m, 3H), 2.1 (s, 3H), 2.1 – 2.0 (m, 1H), 1.9 – 1.8 (m, 1H), 1.4 (s, 9H).

(rac)-tert-Butyl 3-[(E)-3-(dimethylamino)prop-2-enoyl]pyrrolidine-1-carboxylate (28): In a round-bottomed flask, compound 27 (0.234 g, 1.1 mmol) was dissolved in DMF (2.5 mL). To this resulting solution, DMF-DMA (365 µL, 2.75 mmol) was added and the reaction was heated to 140 °C. After 18 h, water was added and the aqueous phase was extracted with EtOAc (x3). The combined organic layers were washed with brine and dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky yellow oil (0.234 g), which was not subjected to any purification and directly used in the next step of reaction.

¹H NMR (400 MHz, DMSO-ｄ₆) δ 7.5 (d, J = 12.7 Hz, 1H), 5.0 (d, J = 12.7 Hz, 1H), 3.4 – 3.3 (m, 1H), 3.3 – 3.1 (m, 3H), 3.1 – 3.0 (m, 3H), 2.8 – 2.7 (m, 2H), 2.0 – 1.7 (m, 2H), 1.4 (s, 9H). UPLC-MS: tᵦ = 1.69 min (generic method); MS (ESI) m/z calcd for C₁₄H₂₅N₂O₃: (M+H)⁺: 269.2.; found: 269.2.

(rac)-tert-Butyl 3-(1H-pyrazol-3-yl)pyrrolidine-1-carboxylate (29): In a round-bottomed flask, compound 28 (0.234 g, 0.87 mmol) was dissolved in EtOH (1 mL). To this resulting solution, hydrazine monohydrate 65% (431 µL, 8.7 mmol) was added and the reaction was heated till 85 °C. After 2 h, solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (CyHex/EtOAc 80:20) to give the pure title compound, as a yellow oil (0.175 g, 85% over three steps).
\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 12.5 (bs, 1H), 7.6 (apps, 1H), 6.1 (d, \(J = 19.3\) Hz, 1H), 3.7 – 3.5 (m, 1H), 3.5 – 3.3 (m, 2H), 3.3 – 3.2 (m, 2H), 2.3 – 2.1 (m, 1H), 2.0 – 1.9 (m, 1H), 1.4 (s, 9H). UPLC-MS: \(t_R = 1.70\) min (apolar method); MS (ESI) m/z calcd for C\(_{12}\)H\(_{20}\)N\(_3\)O\(_2\): (M+H)\(^+\) : 238.1.; found: 238.1.

(rac)-\(\text{tert-Butyl}\) 3-(4-bromo-1H-pyrazol-3-yl)pyrrolidine-1-carboxylate (30): In a round-bottomed flask, compound 29 (0.200 g, 0.84 mmol) was dissolved in DMF (2 mL). To this resulting solution, NBS (0.149 g, 0.84 mmol) was added and the reaction was stirred at room temperature (away from light). After 2 h, water was added and the aqueous phase was extracted with EtOAc (x3). The combined organic layers were washed with brine and dried over Na\(_2\)SO\(_4\) and filtered. Solvent was removed under vacuum, and the resulting oil was subjected to flash chromatography (DCM/MTBE 70:30) to give the pure title compound, as a colorless oil (0.266 g, quantitative yield).

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 13.0 (bs, 1H), 8.0 – 7.8 (m, 1H), 3.7 – 3.6 (m, 1H), 3.5 – 3.3 (m, 3H), 3.3 – 3.2 (m, 1H), 2.2 – 2.1 (m, 1H), 2.1 – 2.0 (m, 1H), 1.4 (s, 9H). UPLC-MS: \(t_R = 2.03\) min (generic method); MS (ESI) m/z calcd for C\(_{12}\)H\(_{19}\)BrN\(_3\)O\(_2\): (M+H)\(^+\) : 316.0.; found: 316.0.

(rac)-\(\text{tert-Butyl}\) 3-[4-bromo-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate (31): In round-bottomed neck flask, at 0 °C under N\(_2\), compound 30 (0.439 g, 1.4 mmol) was dissolved in THF (9 mL) and NaH (60% in mineral oil 0.084 g, 2.1 mmol) was added and the solution was stirred at 0 °C for 30 min. After which 2-(Trimethylsilyl)ethoxymethyl chloride (0.370 mL, 2.1 mmol) was added dropwise and the temperature was raised up to room temperature. After 18 h, the reaction was poured into a NH\(_4\)Cl saturated solution, the aqueous phase was extracted with EtOAc (x3) and the combined organic layers were washed with brine and dried over Na\(_2\)SO\(_4\) and filtered. Removal of the organics gave a sticky oil, which was subjected to flash chromatography (CyHex/MTBE 70:30) to give the pure title compound as two regioisomers, as a colorless oil (0.585 g, 94%). Major isomer: \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.1 (s, 1H), 5.5 (d, \(J = 4.9\) Hz, 1H), 5.3 (s, 1H), 3.9 – 3.7 (m, 0H), 3.7 – 3.6 (m, 1H), 3.6 – 3.3 (m, 5H), 2.4 – 2.3 (m, 0H), 2.3 – 1.9 (m,
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2H), 1.4 (s, 9H), 0.9 – 0.8 (m, 2H), -0.1 (s, 9H). UPLC-MS: \( t_R = 2.17 \) min (generic method); MS (ESI) m/z calcd for \( \text{C}_{18}\text{H}_{33}\text{BrN}_3\text{O}_5\text{Si} \): \( (\text{M}+\text{H})^+ \): 446.1; found: 446.1.

\( \text{(rac)-}\text{tert-Butyl \ 3-[4-(2-aminopyridyl)-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate (33)}: \) In a round-bottomed flask, under \( \text{N}_2 \), compound 31 (0.200 g, 0.45 mmol) was dissolved in dioxane (12 mL) and water (3 mL). To the resulting solution 32 (0.148 g, 0.67 mmol), \( \text{Cs}_2\text{CO}_3 \) (0.585 g, 1.8 mmol) and \( \left[\text{Pd(dppf)}\text{Cl}_2\right] \cdot \text{CH}_2\text{Cl}_2 \) (0.073 g, 0.09 mmol) were added. The reaction was heated to 110 °C. After 1 h, the reaction was diluted with \( \text{EtOAc} \) and filtered through a plug of celite. The organic solution was washed with \( \text{NaHCO}_3 \), water and brine and finally dried over \( \text{Na}_2\text{SO}_4 \) and filtered. Solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/DCM*MeOH NH\(_3\) 1 N 95:5) to give the pure title compound, as an oil (0.137 g, 65%).

Major isomer: \(^1\text{H NMR}\) (400 MHz, DMSO-\( \text{d}_6 \)) \( \delta \): 8.1 (s, 1H), 7.9 (d, \( J = 5.3 \) Hz, 1H), 6.5 (dd, \( J = 5.3, 1.6 \) Hz, 1H), 6.5 – 6.5 (m, 1H), 5.9 (bs, 2H), 5.4 (s, 2H), 3.8 – 3.7 (m, 1H), 3.7 – 3.5 (m, 4H), 3.5 – 3.4 (m, 1H), 3.3 – 3.1 (m, 1H), 2.2 – 1.9 (m, 2H), 1.4 (s, 9H), 0.9 – 0.8 (m, 2H), -0.1 (s, 9H). UPLC-MS: \( t_R = 1.34 \) min (apolar method); MS (ESI) m/z calcd for \( \text{C}_{23}\text{H}_{38}\text{N}_5\text{O}_3\text{Si} \): \( (\text{M}+\text{H})^+ \): 460.3; found: 460.3.

\( \text{(rac)-}\text{tert-Butyl-3-[4-[2-[bis(cyclobutanecarbonyl)amino]-4-pyridyl]-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate (35)}: \) In a round-bottomed flask, at 0 °C under \( \text{N}_2 \), compound 33 (0.460 g, 1.00 mmol) was dissolved in \( \text{DCM} \) (10 mL) and to the resulting solution \( \text{Et}_3\text{N} \) (0.167 mL, 1.2 mmol) and 34 (0.228 g, 2.00 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was washed with \( \text{NaHCO}_3 \), water and brine and finally dried over \( \text{Na}_2\text{SO}_4 \) and filtered. Removal of the organics gave a sticky yellow oil (0.582 g), which was not subjected to any purification and directly used in the next step of reaction.
Major isomer: $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.6 – 8.5 (m, 1H), 8.4 (s, 1H), 7.8 – 7.7 (m, 1H), 7.6 – 7.5 (m, 1H), 7.4 – 7.3 (m, 1H), 5.4 (s, 1H), 3.9 – 3.7 (m, 1H), 3.7 – 3.5 (m, 3H), 3.5 – 3.4 (m, 4H), 3.3 – 3.2 (m, 1H), 2.3 – 2.1 (m, 6H), 2.0 – 1.9 (m, 5H), 1.9 – 1.8 (m, 2H), 1.8 – 1.6 (m, 2H), 1.4 (s, 9H), 0.9 – 0.8 (m, 2H), -0.1 (s, 9H). UPLC-MS: $t_R = 2.47$ min (apolar method); MS (ESI) m/z calcd for C$_{33}$H$_{50}$N$_{5}$O$_5$Si: (M+H)$^+$ : 624.3; found: 624.3.

(rac)-tert-Butyl-3-[4-[2-(cyclobutanecarbonylamino)-4-pyridyl]-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate (36): In a round-bottomed flask, compound 35 (0.582 g, 0.93 mmol) was dissolved in MeOH (18 mL) and to the resulting solution, a solution of NaOH 2 M (2.15 mL, 4.5 mmol) was added. After 1 h, the solution was neutralized till pH 7 with HCl 1 N, extracted with EtOAc, washed with brine and finally dried over Na$_2$SO$_4$ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH$_3$ 1 N 96:4) to give the pure title compound, as an oil (0.446 g, 84% over two steps).

Major isomer: $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.3 (s, 1H), 8.3 – 8.2 (m, 2H), 8.1 (bs, 1H), 7.6 (s, 1H), 7.0 (dd, $J$ = 5.1, 1.6 Hz, 1H), 5.4 (s, 1H), 3.9 – 3.7 (m, 1H), 3.7 – 3.5 (m, 4H), 3.5 – 3.3 (m, 3H), 3.3 – 3.2 (m, 1H), 2.3 – 2.2 (m, 3H), 2.2 – 2.0 (m, 3H), 2.0 – 1.8 (m, 1H), 1.8 – 1.7 (m, 1H), 1.4 (s, 9H), 0.9 – 0.8 (m, 2H), -0.1 (s, 9H). UPLC-MS: $t_R = 1.99$ min (apolar method); MS (ESI) m/z calcd for C$_{28}$H$_{44}$N$_{5}$O$_4$Si: (M+H)$^+$ : 542.3; found: 542.4.

(rac)-N-[4-(3-pyrrolidin-3-yl-1H-pyrazol-4-yl)-2-pyridyl]cyclobutanecarboxamide di-hydrochloride (37): In a round-bottomed flask, compound 36 (0.100 g, 0.18 mmol) was dissolved in a solution of HCl 3 M in MeOH (1.23 mL, 3.7 mmol) at room temperature. The solution was stirred for 3 h. The solvent was removed under vacuum and compound was precipitated with Et$_2$O as dichlorohydrate salt, to give the compound, as a white solid (0.062 g, 89%).

$^1$H NMR (400 MHz, Methanol-$d_4$) $\delta$ 8.4 (s, 1H), 8.3 (d, $J$ = 6.6 Hz, 1H), 7.8 – 7.7 (m, 2H), 4.1 (p, $J$ = 7.0 Hz, 1H), 3.8 – 3.6 (m, 2H), 3.6 – 3.5 (m, 3H), 2.7 – 2.5 (m, 1H), 2.5 – 2.4 (m,
2H), 2.4 – 2.3 (m, 2H), 2.3 – 2.1 (m, 1H), 2.1 – 2.1 (m, 1H), 2.0 – 1.9 (m, 1H). UPLC-MS: 
\( t_R = 1.21 \) min (generic method); MS (ESI) m/z calcd for C\(_{17}\)H\(_{22}\)N\(_{5}\)O: (M+H\(^+\)) : 312.2; found: 312.2.

**(rac)**-N-[4-3-(1-propylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (38): In a round-bottomed flask, at 0 °C under N\(_2\), compound 37 (0.030 g, 0.1 mmol) was dissolved in MeOH (1 mL) and to the resulting solution propionaldehyde (0.007 mL, 0.1 mmol) and Na(CH\(_3\)COO)\(_3\)BH (0.042 g, 0.2 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH\(_4\)Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO\(_3\) and brine and finally dried over Na\(_2\)SO\(_4\) and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH\(_3\) 92:8) to give the pure title compound, as white solid (0.012 g, 34%).

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 12.8 (bs, 1H), 10.2 (s, 1H), 8.2 (d, \( J = 5.0 \) Hz, 2H), 8.2 – 7.5 (m, 1H), 7.1 (dd, \( J = 5.2, 1.6 \) Hz, 1H), 3.7 – 3.5 (m, 1H), 3.4 – 3.3 (m, 2H), 3.0 (t, \( J = 8.3 \) Hz, 1H), 2.8 – 2.7 (m, 1H), 2.6 – 2.5 (m, 2H), 2.4 – 2.3 (m, 2H), 2.3 – 2.2 (m, 3H), 2.2 – 2.0 (m, 2H), 2.0 – 1.9 (m, 2H), 1.9 – 1.7 (m, 1H), 1.5 (h, \( J = 7.4 \) Hz, 2H), 0.9 (t, \( J = 7.4 \) Hz, 3H). \(^{13}\)C NMR (151 MHz, DMSO-\(d_6\)) \( \delta \) 173.8, 152.6, 148.1, 143.1, 117.6, 116.8, 116.7, 110.9, 59.4, 57.4, 53.4, 39.1, 34.6, 30.6, 24.5 (x2), 21.3, 17.7, 11.9. UPLC-MS: \( t_R = 1.39 \) min (generic method); MS (ESI) m/z calcd for C\(_{20}\)H\(_{28}\)N\(_{5}\)O: (M+H\(^+\)) : 354.2; found: 354.2.

**(rac)**-N-[4-3-(1-methylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (39a): In a round-bottomed flask, at 0 °C under N\(_2\), compound 37 (0.073 g, 0.19 mmol) was dissolved in MeOH (1.5 mL) and to the resulting solution formaldehyde (0.014 mL, 0.19 mmol) and Na(CH\(_3\)COO)\(_3\)BH (0.120 g, 0.57 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH\(_4\)Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO\(_3\) and brine and finally dried over Na\(_2\)SO\(_4\) and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH\(_3\) 92:8) to give the pure title compound, as white solid (0.012 g, 34%).
chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.015 g, 24%).

1H NMR (400 MHz, DMSO-d6) δ 12.9 (bs, 1H), 10.2 (s, 1H), 8.2 (d, J = 4.9 Hz, 2H), 8.1 – 7.6 (m, 1H), 7.1 (dd, J = 5.2, 1.6 Hz, 1H), 3.7 – 3.6 (m, 1H), 3.4 – 3.3 (m, 2H), 3.1 – 2.9 (m, 1H), 2.7 – 2.6 (m, 1H), 2.6 (t, J = 8.2 Hz, 1H), 2.3 (s, 4H), 2.3 – 2.2 (m, 3H), 2.2 – 2.0 (m, 2H), 2.0 – 1.9 (m, 2H), 1.9 – 1.7 (m, 1H). 13C NMR (151 MHz, DMSO-d6) δ 174.2, 153.1, 148.5, 143.5, 118.0, 117.0 (x2), 111.3, 62.0, 56.0, 42.2, 40.5, 39.8, 31.7, 29.5, 24.9 (x2), 18.1. UPLC-MS: tR = 1.24 min (generic method); MS (ESI) m/z calcd for C₁₈H₂₄N₅O: (M+H)^+ : 326.2; found: 326.5.

(rac)-N-[4-[3-(1-isopropylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (39b): In a round-bottomed flask, at 0 °C under N₂, compound 37 (0.050 g, 0.16 mmol) was dissolved in MeOH (1 mL) and to the resulting solution, acetone (0.012 mL, 0.16 mmol) and Na(CH₃COO)₃BH (0.068 g, 0.32 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 hour. The solution was quenched with NH₄Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO₃ and brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.017 g, 31%).

1H NMR (400 MHz, DMSO-d6) δ 12.8 (bs, 1H), 10.2 (s, 1H), 8.2 (d, J = 4.9 Hz, 2H), 8.1 – 7.6 (m, 1H), 7.1 (dd, J = 5.2, 1.6 Hz, 1H), 3.7 – 3.5 (m, 1H), 3.4 – 3.3 (m, 1H), 3.1 (t, J = 8.4 Hz, 1H), 2.9 – 2.7 (m, 1H), 2.7 – 2.5 (m, 1H), 2.5 – 2.4 (m, 1H), 2.3 – 2.2 (m, 3H), 2.2 – 2.0 (m, 2H), 2.0 – 1.9 (m, 2H), 1.9 – 1.7 (m, 1H), 1.0 (t, J = 5.9 Hz, 6H). 13C NMR (151 MHz, Chloroform-d) δ 173.9, 152.0, 148.0, 147.1, 144.0, 118.4, 116.1, 112.0, 57.4, 54.8, 50.5, 41.0, 33.4, 31.7, 25.3 (x2), 21.8 (x2), 18.1. UPLC-MS: tR = 1.35 min (generic method); MS (ESI) m/z calcd for C₂₀H₂₈N₅O: (M+H)^+ : 354.2; found: 354.5.
(rac)-N-[4-[3-(1-isobutylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (39c): In a round-bottomed flask, at 0 °C under N₂, compound 37 (0.050 g, 0.13 mmol) was dissolved in MeOH (1 mL) and to the resulting solution, isobutyraldehyde (0.011 mL, 0.13 mmol) and Na(CH₃COO)₃BH (0.055 g, 0.26 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH₄Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO₃ and brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.015 g, 31%).

¹H NMR (400 MHz, DMSO-d₆) δ 12.8 (bs, 1H), 10.2 (s, 1H), 8.2 (d, J = 5.2 Hz, 2H), 7.9 (d, J = 137.0 Hz, 1H), 7.1 (dd, J = 5.2, 1.6 Hz, 1H), 3.7 – 3.5 (m, 1H), 3.4 (p, J = 8.4 Hz, 1H), 3.1 – 3.0 (m, 1H), 2.8 – 2.7 (m, 1H), 2.7 – 2.5 (m, 1H), 2.5 – 2.3 (m, 1H), 2.3 – 2.2 (m, 5H), 2.1 – 2.0 (m, 2H), 2.0 – 1.9 (m, 2H), 1.8 – 1.7 (m, 1H), 1.7 – 1.6 (m, 1H), 0.9 – 0.8 (m, 6H).

¹³C NMR (151 MHz, DMSO-d₆) δ 173.8, 152.6, 148.0, 143.1, 117.5, 116.6, 111.0, 64.1, 59.8, 53.7, 39.3, 35.2, 33.5, 30.6, 27.0, 24.5, 24.5, 20.9, 20.9, 17.7. UPLC-MS: tᵣ = 1.48 min (generic method); MS (ESI) m/z calcd for C₂₁H₃₀N₅O: (M+H)⁺: 368.2; found: 368.5.

(rac)-N-[4-[3-(1-butylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (39d): In a round-bottomed flask, at 0 °C under N₂, compound 37 (0.050 g, 0.16 mmol) was dissolved in MeOH (1 mL) and to the resulting solution, butyraldehyde (0.012 mL, 0.16 mmol) and Na(CH₃COO)₃BH (0.068 g, 0.32 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH₄Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO₃ and brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.017 g, 29%).
\(^1\)H NMR (400 MHz, DMSO-\textit{d}6) \(\delta\) 12.8 (bs, 1H), 10.2 (s, 1H), 8.2 (d, \(J = 5.1\) Hz, 2H), 7.9 (d, \(J = 134.4\) Hz, 1H), 7.1 (dd, \(J = 5.3, 1.6\) Hz, 1H), 3.7 – 3.6 (m, 1H), 3.4 – 3.3 (m, 1H), 3.1 – 3.0 (m, 1H), 2.8 – 2.7 (m, 1H), 2.6 – 2.5 (m, 1H), 2.5 – 2.4 (m, 2H), 2.3 – 2.2 (m, 3H), 2.1 – 2.0 (m, 2H), 2.0 – 1.8 (m, 2H), 1.8 – 1.7 (m, 1H), 1.5 – 1.4 (m, 2H), 1.4 – 1.2 (m, 2H), 0.9 (t, \(J = 7.3\) Hz, 3H). 13\(^C\) NMR (151 MHz, Chloroform-\textit{d}) \(\delta\) 173.9, 152.0, 148.0, 147.1, 143.9, 118.3, 116.1, 112.0, 60.0, 55.7, 52.8, 41.0, 33.4, 31.8, 30.9, 25.3, 25.3, 20.9, 18.1, 14.1(x2). UPLC-MS: \(t_R = 1.51\) min (generic method); MS (ESI) m/z calcd for C\(_{21}\)H\(_{30}\)N\(_3\)O: (M+H)\(^+\) : 368.2; found: 368.5.

\((\text{rac})-N-\{3-[1-(cyclopropylmethyl)pyrrolidin-3-yl]-1H-pyrazol-4-yl\}-2-pyridyl\}cyclobutanecarboxamide (39e):\) In a round-bottomed flask, at 0 °C under N\(_2\), compound 37 (0.073 g, 0.19 mmol) was dissolved in MeOH (1 mL) and to the resulting solution, cyclopropanecarboxaldehyde (0.014 mL, 0.19 mmol) and Na(CH\(_3\)COO)\(_3\)BH (0.080 g, 0.38 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH\(_4\)Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO\(_3\) and brine and finally dried over Na\(_2\)SO\(_4\) and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH\(_3\) 1 N 92:8) to give the pure title compound, as white solid (0.026 g, 37%).

\(^1\)H NMR (400 MHz, DMSO-\textit{d}6) \(\delta\) 12.8 (bs, 1H), 10.2 (s, 1H), 8.2 (d, \(J = 5.2\) Hz, 2H), 8.1 – 7.6 (m, 1H), 7.1 (dd, \(J = 5.2, 1.6\) Hz, 1H), 3.6 (p, \(J = 7.6\) Hz, 1H), 3.4 – 3.3 (m, 1H), 3.1 (t, \(J = 8.4\) Hz, 1H), 2.9 – 2.7 (m, 1H), 2.6 (t, \(J = 8.5\) Hz, 2H), 2.4 – 2.2 (m, 5H), 2.2 – 2.0 (m, 2H), 2.0 – 1.9 (m, 2H), 1.9 – 1.7 (m, 1H), 0.9 – 0.8 (m, 1H), 0.5 – 0.4 (m, 2H), 0.1 – 0.0 (m, 2H). 13\(^C\) NMR (151 MHz, Chloroform-\textit{d}) \(\delta\) 173.9, 152.0, 148.0, 147.2, 143.9, 118.4, 116.2, 112.0, 60.6, 59.8, 53.0, 41.0, 33.3, 31.9, 25.3, 25.3, 18.1, 9.9, 4.2, 4.0. UPLC-MS: \(t_R = 1.40\) min (generic method); MS (ESI) m/z calcd for C\(_{21}\)H\(_{28}\)N\(_3\)O: (M+H)\(^+\) : 366.2; found: 366.5.
(rac)-N-[4-[3-(1-benzylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (39f): In a round-bottomed flask, at 0 °C under N₂, compound 37 (0.073 g, 0.19 mmol) was dissolved in MeOH (1 mL) and to the resulting solution, benzaldehyde (0.028 mL, 0.28 mmol) and Na(CH₃COO)₃BH (0.080 g, 0.38 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH₄Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO₃ and brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.021 g, 27%).

1H NMR (400 MHz, DMSO-d₆) δ 12.9 (appd, J = 24.9 Hz, 1H), 10.2 (s, 1H), 8.2 (d, J = 5.4 Hz, 2H), 7.9 (d, J = 139.6 Hz, 1H), 7.4 – 7.3 (m, 4H), 7.3 – 7.2 (m, 1H), 7.1 (dd, J = 5.2, 1.6 Hz, 1H), 3.8 – 3.5 (m, 3H), 3.4 (p, J = 8.5 Hz, 1H), 3.1 – 2.9 (m, 1H), 2.8 – 2.5 (m, 3H), 2.4 – 2.2 (m, 3H), 2.2 – 2.0 (m, 2H), 2.0 – 1.9 (m, 2H), 1.9 – 1.7 (m, 1H). 13C NMR (151 MHz, Chloroform-d) δ 173.9, 152.0, 148.0, 143.9, 138.1, 129.0, 128.8, 127.7, 118.3, 116.0, 112.0, 60.2, 59.8, 52.8, 41.0, 33.2, 31.9, 25.4, 25.3, 18.1. UPLC-MS: tᵣ = 1.59 min (generic method); MS (ESI) m/z calcd for C₂₄H₂₈N₅O: (M+H)+ : 402.2; found: 402.4.

(rac)-N-[4-[3-(1-propaonoylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (40): In a round-bottomed flask, at 0 °C under N₂, compound 37 (0.073 g, 0.19 mmol) was dissolved in DCM (10 mL) and to the resulting solution, DIPEA (0.132 mL, 0.76 mmol), propionic acid (0.014 mL, 0.19 mmol), HOBT (0.028 g, 0.21 mmol) and EDC (0.047 g, 0.25 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 5 h. The solution was quenched with water, extracted with DCM and the combined organic layers were washed brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/EtOH 90:10) to give the pure title compound, as white solid (0.031 g, 44%).
CHAPTER 2

1H NMR (400 MHz, DMSO-d6) δ 13.0 (appd, J = 15.4 Hz, 1H), 10.3 (d, J = 6.8 Hz, 1H), 8.4 – 8.2 (m, 2H), 8.0 (d, J = 146.2 Hz, 1H), 7.2 – 7.1 (m, 1H), 4.1 – 3.9 (m, 1H), 3.9 – 3.4 (m, 4H), 3.4 – 3.3 (m, 2H), 2.4 – 2.3 (m, 1H), 2.3 – 2.2 (m, 2H), 2.2 – 2.0 (m, 3H), 2.0 – 1.9 (m, 1H), 1.9 – 1.7 (m, 1H), 1.0 (td, J = 7.4, 3.2 Hz, 3H).

13C NMR (151 MHz, DMSO-d6) δ 174.0, 173.9, 171.0, 152.7, 148.1, 129.0, 117.5, 117.1, 117.0, 110.9, 50.9, 45.6, 45.0, 40.1, 30.3, 26.9, 26.7, 24.4, 17.7, 8.9.

UPLC-MS: t_R = 1.49 min (generic method); MS (ESI) m/z calcd for C20H26N5O2: (M+H)^+ : 368.2; found: 368.4.

(rac)-Methyl 3-[4-[2-(cyclobutanecarbonylamino)-4-pyridyl]-1H-pyrazol-3-yl]pyrrolidine-1-carboxylate (41): In a round-bottomed flask, at 0 °C under N2, compound 37 (0.054 g, 0.14 mmol) was dissolved in DCM (1.4 mL) and to the resulting solution, Et3N (0.010 mL, 0.14 mmol), methyl chloroformate (0.030 mL, 0.2 mmol), were added. The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was quenched with NH4Cl, extracted with DCM and the combined organic layers were washed brine and finally dried over Na2SO4 and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/EtOH 90:10) to give the pure title compound, as white solid (0.031 g, 44%). (DCM/DCM*MeOH NH3 1 N 92:8) to give the pure title compound, as white solid (0.021 g, 28%).

1H NMR (400 MHz, DMSO-d6) δ 13.0 (s, 1H), 10.3 (s, 1H), 8.2 (d, J = 5.3 Hz, 2H), 7.9 (d, J = 150.0 Hz, 1H), 7.2 – 7.1 (m, 1H), 3.9 – 3.6 (m, 2H), 3.6 (s, 3H), 3.5 – 3.4 (m, 2H), 3.4 (t, J = 8.3 Hz, 2H), 2.3 – 2.2 (m, 3H), 2.2 – 2.0 (m, 3H), 2.0 – 1.9 (m, 1H), 1.8 – 1.7 (m, 1H).

13C NMR (151 MHz, DMSO-d6) δ 173.9, 154.5, 152.7, 148.1, 148.8, 148.1, 142.8, 129.1, 117.4, 116.9, 110.9, 51.9, 51.1, 45.8, 40.1, 36.5, 31.5, 24.5, 24.5, 17.7.

UPLC-MS: t_R = 1.58 min (generic method); MS (ESI) m/z calcd for C19H24N5O3: (M+H)^+ : 370.2; found: 370.4.

(rac)-Benzyl 3-[methoxy(methyl)carbamoyl]pyrrolidine-1-carboxylate (44): In a round-bottomed flask, under N2, compound 42 (3.00 g, 12 mmol) was dissolved in DCM (48 mL). To this resulting solution, 43 (1.51 g, 15.6 mmol), DIPEA (5.2 mL, 30 mmol), HOBT (1.86 g, 12 mmol) and
EDCI (2.52 g, 13.2 mmol) were added. After 18 h, the reaction was diluted with DCM (20 mL) and the organic layer was washed with water and brine and dried over Na₂SO₄ and filtered. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/MTBE 70:30) to give the pure title compound, as a yellow solid (3.25 g, 93%).

[^1]H NMR (400 MHz, DMSO-d₆) δ 3.68 (s, 3H), 3.51 – 3.41 (m, 1H), 3.42 – 3.29 (m, 2H), 3.29 – 3.18 (m, 2H), 3.11 (s, 3H), 2.09 – 1.97 (m, 1H), 1.97 – 1.80 (m, 1H), 1.39 (s, 9H).

UPLC-MS: t_R = 1.79 min (generic method); MS (ESI) m/z calcd for C₁₂H₂₃N₂O₄: (M+H)^+: 259.3; found: 259.5.

(rac)-Benzyl 3-acetylpyrrolidine-1-carboxylate (45): In a three neck flask, at -78 °C under N₂, compound 44 (3.25 g, 1.74 mmol) was dissolved in THF (28 mL) and a solution of MeMgBr (3 M in Et₂O, 8.3 mL, 25.06 mmol) was added dropwise and then the temperature raised up to room temperature. After 1 h, the reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with EtOAc (x3) and the combined organic layers were washed with brine and filtered. Removal of the organics gave a sticky oil (2.47 g), which was not subjected to any purification and directly used in the next step of reaction.

[^1]H NMR (400 MHz, DMSO-d₆) δ 7.4 – 7.3 (m, 4H), 7.3 – 7.2 (m, 1H), 5.1 (s, 2H), 3.6 – 3.3 (m, 3H), 3.3 – 3.2 (m, 2H), 2.2 (s, 3H), 2.1 – 2.0 (m, 1H), 2.0 – 1.9 (m, 1H).

(rac)-Benzyl-3-[(E)-3-(dimethylamino)prop-2-enoyl]pyrrolidine-1-carboxylatecarboxylate (46): In a round-bottomed flask, under N₂, compound 45 (2.47 g, 10 mmol) was dissolved in DMF (20 mL). To this resulting solution, DMF-DMA (3.3 mL, 2.5 mmol) was added and the reaction was heated to 140 °C. After 18 h, water was added and the aqueous phase was extracted with EtOAc (x3). The combined organic layers were washed with brine and dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky yellow oil (2.45 g), which was not subjected to any purification and directly used in the next step of reaction.

[^1]H NMR (400 MHz, DMSO-d₆) δ 7.5 (d, J = 12.7 Hz, 1H), 7.4 (d, J = 5.8 Hz, 4H), 7.3 – 7.3 (m, 2H), 5.1 (s, 2H), 5.0 (d, J = 12.7 Hz, 1H), 3.5 – 3.3 (m, 3H), 3.1 – 3.0 (m, 2H), 2.8 – 2.7 (m, 2H), 2.1 – 1.9 (m, 1H), 1.9 – 1.8 (m, 1H).
UPLC-MS: $t_R = 1.78$ min (generic method); MS (ESI) m/z calcd for $\text{C}_{17}\text{H}_{23}\text{N}_2\text{O}_3$: (M+H)$^+$: 303.2; found: 303.5.

** rac**- Benzyl 3-(1H-pyrazol-3-yl)pyrrolidine-1-carboxylate (47): In a round-bottomed flask, compound 46 (2.45 g, 8.13 mmol) was dissolved in EtOH (4.6 mL). To this resulting solution, hydrazine monohydrate 65% (1.5 L, 24.4 mmol) was added and the reaction was heated till 85 °C. After 2 h, solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/TBME 30:70) to give the pure title compound, as a yellow oil (1.71 g, 78% over three steps).

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.5 (bs, 1H), 7.6 (s, 1H), 7.4 – 7.3 (m, 4H), 7.3 – 7.2 (m, 1H), 6.1 (apps, 1H), 5.1 (apps, 2H), 3.8 – 3.6 (m, 1H), 3.5 – 3.3 (m, 4H), 2.3 – 2.1 (m, 1H), 2.0 – 1.9 (m, 1H).

UPLC-MS: $t_R = 1.77$ min (generic method); MS (ESI) m/z calcd for $\text{C}_{15}\text{H}_{18}\text{N}_3\text{O}_2$: (M+H)$^+$: 272.1; found: 272.5.

** rac**- Benzyl 3-(4-bromo-1H-pyrazol-3-yl)pyrrolidine-1-carboxylate (48): In a round-bottomed flask, compound 47 (1.71 g, 6.3 mmol) was dissolved in DMF (12 mL). To this resulting solution, NBS (1.11 g, 6.3 mmol) was added and the reaction was stirred at room temperature (away from light). After 2 h, water was added and the aqueous phase was extracted with EtOAc (x3). The combined organic layers were washed with brine and dried over Na$_2$SO$_4$ and filtered. Solvent was removed under vacuum, and the resulting oil was subjected to flash chromatography (DCM/MTBE 70:30) to give the pure title compound, as a colorless oil (2.00 g, 90%).

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 13.0 (s, 1H), 8.0 (s, 1H), 7.4 – 7.3 (m, 4H), 7.3 – 7.2 (m, 1H), 5.1 (s, 2H), 3.8 – 3.6 (m, 1H), 3.6 – 3.3 (m, 4H), 2.3 – 2.1 (m, 1H), 2.1 – 1.9 (m, 1H).

UPLC-MS: $t_R = 2.04$ min (generic method); MS (ESI) m/z calcd for $\text{C}_{15}\text{H}_{17}\text{BrN}_3\text{O}_2$: (M+H)$^+$: 350.0; found: 350.4.
**CHEMISTRY**

(rac)- Benzyl 3-[4-bromo-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate (49): In round-bottomed neck flask, at 0 °C under N\_2, compound 48 (2.64 g, 7.6 mmol) was dissolved in THF (50 mL) and NaH (60% in mineral oil 0.454 g, 11.37 mmol) was added and the solution was stirred at 0 °C for 30 min. After which 2-(Trimethylsilyl)ethoxymethyl chloride (2 mL, 11.37 mmol) was added dropwise and the temperature was raised up to room temperature. After 18 h, the reaction was poured into a NH\_4Cl saturated solution, the aqueous phase was extracted with EtOAc (x3) and the combined organic layers were washed with brine and dried over Na\_2SO\_4 and filtered. Removal of the organics gave a sticky oil, which was subjected to flash chromatography (CyHex/MTBE 70:30) to give the pure title compound as two regioisomers, as a colorless oil (3.25 g, 89%).

Major isomer: \(^1\)H NMR (400 MHz, DMSO-\_d\_6) δ 8.1 (s, 1H), 7.4 – 7.3 (m, 5H), 5.3 (s, 2H), 5.1 – 5.0 (m, 2H), 3.9 – 3.5 (m, 2H), 3.5 – 3.3 (m, 5H), 2.4 – 1.9 (m, 2H), 0.9 – 0.7 (m, 2H), -0.1 (s, 9H). UPLC-MS: \(t_R = 2.06\) min (generic method); MS (ESI) m/z calcd for C\(_{21}\)H\(_{31}\)BrN\(_3\)O\(_3\)Si: (M+H)\(^+\) : 480.1; found: 480.5.

(rac)- Benzyl 3-[4-(2-amino-4-pyridyl)-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate (51): In a round-bottomed flask, under N\_2, compound 49 (0.600 g, 1.25 mmol) was dissolved in dioxane (30 mL) and water (10.4 mL). To the resulting solution 50 (0.412 g, 1.86 mmol), Cs\(_2\)CO\(_3\) (2.03 g, 6.25 mmol) and [Pd(dppf)Cl\(_2\)]-CH\(_2\)Cl\(_2\) (204 g, 0.25 mmol) were added. The reaction was heated to 110 °C. After 18 h, the reaction was diluted with EtOAc and filtered through a plug of celite. The organic solution was washed with NaHCO\(_3\), water and brine and finally dried over Na\_2SO\_4 and filtered. Solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/DCM*MeOH NH\_3 1 N 95:5) to give the pure title compound, as an oil (0.470 g, 76%).

Major isomer: \(^1\)H NMR (400 MHz, DMSO-\_d\_6) δ 8.1 (s, 1H), 7.9 – 7.8 (m, 1H), 7.4 – 7.3 (m, 5H), 6.5 (dd, \(J = 5.3, 1.5\) Hz, 1H), 6.5 – 6.5 (m, 1H), 5.9 (d, \(J = 16.5\) Hz, 2H), 5.3 (s, 2H), 5.1 – 5.0 (m, 2H), 3.9 – 3.6 (m, 2H), 3.6 – 3.3 (m, 5H), 2.3 – 2.0 (m, 2H), 0.9 – 0.8 (m, 2H),
-0.1 (s, 9H). UPLC-MS: $t_R = 1.48$ min (apolar method); MS (ESI) m/z calcd for C$_{26}$H$_{36}$N$_5$O$_3$Si: (M+H)$^+$: 494.3; found: 494.3.

(rac)-Benzyl-3-[4-[2-(tert-butoxycarbonylamino)-4-pyridyl]-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate (52): In a round-bottomed flask, compound 51 (0.600 g, 1.22 mmol) was dissolved in DCM (2.5 mL). To the resulting solution BOC$_2$O (0.319 g, 1.46 mmol), DIPEA (0.302 mL, 61.83 mmol) and DIMAP (1%) were added. After 2 h, the reaction was washed with NH$_4$Cl, and brine, and finally dried over Na$_2$SO$_4$ and filtered. Solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (CyHex/EtOAc 70:30) to give the pure title compound, as an oil (0.465 g, 64%).

Major isomer: $^1$H NMR (400 MHz, DMSO-$_d_6$) δ 9.8 (s, 1H), 8.3 (s, 1H), 8.3 – 8.2 (m, 1H), 7.9 (bs, 1H), 7.4 – 7.2 (m, 5H), 7.2 – 7.1 (m, 1H), 5.4 (s, 2H), 5.1 – 5.0 (m, 2H), 3.9 – 3.7 (m, 2H), 3.6 – 3.4 (m, 5H), 2.3 – 2.0 (m, 2H), 1.5 (s, 9H), 0.9 – 0.8 (m, 2H), -0.1 (s, 9H). UPLC-MS: $t_R = 2.37$ min (apolar method); MS (ESI) m/z calcd for C$_{31}$H$_{44}$N$_5$O$_5$Si: (M+H)$^+$: 594.3; found: 594.3.

(rac)-tert-Butyl N-[4-[3-pyrrolidin-3-yl-1-(2-trimethylsilylethoxymethyl)pyrazol-4-yl]-2-pyridyl]carbamate (53): In a round-bottomed flask, compound 52 (0.050 g, 0.08 mmol) was dissolved in EtOH (1.7 mL). To the resulting solution Pd/C 10% in mineral oil (0.002 g, 0.02 mmol), and Et$_3$SiH (0.038 µL, 0.24 mmol) were added. The reaction was heated to 110 °C. After 4 h, the reaction was diluted with EtOAc and filtered through a plug of celite. Solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/DCM*MEOH NH$_3$ 1 N 95:5) to give the pure title compound, as an oil (0.030 g, 82%).

Major isomer: $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.8 (s, 1H), 8.3 – 8.2 (m, 1H), 7.9 (d, $J = 1.4$ Hz, 1H), 7.1 (dd, $J = 5.2$, 1.6 Hz, 1H), 5.5 (q, $J = 11.3$ Hz, 1H), 5.4 (s, 1H), 3.7 – 3.5 (m,
(rac)-tert-Butyl-N-[4-[3-(1-propylpyrrolidin-3-yl)-1-(2-trimethylsilylethoxymethyl)pyrazol-4-yl]-2-pyridyl]carbamate (54): In a round-bottomed flask, at 0 °C under N₂, compound 53 (0.354 g, 0.77 mmol) was dissolved in MeOH (5 mL) and to the resulting solution propionaldehyde (0.083 mL, 1.15 mmol) and Na(CH₃COO)$_3$BH (0.488 g, 1.32 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 2 h. The solution was quenched with NH₄Cl, extracted with EtOAc and the combined organic layers were washed NaHCO₃ and brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.306 g, 79%).

Major isomer: $^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.7 (s, 1H), 8.2 – 8.2 (m, 1H), 7.9 – 7.9 (m, 1H), 7.1 (dd, 1H), 5.7 (s, 2H), 3.8 – 3.5 (m, 3H), 3.1 (t, $J = 8.3$ Hz, 1H), 2.9 – 2.7 (m, 1H), 2.5 – 2.3 (m, 3H), 2.2 – 2.1 (m, 1H), 2.1 – 1.9 (m, 1H), 1.5 (s, 9H), 1.5 – 1.4 (m, 2H), 0.9 – 0.8 (m, 5H), -0.1 (s, 9H). UPLC-MS: $t_R = 1.53$ min (apolar method); MS (ESI) m/z calcd for C$_{26}$H$_{44}$N$_5$O$_3$Si: (M+H)$^+$: 502.3; found: 502.3.

(rac)-4-[3-(1-Propylpyrrolidin-3-yl)-1H-pyrazol-4-yl]pyridin-2-amine trihydrochloride (55): In a round-bottomed flask, compound 54 (0.306 g, 0.6 mmol) was dissolved in a solution of HCl 3 M in MeOH (4 mL, 12.2 mmol) at room temperature. The solution was stirred for 18 h. The solvent was removed under vacuum and compound was precipitated with Et₂O as trichlorohydrate salt, to give the compound, as a white solid (0.220 g, quantitative yield).

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 13.7 (bs, 1H), 11.0 (bd, $J = 77.8$ Hz, 1H), 8.4 (bd, $J = 15.5$ Hz, 1H), 8.3 – 8.0 (m, 2H), 7.9 (t, $J = 6.8$ Hz, 1H), 7.2 (d, 1H), 7.1 – 6.9 (m, 1H), 4.1 – 3.8
(m, 2H), 3.8 – 3.6 (m, 2H), 3.2 – 3.3 (m, 1H), 2.3 – 2.0 (m, 1H), 1.8 – 1.6 (m, 2H), 0.9 (t, 3H). UPLC-MS: \( t_R = 0.80 \) min (apolar method); MS (ESI) m/z calcd for \( \text{C}_{15}\text{H}_{22}\text{N}_5 \): (M+H)\(^+\) : 272.2; found: 272.3.

\((\text{rac})\)-2-Methyl-N-[4-[3-(1-propylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]propanamide (59a): In a round-bottomed flask, compound 55 (0.070 g, 0.18 mmol) was dissolved in DCM (1.5 mL) and to the resulting solution, Et\(_3\)N (0.050 mL, 0.36 mmol), isobutyryl chloride (0.056 mL, 0.54 mmol), were added. The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was quenched with NH\(_4\)Cl, extracted with DCM and the combined organic layers were washed brine and finally dried over Na\(_2\)SO\(_4\) and filtered. Removal of the organics gave a sticky oil as mixture of triacylated 57a and di-acylated 58a, which was not isolated, but directly solubilized in NaOH 2 M (0.130 mL) in MeOH (1 mL) and stirred at room temperature. After 30 min, the reaction was neutralized till pH 7 with HCl 1 N, washed with NaHCO\(_3\) and brine, and finally dried over Na\(_2\)SO\(_4\) and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH\(_3\) 1 N 92:8) to give the pure title compound, as white solid (0.024 g, 39%).

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 12.8 (bs, 1H), 10.4 (s, 1H), 8.3 – 8.2 (m, 2H), 8.2 – 7.6 (m, 1H), 7.1 (dd, \( J = 5.2, 1.6 \) Hz, 1H), 3.7 – 3.5 (m, 1H), 3.0 (t, \( J = 8.3 \) Hz, 1H), 2.8 – 2.7 (m, 2H), 2.6 – 2.5 (m, 1H), 2.4 (t, \( J = 7.5 \) Hz, 2H), 2.3 – 2.1 (m, 1H), 2.0 – 1.8 (m, 1H), 1.4 (h, \( J = 7.3 \) Hz, 2H), 1.1 (d, \( J = 6.8 \) Hz, 6H), 0.9 (t, \( J = 7.4 \) Hz, 3H). \(^{13}\)C NMR (101 MHz, DMSO-\(d_6\)) \( \delta \) 176.2, 152.7, 148.0, 147.1, 143.1, 117.5, 116.7, 116.6, 111.0, 59.6, 57.5, 53.3, 40.1, 34.5, 30.6, 21.5, 19.4 (x2), 12.0. UPLC-MS: \( t_R = 1.31 \) min (generic method); MS (ESI) m/z calcd for \( \text{C}_{19}\text{H}_{28}\text{N}_5\text{O} \): (M+H)\(^+\) : 342.2; found: 342.3.

\((\text{rac})\)-N-[4-[3-(1-propylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclopropanecarboxamide (59b): In a round-bottomed flask, compound 55 (0.039 g, 0.10 mmol) was dissolved in DCM (1.1 mL) and to the resulting solution, Et\(_3\)N (0.028 mL, 0.2 mmol), cyclopropanecarbonyl chloride (0.030 mL, 0.3 mmol), were added.
The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was quenched with NH₄Cl, extracted with DCM and the combined organic layers were washed brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil as mixture of triacylated 57b and di-acylated 58b, which was not isolated but directly solubilized in NaOH 2 M (0.220 mL) in MeOH (1 mL) and stirred at room temperature. After 30 min, the reaction was neutralized till pH 7 with HCl 1 N, washed with NaHCO₃ and brine, and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.011 g, 33%).

¹H NMR (400 MHz, DMSO-d₆) δ 12.8 (bs, 1H), 10.7 (s, 1H), 8.2 (d, J = 5.2 Hz, 1H), 8.2 (s, 1H), 8.1 – 7.6 (m, 1H), 7.1 (dd, J = 5.2, 1.6 Hz, 1H), 3.7 – 3.5 (m, 1H), 3.1 – 3.0 (m, 1H), 2.8 – 2.6 (m, 1H), 2.4 (t, J = 7.5 Hz, 2H), 2.3 – 2.1 (m, 1H), 2.1 – 2.0 (m, 1H), 2.0 – 1.8 (m, 1H), 1.4 (h, J = 7.3 Hz, 2H), 0.9 – 0.7 (m, 7H).

¹³C NMR (101 MHz, DMSO-d₆) δ 172.6, 152.5, 148.0, 143.1, 117.5, 115.1, 110.9, 59.5, 57.5, 53.4, 30.6, 21.5, 14.2, 12.0, 7.6.

UPLC-MS: tᵣ = 1.27 min (generic method); MS (ESI) m/z calcd for C₁₉H₂₆N₅O: (M+H)+: 340.2; found: 340.3.

(rac)- N-[4-[3-(1-Propylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]benzamide (59c): In a round-bottomed flask, compound 55 (0.055 g, 0.15 mmol) was dissolved in DCM (1.5 mL) and to the resulting solution, Et₃N (0.041 mL, 0.3 mmol), benzoyl chloride (0.051 mL, 0.45 mmol), were added.

The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was quenched with NH₄Cl, extracted with DCM and the combined organic layers were washed brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil as mixture of triacylated 57c and di-acylated 58c which was not isolated but directly solubilized in NaOH 2 M (0.130 mL) in MeOH (1 mL) and stirred at room temperature. After 30 min, the reaction was neutralized till pH 7 with HCl 1 N, washed with NaHCO₃ and brine, and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.008 g, 14%).

¹H NMR (400 MHz, DMSO-d₆) δ 10.8 (s, 1H), 8.3 (d, J = 5.2 Hz, 1H), 8.3 (d, J = 1.5 Hz, 1H), 8.1 – 8.0 (m, 2H), 7.9 (s, 1H), 7.6 – 7.6 (m, 1H), 7.6 – 7.5 (m, 2H), 7.2 (dd, J = 5.2, 1.6 Hz, 1H), 4.7 – 4.3 (m, 2H), 4.3 – 4.0 (m, 2H), 3.8 – 3.5 (m, 1H), 3.2 – 2.8 (m, 1H), 2.5 – 2.3 (m, 1H), 1.4 – 1.1 (m, 2H), 0.8 – 0.5 (m, 7H).
Hz, 1H), 3.7 (dq, J = 9.9, 7.6 Hz, 1H), 3.0 (t, J = 8.3 Hz, 1H), 2.8 – 2.7 (m, 1H), 2.6 – 2.5 (m, 2H), 2.4 – 2.2 (m, 3H), 2.0 – 1.9 (m, 1H), 1.5 (h, J = 7.4 Hz, 2H), 0.9 (t, J = 7.4 Hz, 3H).

$^{13}$C NMR (151 MHz, Chloroform-$d$) δ 166.1, 152.1, 148.3, 147.2, 144.1, 134.5, 132.4, 129.0, 127.3, 118.7, 116.0, 112.2, 60.0, 57.8, 52.7, 33.3, 31.8, 22.0, 12.2.

UPLC-MS: $t_R = 1.52$ min (generic method); MS (ESI) m/z calcd for C$_{22}$H$_{26}$N$_5$O: (M+H)$^+$ : 376.2; found: 376.3.

(rac)-$N$-[4-[3-(1-Propylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclohexanecarboxamide (59d): In a round-bottomed flask, compound 55 (0.055 g, 0.15 mmol) was dissolved in DCM (1.5 mL) and to the resulting solution, Et$_3$N (0.041 mL, 0.3 mmol), cyclohexanecarbonyl chloride (0.030 mL, 0.3 mmol), were added. The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was quenched with NH$_4$Cl, extracted with DCM and the combined organic layers were washed brine and finally dried over Na$_2$SO$_4$ and filtered. Removal of the organics gave a sticky oil as mixture of triacylated 57d and di-acylated 58d, which was not isolated but directly solubilized in NaOH 2 M (0.130 mL) in MeOH (1 mL) and stirred at room temperature. After 30 min, the reaction was neutralized till pH 7 with HCl 1 N, washed with NaHCO$_3$ and brine, and finally dried over Na$_2$SO$_4$ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH$_3$ 1 N 92:8) to give the pure title compound, as white solid (0.010 g, 17%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 10.3 (bs, 1H), 8.4 – 8.1 (m, 2H), 7.9 (s, 1H), 7.1 (s, 1H), 3.7 – 3.5 (m, 1H), 3.0 (t, J = 8.1 Hz, 1H), 2.8 – 2.6 (m, 1H), 2.4 – 2.3 (m, 3H), 2.3 – 2.2 (m, 1H), 2.0 – 1.8 (m, 1H), 1.8 – 1.7 (m, 4H), 1.7 – 1.5 (m, 1H), 1.5 – 1.3 (m, 4H), 1.3 – 1.1 (m, 4H), 0.9 – 0.8 (m, 3H). $^{13}$C NMR (151 MHz, DMSO-$d_6$) δ 175.3, 152.7, 148.0, 143.1, 117.5, 116.7, 110.9, 59.6, 57.5, 53.4, 44.3, 40.1, 34.3, 30.7, 29.1 (x2), 25.4, 25.2, 21.5, 12.0.

UPLC-MS: $t_R = 1.67$ min (generic method); MS (ESI) m/z calcd for C$_{22}$H$_{32}$N$_5$O: (M+H)$^+$ : 382.3; found: 382.3.
(rac)- **tert-Butyl 3-[4-[2-[benzyl(cyclobutane carboxyl)amino]-4-pyridyl]-1-(2-trimethylsilylethoxymethyl)pyrazol-3-yl]pyrrolidine-1-carboxylate** (62): In a round-bottomed flask, under N₂, compound 36 (0.376 g, 0.70 mmol) was dissolved in DMF (14 mL) and to the resulting solution, Cs₂CO₃ (0.682 g, 2.1 mmol) was added. After 30 min, benzyl bromide (0.250 mL, 2.1 mmol) was added and the reaction was stirred at room temperature for 18 h. The reaction was extracted with Et₂O, washed with brine, and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH 96:4) to give the pure title compound as two regioisomers, as an oil (0.418 g, 94%).

Major isomer: ¹H NMR (400 MHz, DMSO-d₆) δ 8.4 (d, J = 5.2 Hz, 1H), 8.3 (d, J = 3.5 Hz, 1H), 7.5 – 7.4 (m, 1H), 7.4 – 7.1 (m, 6H), 5.6 – 5.5 (m, 1H), 5.1 – 5.0 (m, 1H), 3.8 – 3.5 (m, 4H), 2.3 – 2.1 (m, 4H), 2.1 – 1.6 (m, 7H), 1.4 (dd, J = 7.4, 4.2 Hz, 9H), 0.9 – 0.8 (m, 2H), -0.1 (s, 9H). UPLC-MS: tᵣ = 1.39 min (apolar method); MS (ESI) m/z calcd for C₃₅H₅₀N₅O₄Si: (M+H)⁺: 632.3; found: 632.6.

(rac)- **N-Benzyl-N-[4-(3-pyrrolidin-3-yl-1H-pyrazol-4-yl)-2-pyridyl]cyclobutane carboxamide; dihydrochloride** (63): In a round-bottomed flask, at 0 °C compound 62 (0.418 g, 0.6 mmol) was dissolved in a solution of HCl 3 M in dioxane (4 mL, 13.2 mmol). The solution was stirred for 3 h, at room temperature. The solvent was removed under vacuum and compound was precipitated with Et₂O as dichlorohydrate salt, to give the compound as a white solid (0.348 g, quantitative yield).

Major rotamer: ¹H NMR spectra at room temperature showed a complex mixture of rotamers. UPLC-MS: tᵣ = 1.53 min (generic method); MS (ESI) m/z calcd for C₂₄H₂₄N₅O: (M+H)⁺: 402.2; found: 402.5.
(rac)-N-Benzyl-N-[4-[3-(1-propylpyrrolidin-3-yl)-1H-pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (60): In a round-bottomed flask, at 0 °C under N₂, compound 63 (0.320 g, 0.67 mmol) was dissolved in DCE (1 mL) and to the resulting solution propionaldehyde (0.072 mL, 1 mmol) and Na(CH₃COO)₃BH (0.303 g, 1.44 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH₄Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO₃ and brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1N 92:8) to give the pure title compound, as white solid (0.138 g, 46%).

¹H NMR (400 MHz, DMSO-d₆) δ 12.9 (s, 1H), 8.4 (d, J = 5.2 Hz, 1H), 7.9 (s, 1H), 7.4 – 7.1 (m, 7H), 5.0 (s, 2H), 3.5 – 3.4 (m, 1H), 3.4 – 3.3 (m, 1H), 2.8 (t, J = 8.3 Hz, 1H), 2.7 – 2.6 (m, 1H), 2.5 – 2.4 (m, 1H), 2.4 – 2.3 (m, 2H), 2.2 – 2.1 (m, 2H), 2.1 – 2.0 (m, 1H), 2.0 – 1.8 (m, 1H), 1.8 – 1.6 (m, 4H), 1.4 (h, J = 7.3 Hz, 2H), 0.9 (t, J = 7.4 Hz, 3H).

UPLC-MS: tᵣ = 1.68 min (generic method); MS (ESI) m/z calcd for C₂₇H₃₄N₅O: (M+H)⁺ : 444.3; found: 444.5.

(rac)-Benzy l 3-(4-bromo-1-methyl-pyrazol-3-yl)pyrrolidine-1-carboxylate (66): In round-bottomed neck flask, at 0 °C under N₂, compound 48 (2.01 g, 5.8 mmol) was dissolved in DMF (10 mL) and to the resulting solution Cs₂CO₃ (2.82 g, 9.15 mmol) and iodomethane (0.357 mL, 5.8 mmol) were added and the solution was stirred at 0 °C for 1 h., The reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with EtOAc (x3) and the combined organic layers were washed with brine and dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil as a mixture of two regioisomers, which was subjected to flash chromatography (CyHex/EtOAc 50:50) to give the pure regioisomer 66 as a colorless oil (1.49 g, 71%).

¹H NMR (400 MHz, DMSO-d₆) δ 7.9 (s, 1H), 7.4 – 7.3 (m, 4H), 7.3 – 7.3 (m, 1H), 5.1 (s, 2H), 3.8 (s, 3H), 3.8 – 3.6 (m, 1H), 3.6 – 3.3 (m, 4H), 2.3 – 2.1 (m, 1H), 2.1 – 1.9 (m, 1H).

UPLC-MS: tᵣ = 2.24 min (generic method); MS (ESI) m/z calcd for C₁₆H₁₉BrN₃O₂: (M+H)⁺ : 364.1; found: 364.5.
(rac)- Benzyl 3-[4-(2-amino-4-pyridyl)-1-methyl-pyrazol-3-yl]pyrrolidine-1-carboxylate (67): In a round-bottomed flask, under N₂, compound 66 (0.500 g, 1.37 mmol) was dissolved in dioxane (34.5 mL) and water (11.5 mL). To the resulting solution 32 (0.454 g, 2.06 mmol), Cs₂CO₃ (2.11 g, 6.50 mmol) and [Pd(dppf)Cl₂]-CH₂Cl₂ (0.223 g, 0.27 mmol) were added. The reaction was heated to 110 °C. After 18 h, the reaction was diluted with EtOAc and filtered through a plug of celite. The organic solution was washed with NaHCO₃, water and brine and finally dried over Na₂SO₄ and filtered. Solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 95:5) to give the pure title compound, as an oil. (0.228 g, 44%)

¹H NMR (400 MHz, DMSO- d₆) δ 7.9 (s, 1H), 7.9 (d, J = 5.3 Hz, 1H), 7.4 – 7.2 (m, 5H), 6.5 (dd, J = 5.3, 1.5 Hz, 1H), 6.5 (d, J = 1.5 Hz, 1H), 5.9 (s, 2H), 5.1 – 5.0 (m, 2H), 3.8 (s, 3H), 3.8 – 3.3 (m, 5H), 2.3 – 2.1 (m, 1H), 2.1 – 2.0 (m, 1H).0.228 g, 44%).

(rac)- Benzyl 3-[4-[2-(cyclobutanecarbonylamino)-4-pyridyl]-1-methyl-pyrazol-3-yl]pyrrolidine-1-carboxylate (68): In a round-bottomed flask, compound 67 (0.329 g, 0.87 mmol) was dissolved in DCM (1 mL) and to the resulting solution, Et₃N (0.266 mL, 1.91 mmol), 34 (0.198 mL, 1.74 mmol), were added. The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was quenched with NH₄Cl, extracted with DCM and the combined organic layers were washed brine and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil as di-acylated intermediate which were not isolated but directly solubilized in NaOH 2 M (2 mL) in MeOH (18 mL) and stirred for at room temperature. After 30 min, the reaction was neutralized till pH 7 with HCl 1 N, washed with NaHCO₃ and brine, and finally dried over Na₂SO₄ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.362 g, 87%).

¹H NMR (400 MHz, DMSO-d₆) δ 10.3 (s, 1H), 8.2 (d, J = 5.2 Hz, 2H), 8.1 (s, 1H), 7.4 – 7.3 (m, 5H), 7.1 (dd, J = 5.3, 1.6 Hz, 1H), 5.1 – 5.0 (m, 2H), 3.8 (s, 3H), 3.8 – 3.6 (m, 2H), 3.6 – 3.3 (m, 4H), 2.3 – 2.2 (m, 3H), 2.1 – 2.0 (m, 3H), 2.0 – 1.7 (m, 2H).
UPLC-MS: $t_R = 2.21$ min (generic method); MS (ESI) m/z calcd for $C_{26}H_{30}N_{5}O_3$: (M+H)$^+$: 460.2; found: 460.3.

(rac)-N-[4-(1-methyl-3-pyrrolidin-3-yl-pyrazol-4-yl)-2-pyridyl]cyclobutanecarboxamide (69): In a round-bottomed flask, compound 68 (0.165 g, 0.36 mmol) was dissolved in THF/MeOH 1:1 (14 mL). To the resulting solution 1,4-cyclohexadiene (0.340 mL, 3.6 mmol) and Pd/C 10% in mineral oil (0.165 g) were added. The reaction was heated to 60 °C. After 4 h, the reaction was diluted with EtOAc and filtered through a plug of celite. Solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 95:5) to give the pure title compound, as an oil (0.065 g, 55%).

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 10.3 (s, 1H), 8.3 (d, $J = 5.2$ Hz, 1H), 8.2 – 8.2 (m, 1H), 8.1 (s, 1H), 7.1 (dd, $J = 5.2$, 1.7 Hz, 1H), 3.8 (s, 3H), 3.7 (p, $J = 7.7$ Hz, 1H), 3.5 – 3.3 (m, 4H), 3.3 – 3.2 (m, 3H), 2.4 – 2.3 (m, 1H), 2.3 – 2.2 (m, 2H), 2.1 – 2.1 (m, 2H), 2.0 – 1.9 (m, 2H), 1.9 – 1.7 (m, 1H).

UPLC-MS: $t_R = 1.35$ min (generic method); MS (ESI) m/z calcd for $C_{18}H_{24}N_{5}O$: (M+H)$^+$: 326.2; found: 326.3.

(rac)-N-[4-[1-methyl-3-(1-propylpyrrolidin-3-yl)pyrazol-4-yl]-2-pyridyl]cyclobutanecarboxamide (64): In a round-bottomed flask, at 0 °C under N₂, compound 69 (0.030 g, 0.09 mmol) was dissolved in MeOH (1 mL) and to the resulting solution propionaldehyde (0.007 mL, 0.09 mmol) and Na(CH$_3$COO)$_3$BH (0.038 g, 0.18 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH$_4$Cl, extracted with EtOAc and the combined organic layers were washed NaHCO$_3$ and brine and finally dried over Na$_2$SO$_4$ and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH₃ 1 N 92:8) to give the pure title compound, as white solid (0.011 g, 33%).

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 10.2 (s, 1H), 8.2 (d, $J = 5.2$ Hz, 2H), 8.0 (s, 1H), 7.1 (dd, $J = 5.1$, 1.7 Hz, 1H), 3.8 (s, 3H), 3.6 – 3.5 (m, 1H), 3.4 – 3.3 (m, 1H), 3.0 (t, $J = 8.3$ Hz, 1H),
2.8 – 2.7 (m, 1H), 2.5 (s, 1H), 2.4 – 2.3 (m, 3H), 2.3 – 2.2 (m, 3H), 2.2 – 2.0 (m, 2H), 2.0 – 1.9 (m, 2H), 1.9 – 1.7 (m, 1H), 1.4 (h, J = 7.4 Hz, 2H), 0.9 (t, J = 7.4 Hz, 3H). 13C NMR (101 MHz, DMSO-d$_6$) δ 173.8, 152.6, 151.2, 148.0, 142.7, 131.0, 117.5, 117.3, 110.8, 59.6, 57.7, 53.6, 40.2, 38.6, 35.2, 30.6, 24.5 (x2), 21.5, 17.7, 12.0.

UPLC-MS: $t_R = 1.53$ min (generic method); MS (ESI) m/z calcd for C$_{21}$H$_{30}$N$_5$O: (M+H)$^+$: 368.2; found: 368.3.

(rac)-4-Bromo-3-pyrrolidin-3-yl-1H-pyrazole;hydrochloride (70): In a round-bottomed flask, at 0 °C compound 31 (0.600 g, 1.34 mmol) was dissolved in a solution of HCl 3 M in MeOH (9 mL, 26.9 mmol) and stirred at room temperature. After 3 h, the solvent was removed under vacuum and the compound was precipitated with Et$_2$O as dichlorohydrate salt, to give compound as a white solid (0.335 g, quantitative yield) which was not subjected to any purification and directly used in the next step of reaction.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.5 (d, J = 48.7 Hz, 2H), 7.9 (s, 1H), 3.6 – 3.4 (m, 2H), 3.4 – 3.1 (m, 3H), 2.4 – 2.2 (m, 1H), 2.1 – 1.9 (m, 1H).

UPLC-MS: $t_R = 0.88$ min (generic method); MS (ESI) m/z calcd for C$_7$H$_{11}$BrN$_3$: (M+H)$^+$: 216.0; found: 216.3.

(rac)- [3-(4-Bromo-1H-pyrazol-3-yl)pyrrolidin-1-yl]-cyclopropyl-methanone (72): In a round-bottomed flask, compound 70 (0.335 g, 1.34 mmol) was dissolved in DCM (13.5 mL) and to the resulting solution, Et$_3$N (0.373 mL, 2.68 mmol), 71 (0.242 mL, 2.68 mmol), were added. The temperature was raised up to room temperature and reaction was stirred for 1 h. The solution was quenched with NH$_4$Cl, extracted with DCM and the combined organic layers were washed brine and finally dried over Na$_2$SO$_4$ and filtered. Removal of the organics gave a sticky oil as di-acylated intermediate, which were not isolated but directly solubilized in NaOH 2 M (2.1 mL) in MeOH (19 mL) and stirred for at room temperature. After 30 min, the reaction was neutralized till pH 7 with HCl 1 N, washed with NaHCO$_3$ and brine, and finally dried over Na$_2$SO$_4$ and filtered. Removal of the organics gave a sticky oil (0.245 g) which was not subjected to any purification and directly used in the next step of reaction.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 13.0 (bs, 1H), 8.0 – 7.7 (appm, 1H), 4.1 – 4.0 (m, 1H), 4.0 – 3.4 (m, 4H), 2.4 – 2.3 (m, 1H), 2.3 – 1.9 (m, 2H), 1.8 – 1.7 (m, 1H), 0.8 – 0.6 (m, 4H).
CHAPTER 2

UPLC-MS: \( t_R = 1.49 \text{ min} \) (generic method); MS (ESI) m/z calcd for \( \text{C}_{11}\text{H}_{15}\text{BrN}_3\text{O}^+ \): 284.0; found: 284.3.

(rac)-[3-(4-Bromo-1-methyl-pyrazol-3-yl)pyrrolidin-1-yl]-cyclopropyl-methanone (73): In round-bottomed neck flask, at 0 °C under \( \text{N}_2 \), compound 48 (0.245 g, 0.86 mmol) was dissolved in DMF (8.6 mL) and to the resulting solution \( \text{Cs}_2\text{CO}_3 \) (0.419 g, 1.29 mmol) and iodomethane (0.053 mL, 0.86 mmol) were added and the solution was stirred at 0 °C for 1 h. The reaction was poured into a \( \text{NH}_4\text{Cl} \) saturated solution, the aqueous phase was extracted with \( \text{Et}_2\text{O} \) (x3) and the combined organic layers were washed with brine and dried over \( \text{Na}_2\text{SO}_4 \) and filtered. Removal of the organics gave a sticky oil as a mixture of two regioisomers, which was subjected to flash chromatography (CyHex/EtOAc 70:30) to give the pure regioisomer 73 as a colorless oil (0.150 g, 59% over two steps).

\(^1\text{H} \text{NMR} \) (400 MHz, DMSO-\( \text{d}_6 \)) \( \delta \) 7.9 (d, \( J = 6.3 \text{ Hz} \), 1H), 3.8 (d, \( J = 3.3 \text{ Hz} \), 3H), 3.8 – 3.6 (m, 1H), 3.6 – 3.4 (m, 1H), 3.4 – 3.3 (m, 3H), 2.3 – 2.1 (m, 2H), 2.0 – 1.9 (m, 1H), 1.8 – 1.7 (m, 1H), 0.8 – 0.6 (m, 4H).

UPLC-MS: \( t_R = 1.62 \text{ min} \) (generic method); MS (ESI) m/z calcd for \( \text{C}_{12}\text{H}_{17}\text{BrN}_3\text{O}^+ \): 298.0; found: 298.3.

(rac)-tert-Butyl-N-[4-[3-[1-(cyclopropanecarbonyl)pyrrolidin-3-yl]-1-methyl-pyrazol-4-yl]-2-pyridyl]carbamate (75): In a round-bottomed flask, under \( \text{N}_2 \), compound 73 (0.100 g, 0.33 mmol) was dissolved in dioxane (8.2 mL) and water (2.7 mL). To the resulting solution 74 (0.158 g, 0.49 mmol), \( \text{Cs}_2\text{CO}_3 \) (0.429 g, 1.32 mmol) and \( \text{[Pd(dppf)Cl}_2\text{-CH}_2\text{Cl}_2} \) (0.053 g, 0.06 mmol) were added. The reaction was heated to 110 °C. After 18 h, the reaction was diluted with EtOAc and filtered through a plug of celite. The organic solution was washed with \( \text{NaHCO}_3 \), water and brine and finally dried over \( \text{Na}_2\text{SO}_4 \) and filtered. Solvent was removed under vacuum and the resulting oil (0.075 g) which was not subjected to any purification and directly used in the next step of reaction.

\(^1\text{H} \text{NMR} \) (400 MHz, DMSO-\( \text{d}_6 \)) \( \delta \) 9.7 (d, \( J = 7.3 \text{ Hz} \), 1H), 8.3 – 8.2 (m, 1H), 8.1 (d, \( J = 7.8 \text{ Hz} \), 1H), 8.0 – 7.8 (m, 1H), 7.1 (ddd, \( J = 7.0, 5.2, 1.6 \text{ Hz} \), 1H), 3.8 (d, \( J = 3.9 \text{ Hz} \), 3H), 3.8 – 3.7 (m, 2H), 3.7 – 3.4 (m, 3H), 2.4 – 2.1 (m, 1H), 2.1 – 1.8 (m, 1H), 1.8 – 1.7 (m, 1H), 1.5 (s, 9H), 0.8 – 0.6 (m, 4H).
UPLC-MS: $t_R = 1.90$ min (generic method); MS (ESI) m/z calcd for C$_{22}$H$_{30}$N$_5$O$_3$: (M+H)$^+$: 412.2; found: 412.3.

*(rac)*-tert-Butyl N-[4-[3-[(cyclopropanecarbonyl)pyrrolidin-3-yl]-1-methyl-pyrazol-4-yl]-2-pyridyl]-N-[(4-fluorophenyl)methyl]carbamate (77): In a round-bottomed flask, under N$_2$, compound 75 (0.075 g, 0.18 mmol) was dissolved in DMF (3.6 mL). To the resulting solution Cs$_2$CO$_3$ (0.175 g, 0.54 mmol) and TBAI (0.199 g, 0.54 mmol) were added and the reaction was stirred at room temperature. After 30 min, 4-fluorobenzyl bromide (0.102 g, 0.54 mmol) was added and the reaction was stirred for 18 h. The mixture was then poured into water and extracted with Et$_2$O. The combined organic layers were washed with brine and dried over Na$_2$SO$_4$ and filtered. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (DCM/MeOH 98:2) to give the pure title compound, as a colorless oil (0.078 g, 30% over two steps).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.4 – 8.3 (m, 1H), 8.1 (d, $J = 7.7$ Hz, 1H), 7.7 (d, 1H), 7.4 – 7.3 (m, 2H), 7.2 – 7.2 (m, 1H), 7.1 (t, $J = 8.8$ Hz, 2H), 5.1 (d, $J = 3.7$ Hz, 2H), 4.1 – 3.9 (m, 1H), 3.8 (d, $J = 3.7$ Hz, 3H), 3.8 – 3.7 (m, 1H), 3.7 – 3.5 (m, 1H), 2.4 – 2.1 (m, 2H), 1.8 – 1.7 (m, 1H), 1.3 (s, 9H), 0.7 – 0.6 (m, 4H).

UPLC-MS: $t_R = 2.42$ min (apolar method); MS (ESI) m/z calcd for C$_{29}$H$_{35}$FN$_5$O$_3$: (M+H)$^+$: 520.3; found: 520.3.

*(rac)*-Cyclopropyl-[3-[4-[(4-fluorophenyl)methylamino]-4-pyridyl]-1-methyl-pyrazol-3-yl]pyrrolidin-1-yl)methanone;hydrochloride (78): In a round-bottomed flask, at 0 °C compound 77 (0.067 g, 0.12 mmol) was dissolved in a solution of HCl 3 M in MeOH (0.43 mL, 1.3 mmol) and stirred at room temperature. After 6 h, the solvent was removed under vacuum and compound was precipitated with Et$_2$O as hydrochloride salt, giving the pure title compound 78 as a white solid (0.042 g, 71%).
\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\): 8.0 – 7.9 (m, 2H), 7.4 – 7.3 (m, 2H), 7.2 – 7.1 (m, 2H), 7.1 – 7.0 (m, 1H), 6.6 – 6.5 (m, 2H), 4.5 – 4.4 (m, 2H), 4.1 – 3.9 (m, 1H), 3.9 – 3.8 (m, 3H), 3.7 – 3.5 (m, 3H), 2.3 – 2.1 (m, 2H), 2.0 – 1.9 (m, 1H), 1.8 – 1.7 (m, 1H), 0.8 – 0.6 (m, 4H).

UPLC-MS: \(t_R = 1.79\) min (generic method); MS (ESI) \(m/z\) calcd for \(\text{C}_{24}\text{H}_{27}\text{FN}_5\text{O}\): (M+H)\(^+\): 420.2; found: 420.3.

\((\text{rac})\)-tert-Butyl \((1S,5R)-3\text{-oxo-2-(2,2,2\text{-trifluoroacetyl})-8-azabicyclo[3.2.1]octane-8-carboxylate} (81)\): A solution of N-Boc-nortropinone (79) (1.00 g, 4.4 mmol) in dry tetrahydrofuran (9 mL) under N\(_2\) was cooled to -70°C with stirring. The solution was then treated with a 2M solution of LDA (2.2 mL, 4.4 mmol) in THF dropwise over 30 min. The mixture was then stirred for 30 min at -70°C, then treated dropwise with ethyl trifluoroacetate (80) (0.528 mL, 4.4 mmol) and the mixture was allowed to stir for 2h. The reaction was quenched by adding water and 2M HCl solution until pH = 6 and the resulting aqueous layer was extracted with AcOEt. The organic phase was dried over Na\(_2\)SO\(_4\), filtered and concentrated under vacuum to afford a brown oil. The title compound was obtained, after purification by silica gel flash-column chromatography with 10% AcOEt in Cyclohexane as the eluent, as a brown oil (0.303 g, 21%). \(^1\)H NMR spectrum showed the corresponding enol form: \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\): 13.71 (bs, 1H), 4.81 (app-bs, 1H), 4.29 (t, \(J = 6.3\) Hz, 1H), 3.06 – 2.84 (m, 1H), 2.44 (d, \(J = 19.1\) Hz, 1H), 2.24 – 1.94 (m, 2H), 1.83 (t, \(J = 9.4\) Hz, 1H), 1.78 – 1.64 (m, 1H), 1.37 (s, 9H); UPLC-MS: \(t_R = 2.11\) min (generic method); MS (ESI) \(m/z\) calcd for \(\text{C}_{14}\text{H}_{17}\text{F}_{3}\text{NO}_4\) (M)\(^+\): 320.3, found: 320.2.

\((\text{rac})\)-3-((4R,7S)-9-(tert-Butoxycarbonyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-4,7-epiminocyclohepta[c]pyrazol-1(4H)-yl)benzoic acid (83): To a solution of 3-hydrazinobenzoic acid 82 (0.289 g, 1.9 mmol) in AcOH (12 mL), 81 (0.606 g, 1.9 mmol) was added dropwise, and the mixture stirred at room temperature for 3 h. H\(_2\)O (20 mL) was added and the resultant precipitate collected by filtration and purified by trituration with water to yield the title compound as a beige solid (0.744 g, 90%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\): 13.36 (bs, 1H), 8.10 (bs, 1H), 8.06 – 7.99 (m, 1H), 7.96 – 7.86 (m, 1H), 7.69 (t, \(J = 7.9\) Hz, 1H), 5.00 (bs, 1H), 4.50 (dd, \(J = 7.3, 4.5\) Hz, 1H), 3.49 – 3.36 (m, 1H), 2.76 (d, \(J = 16.5\) Hz, 1H), 2.30 – 2.04 (m, 2H), 1.90 – 1.79 (m, 1H), 1.79 – 1.65 (m, 1H), 1.41 (bs, 9H); UPLC-
MS: $t_R = 1.95$ min (generic method); MS (ESI) $m/z$ calcd for C$_{21}$H$_{23}$F$_3$N$_3$O$_4$ (M+H)$^+$: 438.4, found: 438.5.

**(rac)-**tert-Butyl (4R,7S)-1-(3-(benzo[d][1,3]dioxol-5-yl(methyl)carbamoyl)phenyl)-3-(trifluoromethyl)-1,4,5,6,7,8-hexahydro-4,7-epiminocyclohepta[c]pyrazole-9-carboxylate (85): To a solution of 83 (0.200 g, 0.46 mmol) in DMF (3.5 mL), 1,3-Benzodioxol-5-yl(methyl)ammonium chloride 84 (0.120 g, 0.64 mmol), HATU (0.350 g, 0.92 mmol) and DIPEA (0.240 mL, 1.38 mmol) were added, and the mixture stirred at room temperature for 4 h. The solution was concentrated under reduced pressure and the resultant residue partitioned between Et$_2$O and sat. aq. NH$_4$Cl. The organic phase was separated, washed with brine, dried over anhydrous Na$_2$SO$_4$, filtered and the solvent evaporated under reduced pressure. The title compound was obtained, after purification by silica gel flash-column chromatography with Cyclohexane/EtOAc (70:30) as the eluent, as a brown oil (0.141 g, 54%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.63 – 7.48 (m, 2H), 7.49 – 7.34 (m, 2H), 6.98 (d, $J$ = 2.1 Hz, 1H), 6.77 (d, $J$ = 8.2 Hz, 1H), 6.65 (dd, $J$ = 8.2, 2.1 Hz, 1H), 6.00 (d, $J$ = 1.7 Hz, 2H), 4.96 (s, 1H), 4.45 (dd, $J$ = 8.0, 4.5 Hz, 1H), 3.34 (s, 3H), 3.19 – 2.99 (m, 1H), 2.41 (d, $J$ = 16.4 Hz, 1H), 2.30 – 2.18 (m, 1H), 2.18 – 2.03 (m, 1H), 1.83 (s, 1H), 1.73 – 1.57 (m, 1H), 1.35 (bs, 9H); UPLC-MS: $t_R = 2.39$ min (generic method); MS (ESI) $m/z$ calcd for C$_{29}$H$_{30}$F$_3$N$_4$O$_5$ (M+H)$^+$: 571.6, found: 571.3.

**(rac)-**N-(Benz[d][1,3]dioxol-5-yl)-N-methyl-3-((4R,7S)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-4,7-epiminocyclohepta[c]pyrazol-1(4H)-yl)benzamide hydrochloride (86): Compound 85 (0.141 g, 0.23 mmol) was dissolved in a HCl solution in MeOH (3M, 3.8 mL). Mixture was stirred for 5h at room temperature and solvent was evaporated. The title compound was obtained, after trituration with Et$_2$O, as a pale purple solid (0.110 g) and was used in the next step without any other purification.

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.96 (bs, 1H), 9.47 (bs, 1H), 7.64 – 7.52 (m, 2H), 7.47 (t, $J$ = 7.8 Hz, 1H), 7.39 (d, $J$ = 7.7 Hz, 1H), 6.98 (d, $J$ = 2.1 Hz, 1H), 6.80 (d, $J$ = 8.2 Hz, 1H), 6.68 (dd, $J$ = 8.0, 2.1 Hz, 1H), 6.01 (s, 2H), 5.01 (d, $J$ = 4.5 Hz, 1H), 4.38 (bs, 1H), 3.34 (s,
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3H), 3.30 – 3.21 (m, 1H), 2.89 (d, J = 16.8 Hz, 1H), 2.36 – 2.19 (m, 3H), 2.14 – 2.02 (m, 1H), 1.98 – 1.86 (m, 1H); UPLC-MS: t_R = 1.64 min (generic method); MS (ESI) m/z calcd for C_{24}H_{22}F_{3}N_{4}O_{3} (M)^+: 471.6, found: 471.3.

(rac)-9-N-(1,3-Benzodioxol-5-yl)-N-methyl-3-[11-methyl-3-(trifluoromethyl)-4,5,11 triazatricyclo[6.2.1.0^{2,6}]undeca-2(6),3-dien-5-yl]benzamide (89): In a round-bottomed flask, at 0 °C under N_2, compound 86 (0.035 g, 0.07 mmol) was dissolved in MeOH (2.3 mL) and to the resulting solution 87 (0.014 mL, 0.19 mmol) and Na(CH_3COO)_3BH (0.022 g, 0.1 mmol) were added. The temperature was raised up to room temperature and reaction was stirred for 18 h. The solution was quenched with NH_4Cl, extracted with EtOAc and the combined organic layers were washed with NaHCO_3 and brine and finally dried over Na_2SO_4 and filtered. Removal of the organics gave a sticky oil which was subjected to flash chromatography (DCM/DCM*MeOH NH_3 1:1:92:8) to give the pure title compound, as white solid (0.017 g, 50% over two steps).

^1H NMR (400 MHz, DMSO-d_6) δ 7.6 – 7.5 (m, 2H), 7.5 – 7.3 (m, 2H), 7.0 (d, J = 2.1 Hz, 1H), 6.8 (d, J = 8.2 Hz, 1H), 6.7 – 6.6 (m, 1H), 6.0 (s, 2H), 4.0 – 3.9 (m, 1H), 3.5 – 3.4 (m, 1H), 3.0 – 2.9 (m, 1H), 2.2 (s, 3H), 2.2 – 2.0 (m, 3H), 1.7 – 1.6 (m, 1H), 1.5 – 1.4 (m, 1H).

^19F NMR (565 MHz, DMSO-d_6) δ -58.7. ^13C NMR (151 MHz, DMSO-d_6) δ 168.2, 147.6, 145.8, 138.2, 137.7 (q, J = 38.2 Hz), 137.6, 137.2, 129.3, 127.6, 124.6, 123.8, 122.0, 121.1 (q, J = 225.1 Hz), 119.4, 119.3, 108.4, 108.1, 101.6, 56.1, 55.9, 38.0, 35.1, 34.7, 28.3, 27.5. UPLC-MS: t_R = 0.65 min (generic method); MS (ESI) m/z calcd for C_{25}H_{24}F_{3}N_{4}O_{3} (M)^+: 485.2, found: 485.3.

(rac)-N-(benzo[d][1,3]dioxol-5-yl)-3-((4R,7S)-9-(((3,5-dimethylisoxazol-4-yl)sulfonyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-4,7-epiminocyclohepta[c]pyrazol-1(4H)-yl)-N-methylbenzamide (90): To a solution of 86 (0.030 g, 0.06 mmol) in DCM (1 mL), TEA (0.014 mL, 0.10 mmol) was added and mixture cooled to 0°C (ice-bath). 3,5-dimethylisoxazole-4-sulfonyl chloride (0.012 g, 0.06 mmol) was added and mixture stirred
at room temperature for 4 h. Sat. aq. NH₄Cl (5 mL) was added and aqueous layer was extracted with EtOAc (3x15 mL). Collected organic layers were washed with water (20 mL) and brine (20 mL) dried with Na₂SO₄, filtered and solvent evaporated. The title compound was obtained, after purification silica gel flash-column chromatography with Cyclohexane/EtOAc (40/60) as the eluent, as a white solid (0.014 g, 37%): ¹H NMR (400 MHz, DMSO-d₆) δ 7.69 – 7.16 (m, 4H), 6.97 (d, J = 2.0 Hz, 1H), 6.77 (d, J = 8.2 Hz, 1H), 6.65 (d, J = 7.6 Hz, 1H), 5.99 (app-d, J = 1.5 Hz, 1H), 5.02 (d, J = 5.4 Hz, 1H), 4.66 – 4.36 (m, 1H), 3.32 (s, 3H), 3.00 (dd, J = 16.5, 4.1 Hz, 1H), 2.67 – 2.53 (m, 4H), 2.27 (s, 3H), 2.25 – 2.15 (m, 1H), 2.09 (tt, J = 11.1, 6.0 Hz, 1H), 1.96 – 1.86 (m, 1H), 1.76 (dt, J = 14.7, 8.0 Hz, 1H); ¹⁹F NMR (565 MHz, CDCl₃) δ -60.1; ¹³C NMR (151 MHz, CDCl₃) δ 173.6, 168.9, 157.9, 148.4, 146.7, 138.5, 138.1, 137.5, 137.3 (q, J = 38.2 Hz), 136.8, 129.3, 128.7, 124.6, 123.1, 121.6 (q, J = 225.1 Hz), 120.8, 120.5, 116.3, 108.6, 108.0, 101.9, 54.3, 54.0, 39.0, 36.8, 33.0, 29.4, 12.8, 10.9; UPLC-MS: tᵣ = 5.24 min; MS (ESI) m/z calcd for C₂₉H₂₇F₃N₅O₆S (M+H)+: 630.2, found: 630.3. HRMS-ESI (m/z): (M+H)+ calcd for C₂₉H₂₇F₃N₅O₆S: 630.1634; found: 630.1632.

(rac)-tert-Butyl-5-(3-ethoxycarbonylphenyl)-3-(trifluoromethyl)-4,5,11triazatricyclo[6.2.1.0²,6]undeca-2(6),3-diene-11-carboxylate (91): In a round-bottom flask, at 0°C under N₂, 83 (0.469 g, 1.0 mmol) was suspended in DMF (2 mL). The resulting solution was treated with K₂CO₃ (0.220 g, 1.6 mmol) and then stirred for 20 min at the same temperature. Iodoethane (0.249 g, 1.6 mmol) was added and the temperature was raised up to room temperature. After 2 h, the reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with Et₂O and the combined organic layers dried over Na₂SO₄. Removal of the organics gave a sticky oil (0.442 g), which was not subjected to any purification and directly used in the next step of reaction. ¹H NMR (400 MHz, DMSO-d₆) δ 8.10 (t, J = 1.9 Hz, 1H), 8.04 (dt, J = 7.8, 1.3 Hz, 1H), 7.97 – 7.87 (m, 1H), 7.71 (t, J = 7.9 Hz, 1H), 4.99 (app-s, 1H), 4.49 (t, J = 6.1 Hz, 1H), 4.36 (q, J = 7.1 Hz, 2H), 3.42-3.33 (m, 1H), 2.73 (dd, J = 16.3, 1.3 Hz, 1H), 2.27 – 2.03 (m, 2H), 1.90-1.78 (m, 1H), 1.76 – 1.63 (m, 1H), 1.44-1.23 (m, 12H). UPLC-MS: tᵣ = 2.12 min (Apolar method); MS (ESI) m/z calcd for C₂₃H₂⁷F₃N₃O₄ (M+H)+: 466.2, found: 466.5.
(rac)-Ethyl-3-[3-(trifluoromethyl)-4,5,11-triazatricyclo[6.2.1.02,6]undeca-2(6),3-dien-5-yl]benzoate;hydrochloride (92): Compound 91 (0.442 g, 0.95 mmol) was dissolved in a solution of HCl 3.0M in MeOH (3.17 mL, 9.5 mmol) at room temperature. The solution was stirred for 1 h. The solvent was removed under vacuum and compound was precipitated with Et₂O as chlorohydrate salt, to give the compound, as a white solid (0.329 g, 86% over two steps). The presence of 13% of the methyl ester compound deriving from the transesterification reaction between compound 65 and MeOH in the presence of HCl was observed. ¹H NMR (400 MHz, DMSO-d₆) δ 9.82 (bs, 2H), 8.15 – 8.04 (m, 2H), 7.96 – 7.89 (m, 1H), 7.76 (t, J = 7.9 Hz, 1H), 5.03 (app-d, J = 4.9 Hz, 1H), 4.47-4.33 (m, 3H), 3.52 (dd, J = 16.8, 4.7 Hz, 1H), 3.11 – 3.01 (m, 1H), 2.34 – 2.18 (m, 2H), 2.08 (t, J = 10.1 Hz, 1H), 2.01-1.87 (m, 1H), 1.34 (t, J = 7.1 Hz, 3H). UPLC-MS: tᵣ = 1.88 min (Generic method); MS (ESI) m/z calcd for C₁₈H₁₈F₃N₃O₂ (M+H)⁺: 366.1, found: 366.1.

(rac)-Ethyl-3-[11-(3,5-dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11 triazatricyclo[6.2.1.02,6]undeca-2(6),3-dien-5-yl]benzoate (93):

In a round-bottom flask, at 0°C under N₂, compound 92 (0.329 g, 0.82 mmol) was dissolved in DCM (4.5 ml). The resulting solution was treated with Et₃N (0.228 mL, 1.6 mmol) and then 3,5-dimethylisoxazole-4-sulfonyl chloride (88) (0.176 g, 0.9 mmol) was added and the temperature was raised up to room temperature. After 18 h, the reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with DCM and the combined organic layers dried over Na₂SO₄. Removal of the organics gave a sticky oil (0.427 g), which was not subjected to any purification and directly used in the next step of reaction. The presence of 13% of the methyl ester compound deriving from the transesterification reaction between compound 65 and MeOH in the presence of HCl was observed. ¹H NMR (400 MHz, DMSO-d₆) δ 8.05 (dd, J = 7.3, 1.4 Hz, 2H), 7.91 – 7.85 (m, 1H), 7.76 – 7.68 (m, 1H), 5.07 (d, J = 5.5 Hz, 1H), 4.62 – 4.56 (m, 1H), 4.37 (q, J = 7.1 Hz, 2H), 3.23 (dd, J = 16.6, 4.7 Hz, 1H), 2.89 – 2.80 (m, 1H), 2.60 (s, 3H), 2.28 (s, 3H), 2.24 – 2.15 (m, 1H), 2.16-2.04 (m, 1H), 1.97-1.88 (m, 1H), 1.8-1.75 (m, 1H), 1.35 (t, J = 7.1 Hz, 3H). UPLC-MS: tᵣ = 1.75 min (Generic method); MS (ESI) m/z calcd for C₂₃H₂₄F₃N₃O₅S: (M+H)⁺:525.1; found: 524.9.
(rac)-3-[11-(3,5-Dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11-triazatricyclo[6.2.1.0²,6]undeca-2(6),3-dien-5-yl]benzoic acid (94):

In a round-bottom flask, compound 93 (0.427 g, 0.81 mmol) was dissolved in THF/H$_2$O 1:1 (5.4 mL). To this resulting solution LiOH (0.039 g, 1.63 mmol) was added and the mixture was stirred at room temperature. After 4 h, the reaction was acidified till pH 3 with HCl 1.0 M and then the aqueous phase was extracted with EtOAc. The combined organic layers were washed with brine and dried over Na$_2$SO$_4$. Removal of the organics gave a sticky oil (0.401 g), which was not subjected to any purification and directly used in the next step of reaction. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.38 (bs, 1H), 8.09 – 7.99 (m, 2H), 7.87–7.83 (m, 1H), 7.69 (t, J = 7.9 Hz, 1H), 5.07 (d, J = 5.4 Hz, 1H), 4.64 – 4.55 (m, 1H), 3.24 (dd, J = 16.5, 4.7 Hz, 2H), 2.87 (dd, J = 16.6, 1.3 Hz, 1H), 2.60 (s, 3H), 2.27 (s, 3H), 2.23 – 2.05 (m, 2H), 1.97-1.89 (m, 1H), 1.86 – 1.74 (m, 1H). UPLC-MS: $t_R$ = 1.84 min (Generic method); MS (ESI) m/z calcd for C$_{21}$H$_{18}$F$_3$N$_4$O$_5$: (M-H) $^-$:495.1.; found: 495.2.

(rac)-3-[11-(3,5-Dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11-triazatricyclo[6.2.1.0²,6]undeca-2(6),3-dien-5-yl]-N-(2-methyl-1,3-benzoxazol-6-yl)benzamide (96a):

In a round-bottom flask, at 0°C under N$_2$, compound 94 (0.100 g, 0.20 mmol) was dissolved in DMF (1.7 mL). The resulting solution was treated with DIPEA (0.084 ml, 0.48 mmol) and HATU (0.084 g, 0.22 mmol) and then stirred for 20 min at the same temperature. 95a (0.033 g, 0.22 mmol) was added and the temperature was raised up to room temperature. After 1 h, the reaction was poured into a NH$_4$Cl saturated solution, the aqueous phase was extracted with Et$_2$O and the combined organic layers were washed with brine and dried over Na$_2$SO$_4$. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (cyclohexane/AcOEt 50:50) to give the pure title compound, as a yellow solid (0.110 g, 59% over three steps).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 10.60 (bs, 1H), 8.23 (t, J = 1.2 Hz, 1H), 8.17 – 8.04 (m, 2H), 7.82 (ddd, J = 8.1, 2.2, 1.1 Hz, 1H), 7.74 (t, J = 7.9 Hz, 1H), 7.68 – 7.59 (m, 2H), 5.75
(rac)-3-[[11-(3,5-Dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11-triazatricyclo[6.2.1.0^2,6]undeca-2(6),3-dien-5-yl]-N-methyl-N-(2-methyl-1,3-benzoazol-6-yl)benzamide (97a):

In a round-bottom flask, at 0°C under N_2, compound 96a (0.110 g, 0.17 mmol) was dissolved in DMF (1.7 mL). The resulting solution was treated with Cs_2CO_3 (0.102 mg, 0.31 mmol) and CH_3I (0.013 mL, 0.2 mmol) the temperature was raised up to room temperature. After 18 h, the reaction was poured into a NH_4Cl saturated solution, the aqueous phase was extracted with Et_2O and the combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (cyclohexane/AcOEt 20:80) to give the pure title compound, as a white solid (0.050 g, 46%).

^1^H NMR (400 MHz, DMSO-d_6) δ 7.69 (d, J = 1.9 Hz, 1H), 7.56 – 7.45 (m, 2H), 7.47 – 7.36 (m, 3H), 7.19 (dd, J = 8.4, 2.0 Hz, 1H), 4.99 (d, J = 5.5 Hz, 1H), 4.42 – 4.34 (m, 1H), 3.44 (s, 3H), 2.85 – 2.75 (m, 1H), 2.58 (s, 3H), 2.57 (s, 3H), 2.43 – 2.29 (m, 1H), 2.26 (s, 3H), 2.25 – 2.00 (m, 2H), 1.93 – 1.83 (m, 1H), 1.74 – 1.62 (m, 1H).

^13^C NMR (151 MHz, DMSO) δ 173.5, 167.2, 165.2, 157.2, 150.2, 141.0, 139.6, 138.0, 137.6, 137.3, 135.4 (q, J = 37.4 Hz), 129.4, 128.2, 124.1, 124.1, 122.5, 121.4 (q, J = 268.8 Hz), 120.0, 119.0, 115.6, 110.0, 53.7, 53.3, 38.3, 35.9, 31.6, 28.5, 14.1, 12.4, 10.4. ^19^F NMR (565 MHz, DMSO) δ -59.9. UPLC-MS: t_R = 5.01 min; MS (ESI) m/z calcd for C_{30}H_{28}F_{3}N_{6}O_{5}S: (M+H)^+ : 641.2.; found: 641.2. HRMS-ESI (m/z): (M+H)^+ calcd for C_{30}H_{28}F_{3}N_{6}O_{5}S: 641,1794; found: 641.1798.
(rac)-3-[11-(3,5-Dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11-
triazatricyclo[6.2.1.02,6]undeca-2(6),3-dien-5-yl]-N-(2-methoxypyrimidin-5-
yl)benzamide (96b):

In a round-bottom flask, at 0°C under N₂, compound 94 (0.074 g, 0.15 mmol) was dissolved in DMF (1.1 mL). The resulting solution was treated with DIPEA (0.057 ml, 0.33 mmol) and HATU (0.065 g, 0.17 mmol) and then stirred for 20 min at the same temperature. 95b (0.021 g, 0.17 mmol) was added and the temperature was raised up to room temperature. After 1 h, the reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with Et₂O and the combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (cyclohexane/AcOEt 30:70) to give the pure title compound, as a yellow solid (0.068 g, 38% over three steps).

¹H NMR (400 MHz, DMSO-d₆) δ 10.63 (bs, 1H), 8.92 (s, 2H), 8.13 (t, J = 1.9 Hz, 1H), 8.09 (dt, J = 7.8, 1.4 Hz, 1H), 7.86-7.83 (m, 1H), 7.75 (t, J = 7.9 Hz, 1H), 5.07 (d, J = 5.4 Hz, 1H), 4.61-4.58 (m, 1H), 3.93 (s, 3H), 3.29-3.26 (m, 1H), 2.89 (d, J = 16.4 Hz, 1H), 2.61 (s, 3H), 2.29 (s, 3H), 2.28 – 2.15 (m, 1H), 2.14-2.02 (m, 1H), 1.99 – 1.88 (m, 1H), 1.86 – 1.73 (m, 1H). UPLC-MS: tᵣ = 1.19 min (apolar method); MS (ESI) m/z calcd for C₂₆H₂₅F₃N₇O₅S: (M+H)⁺:604.1.; found:604.1.

(rac)-3-[11-(3,5-Dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11-
triazatricyclo[6.2.1.02,6]undeca-2(6),3-dien-5-yl]-N-(2-methoxypyrimidin-5-yl)-N-
methyl-benzamide (97b):

In a round-bottom flask, at 0°C under N₂, compound 96b (0.068 g, 0.11 mmol) was dissolved in DMF (1.1 mL). The resulting solution was treated with Cs₂CO₃ (0.071 mg, 0.21 mmol) and CH₃I (0.011 mL, 0.17 mmol) the temperature was raised up to room temperature. After 18 h, the reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with Et₂O and the combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (cyclohexane/AcOEt 30:70) to give the pure title compound, as a white solid (0.041 g, 60%).
\(^1\)H NMR (400 MHz, DMSO-d6) δ 8.55 (s, 2H), 7.78 – 7.18 (m, 4H), 5.03 (d, J = 5.6 Hz, 1H), 4.52 (app-s, 1H), 3.85 (s, 3H), 3.38 (s, 3H), 3.10 – 2.94 (m, 1H), 2.60 (s, 3H), 2.27 (s, 3H), 2.24 – 2.15 (m, 1H), 2.14 – 2.02 (m, 1H), 1.94 – 1.87 (m, 1H), 1.80 – 1.72 (m, 1H). \(^1\)C NMR (151 MHz, DMSO-d6) δ 173.5, 168.3, 162.7, 158.0, 157.2, 138.1, 137.7, 136.9, 135.5 (q, J = 37.1 Hz), 133.9, 129.7, 128.2, 124.2, 121.5 (q, J = 268.8 Hz), 122.5, 120.2, 115.6, 55.0, 53.8, 53.4, 40.1, 39.9, 37.6, 35.9, 31.9, 28.5, 12.4, 10.4. \(^{19}\)F NMR (565 MHz, DMSO) δ -59.8. UPLC-MS: \(t_R = 4.78\) min; MS (ESI) m/z calcd for C\(_{27}\)H\(_{27}\)F\(_3\)N\(_7\)O\(_5\)S: (M+H)\(^+\):618.2; found: 618.2. HRMS-ESI (m/z): (M+H)\(^+\) calcd for C\(_{30}\)H\(_{27}\)F\(_3\)N\(_6\)O\(_5\)S: 618.1746; found: 618.1743.

**\(\textit{rac}\)-3-[11-(3,5-Dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11-triazatricyclo[6.2.1.0\(_2\),6]undeca-2(6),3-dien-5-yl]-N-(2-methylpyrazolo[1,5-al]pyrimidin-6-yl)benzamide (96c):**

In a round-bottom flask, at 0°C under N\(_2\), compound 94 (0.074 g, 0.15 mmol) was dissolved in DMF (1.1 mL). The resulting solution was treated with DIPEA (0.057 mL, 0.33 mmol) and HATU (0.064 g, 0.17 mmol) and then stirred for 20 min at the same temperature. 95c (0.024 g, 0.17 mmol) was added and the temperature was raised up to room temperature. After 1 h, the reaction was poured into a NH\(_4\)Cl saturated solution, the aqueous phase was extracted with Et\(_2\)O and the combined organic layers were washed with brine and dried over Na\(_2\)SO\(_4\). The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (cyclohexane/AcOEt 30:70) to give the pure title compound, as a yellow solid (0.060 g, 31% over three steps).

\(^1\)H NMR (400 MHz, DMSO-d6) δ 10.76 (bs, 1H), 9.46 (dd, J = 2.3, 0.9 Hz, 1H), 8.71 (d, J = 2.4 Hz, 1H), 8.16 (t, J = 2.0 Hz, 1H), 8.11 (dt, J = 7.8, 1.3 Hz, 1H), 7.90 – 7.81 (m, 1H), 7.77 (t, J = 7.9 Hz, 1H), 6.53 (app-s, 1H), 5.08 (app-d, J = 5.5 Hz, 1H), 4.64 – 4.54 (m, 1H), 3.31 – 3.24 (m, 1H), 2.91 (d, J = 16.3 Hz, 1H), 2.61 (s, 3H), 2.43 (s, 3H), 2.29 (s, 3H), 2.28 – 2.16 (m, 1H), 2.17 – 2.05 (m, 1H), 1.99 – 1.88 (m, 2H), 1.86 – 1.74 (m, 1H), 1.17 (t, J = 7.1 Hz, 1H). UPLC-MS: \(t_R = 1.26\) min (apolar method); MS (ESI) m/z calcd for C\(_{28}\)H\(_{26}\)F\(_3\)N\(_7\)O\(_4\)S: (M+H)\(^+\): 627.2; found: 627.2.
(rac)-3-[11-(3,5-Dimethylisoxazol-4-yl)sulfonyl-3-(trifluoromethyl)-4,5,11-triazatricyclo[6.2.1.02,6]undeca-2(6),3-dien-5-yl]-N-methyl-N-(2-methylpyrazolo[1,5-al]pyrimidin-6-yl)benzamide (97c):

In a round-bottom flask, at 0°C under N₂, compound 96c (0.060g, 0.09 mmol) was dissolved in DMF (0.9 mL). The resulting solution was treated with Cs₂CO₃ (0.053 mg, 0.16 mmol) and CH₃I (0.008 mL, 0.13 mmol) the temperature was raised up to room temperature. After 18 h, the reaction was poured into a NH₄Cl saturated solution, the aqueous phase was extracted with Et₂O and the combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was removed under vacuum and the resulting oil was subjected to flash chromatography (cyclohexane/AcOEt 70:30) to give the pure title compound, as a white solid (0.026 g, 45%).

¹H NMR (400 MHz, DMSO-d₆) δ 9.21 (d, J = 2.3 Hz, 1H), 8.42 (bs, 1H), 7.64 – 7.40 (m, 4H), 6.49 (s, 1H), 5.00 (d, J = 5.4 Hz, 1H), 4.36 (app-bs, 1H), 3.44 (s, 3H), 2.94 – 2.75 (m, 1H), 2.59 (s, 3H), 2.38 (s, 3H), 2.26 (s, 3H), 2.23 – 2.00 (m, 3H), 1.93 – 1.83 (m, 1H), 1.73 – 1.58 (m, 1H).

¹³C NMR (151 MHz, DMSO-d₆) δ 173.5, 168.5, 157.2, 155.6, 149.8, 146.8, 138.0, 137.5, 137.0, 135.5 (q, J = 37.1 Hz), 133.8, 129.8, 128.2, 126.9, 124.4, 124.1, 122.2 (d, J = 22.7 Hz), 120.5, 120.1, 118.8, 115.6, 95.8, 53.9, 53.3, 37.8, 35.9, 31.6, 29.0, 28.5, 14.2, 12.4, 10.4. ¹⁹F NMR (565 MHz, DMSO-d₆) δ -58.9. UPLC-MS: tᵣ = 4.88 min; MS (ESI) m/z calcd for C₂₉H₂₈F₃N₈O₄S: (M+H)⁺:641.2.; found:641.2 . HRMS-ESI (m/z): (M+H)⁺ calcd for C₂₉H₂₈F₃N₈O₄S: 641,1906; found: 641.1896.
2.5 Bibliography


3. Results and Discussion

3.1 High-Throughput Screening (HTS) of IIT compound collection

ARN9364 and ARN5562 were identified as hits in a high-throughput screening (HTS) of the IIT compound collection consisting of 11,334 commercial small molecules. The screening was conducted at the Istituto Giannina Gaslini (IGG, Genoa), using a phenotypic cell assay developed to detect and measure the CFTR chloride channel function.

The primary screening assay was carried out using two cell lines: Fischer Rat Thyroid (FRT) epithelial cells and human bronchial CFBE41o- cells, co-expressing F508del-CFTR and the halide-sensitive yellow fluorescent protein (HS-YFP) mutant H148Q/I152L, in a 96-well microplate format (Figure 3.1). For this purpose, YFP mutants were identified whose fluorescence is strongly quenched by iodide, a halide that is efficiently transported by CFTR.

The FRT cell line was used for the first time in 1990’s to study CFTR function and later on these cells were found useful for the identification of CFTR modulators. FRT cells are of epithelial origin, have low levels of anion permeability, rapid growth and strong adherence to the cell culture supports, which make them resistant to all procedures required by the screening. Moreover, since significant levels of the mutant protein can reach the plasma membrane (probably as a result of more relaxed cell quality control), FRT cells have been reported to allow easier detection of correctors, as compared to other cell models, such as CFBE41o-.

On the contrary, CFBE41o- cells display a more stringent cell quality control system compared to FRT cells, showing a tighter internal biological quality control.

The primary aim of the HTS campaign was the discovery of small molecule correctors. Compounds of the IIT library were tested in duplicate at 10 µM in FRT cells, and at 5 µM in CFBE41o- cells, following a previously reported protocol. FRT cells were incubated for 24 h at 37°C with compounds, washed and then stimulated acutely with a cocktail of a cAMP agonist plus genistein, as a potentiator. CFTR activity in the cell membrane was calculated from the rate of YFP fluorescence quenching caused by extracellular addition and therefore influx of iodide (Figure 3.1).
The screening identified 117 initial hits, i.e., compounds potentially able to significantly correct the F508del-CFTR trafficking defect in FRT cells.

A second HTS for F508del correctors was carried out on CFBE41o- cells, a model closer to the native tissue from CF patients. The screening was run in duplicate using two separate cell preparations, and the compounds were tested at 5 μM, following similar screening conditions and data analysis to those described for FRT cells. As expected, less hits (11 in total) were found in CFBE41o- cells compared to the HTS performed on FRT cells (Figure 3.2).
All corrector hits identified in the two primary screenings were re-tested at different concentrations in order to confirm activity and obtain information on potency and efficacy. When tested on FRT cells, a large fraction of initial hits was confirmed. Interestingly, some compounds had rescue activity comparable to or even higher than that of VX-809, with a clear dose-dependence in the range of concentrations between 0.63 and 20 μM (data not shown). All identified hits were also re-tested in CFBE41o- cells. In agreement with the results of the primary screenings, most compounds active in FRT cells were inactive in the bronchial cell line (in the range of concentrations between 0.47 and 15 μM). Interestingly, few hits showed a confirmed activity as correctors in both cell types (ARN9364 and ARN5562 were among them). This was a promising finding since compounds with activity in two different cell types are more likely to be effective as correctors also in secondary screening assays (e.g., primary bronchial epithelial cells from CF patients).^9

### 3.2 Biological results of ARN9364 and its analogs

Among the chemically different compounds identified initially as primary hits, due to its initial promising biological activity ARN9364 (CAS: 1794126-00-3, Figure 3.3) was selected as a starting point for this PhD project.

In Figure 3.3, the dose-response data in both FRT and CFBE41o- cells of ARN9364 are reported. Biological data obtained during the HTS campaign and post-hit identification were in agreement, showing for ARN9364 a promising initial activity profile in the micromolar range at the tested concentrations (0.625 μM - 20 μM), reasonably suitable to be considered as a starting point for a SAR study.
CHAPTER 3

**HTS data**

Figure 3.3: Biological activity of ARN9364 after HTS campaign (top) and post-hit identification (bottom) [Compound’s batches were tested in triplicate in independent experiments].

The hit compound was first re-synthesized (compound 26, Figure 3.4) to confirm its chemical structure. After UPLC/MS and NMR analyses, we argued that the reported structure of purchased ARN9364 was not consistent with experimental data. This hypothesis was further confirmed by biological data obtained in the YFP assay on F508del-CFTR FRT and CFBE41o- cells (Chapter 2, paragraph 2.1). The re-synthesized compound 26 turned out to be inactive, while the screening hit confirmed its activity (Figure 3.4).

Supported by an accurate NMR analysis a new structure was assigned to the purchased compound, as depicted in Figure 3.5. To confirm our hypothesis, the new compound (38) was synthetized and tested showing a complete overlap with the library sample, tested initially in the HTS (Figure 3.4).

**Re-tested hit data**

Figure 3.4: Biological data of synthetized compounds 26 and 38 vs. library sample of ARN9364 [A = DMSO; compounds were tested in triplicate in independent experiments].
At this point, in order to elucidate the Structure–Activity Relationship (SAR) patterns around this novel chemo-type, new derivatives were designed, synthesized (see Chapter 2, paragraph 2.2) and tested, in particular focusing our attention on the pyrrolidine, pyrazole and amino-pyridine moieties. All the synthesized analogs were characterized by UPLC/MS and NMR analysis and subjected to *in vitro* biological assays to assess their activity in rescuing mutated F508del-CFTR. The biological assays were performed at the Istituto Giannina Gaslini (IGG) using the previously described phenotypic cell-based assay on CFBE41o- cells, co-expressing F508del-CFTR and the halide-sensitive yellow fluorescent protein. The synthesized racemic compounds were tested in triplicate in the concentrations range 20 µM – 1.25 µM.

As the first step of the SAR evolution, we started to explore the impact of different *N*-substitutions on the pyrrolidine moiety (R₁) and the effect of *N*-methylation on the pyrazole (R₂) on the biological activity (Figure 3.6).

Starting from the preliminary activity in the micromolar range of the initial hit 38, the replacement of the *N*-propyl chain on the pyrrolidine ring with other alkyl groups with different lengths and ramifications (compounds 39a-f) resulted in low or marginal increase in activity at the tested concentrations. In fact, while analogs 39a (R₁ = Me), 39b (R₁ = i-Pr) and 39d (R₁ = n-Bu) showed no improvement in activity at any tested concentration, the insertion of a branched substituent, such as the iso-butyyl group, in 39c, led to a marginal, but clear increase in potency also at the lower concentrations, in particular at 1.25 µM - 2.5 µM.
Figure 3.6: Structures and biological activity of racemic compounds 26, 37, 38, 39a-f, 40-41 and 64 [A = DMSO; all compounds were tested in triplicate in independent experiments].
Unfortunately, constraining the two terminal methyl groups of 39c into a cyclic moiety, as for methylene-cyclopropyl substituted analog 39e, resulted in loss of activity at the lowest concentrations. On the contrary, the insertion of a benzylic residue on the pyrrolidine nitrogen, as in 39f, increased the activity at 20µM, but did not give any effect at low concentrations when compared to initial hit 38.

Interestingly, the corresponding un-substituted derivative 37 showed a promising increase in potency at the lowest concentrations. Based on the limited exploration of this portion of the molecule, the data obtained for the unsubstituted pyrrolidine analog 37 could be further evaluated in order to possibly highlight the effect of this hydrogen-bonding donor group, in terms of new interactions with the biological target.

The replacement of the n-propyl group on the pyrrolidine nitrogen with a carboxyethyl moiety, as for amide 40, resulted in a loss of activity. This outcome was further confirmed by the complete lack of rescuing effect in CFBE410- cells elicited by compound 26, whose structure was erroneously attributed to the initial hit (see Chapter 2). Similarly, the methyl carbamate analog 41 was devoid of any activity in the tested cell line.

Disappointedly, the insertion of a methyl group on the pyrazole in N1-position (compound 64) led to a drop in activity at the highest concentrations, compared with the initial hit 38.

Based on these preliminary data and considering the marginal increase in activity of these first derivatives, we decided to explore the amino-pyridine side of the molecule. Few analogs bearing structurally and electronically different acyl groups were synthesized trying to improve the activity and further elucidate the SAR. Biological results for racemic compounds 59a-d, 60 and 78 are reported in Figure 3.7.
At first, trying to evaluate the effect of an acyclic acyl group on the amino moiety of the pyridine, the cyclobutane ring was replaced with an iso-propyl residue (59a). This modification resulted in a similar activity profile, with a promising rescuing effect of F5080del-CFTR activity at 2.5 μM.

While the modification with a cyclopropyl moiety (59b) gave a slight improvement in activity at lowest concentrations (2.5 μM - 1.25 μM), the insertion of a cyclohexyl ring (59d) did not contribute to any positive effect on activity at any tested concentration, when compared to hit 38. The replacement with the less flexible phenyl ring, as in compound 59c,
RESULTS AND DISCUSSION

resulted in a loss of activity at low concentrations, but interestingly an evident increase in potency was displayed at the highest tested concentration (20 \( \mu \)M).

A further modification by insertion of a benzylic moiety on the amino pyridine nitrogen of the hit compound 38, as for analog 60, led to a completely loss of activity at all tested concentrations.

A careful analysis of ARN9364 close analogs present in the IIT compound collection revealed that another derivative showed a minimal activity on F508del-CFTR FRT cells in the HTS. Assuming this preliminary biological effect worth of further evaluation, the compound was then resynthesized and confirmed for its structure (Chapter 2, paragraph 2.2). Unfortunately, analog 78, bearing an acyl group on the pyrrolidine moiety and a para-fluoro benzyl group on the amino-pyridine portion, did not confirmed its activity as seen in the initial HTS and turned out to be inactive across the whole concentrations range.

Along with the biological data, preliminary studies were conducted on few analogs to evaluate their resistance to oxidative metabolism. In vitro liver microsomal stability tests were carried out on five different species (mouse, rat, dog, monkey and human) mainly detecting Phase I metabolic modifications (Table 3.1). Hit compound 38 showed high stability in 4 out of 5 tested species. In particular, the compound displayed a half-life (\( t_{1/2} \)) values higher than 60 minutes in dog, monkey and human liver microsomes (with good/high percentage of remaining compound at 60 minutes).

### Table 3.1: Liver microsomal stabilities of synthesized compounds 38, 39b and 41.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Mouse LM_NADPH ( t_{1/2} ) (min)(^{a,b} )</th>
<th>Rat LM_NADPH ( t_{1/2} ) (min)(^{a,b} )</th>
<th>Dog LM_NADPH ( t_{1/2} ) (min)(^{a,b} )</th>
<th>Monkey LM_NADPH ( t_{1/2} ) (min)(^{a,b} )</th>
<th>Human LM_NADPH ( t_{1/2} ) (min)(^{a,b} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>13±3</td>
<td>49±5</td>
<td>&gt;60 (64%)(^{c} )</td>
<td>&gt;60 (51%)(^{c} )</td>
<td>&gt;60 (90%)(^{c} )</td>
</tr>
<tr>
<td>39b</td>
<td>17±6</td>
<td>&gt;60 (58%)(^{c} )</td>
<td>&gt;60 (62%)(^{c} )</td>
<td>42±5</td>
<td>&gt;60 (86%)(^{c} )</td>
</tr>
<tr>
<td>41</td>
<td>unst.(^{d} )</td>
<td>47±9</td>
<td>&gt;60 (79%)(^{c} )</td>
<td>34±6</td>
<td>&gt;60 (82%)(^{c} )</td>
</tr>
</tbody>
</table>

\(^{a}\): 4.6 \( \mu \)M in liver microsomes (LMs) with NADPH, as co-factors, 0.1%DMSO; \(^{b}\): data collected as \( n \geq 3 \); \(^{c}\): % compound remaining at 60 min; \(^{d}\): compound unstable in LMs without co-factors.
The modification of the \textit{N}-substitution from an \textit{n}-propyl-chain to the \textit{iso}-propyl group (compound 39b) resulted in an overall profile similar to 38, with a slight increase in the microsomal stability in rat, and a modest decrease in $t_{1/2}$ in monkey.

The introduction of a carbamate moiety on the pyrrolidine ring led to a compound 41 with a comparable profile to 38 in all the tested species, except for mouse LMs where the analog surprisingly turned out to be quite unstable.

Finally, the kinetic solubility in buffer (PBS) at pH 7.4 of 38 was also determined: the compound showed good solubility, i.e. $> 250 \mu$M.

### 3.3 Biological results of ARN5562 and its analogs

A second part of this project, aimed at the discovery of novel potent CFTR correctors, was focused on the design and synthesis of close analogs of a compound deriving from another hit identified in the same HTS. In this case, the initial hit ARN5562 (Figure 3.8) showed a chemically distinct structure from the previously described ARN9364, and an initial promising biological activity (Figure 3.8). Compound ARN5562, with its encouraging efficacy and potency, represented an important starting point for the search of novel correctors.

![Structure and initial activity data of ARN5562](image)

Figure 3.8: Structure and initial activity data of ARN5562 [compound’s batches were tested in triplicate in independent experiments].
All newly synthetized analogs were characterized and subjected to in vitro biological assays to assess their activity in rescuing mutated CFTR. The biological assays were performed at the Istituto Giannina Gaslini (IGG, Genoa) using the phenotypic cell based assay described before, on CFBE41o- cells, co-expressing F508del-CFTR and the halide-sensitive yellow fluorescent protein. The synthetized compounds were tested in triplicate in the concentration range 20 μM – 82 nM.

Rounds of chemical modifications, exploring primarily both the heterocyclic moiety on the right-end side and the substitutions on the pyrazole ring of ARN5562, provided the information to build Structure-Activity Relationships (Figure 3.9) and to identify two distinct structural classes of correctors.

![Figure 3.9: General structure and points of modification to expand SARs of ARN5562.](image)

A preliminary investigation of the SARs around this novel chemical class by replacement of the 1,4-dioxane ring with an unsubstituted 5-membered dioxole group, and cyclization between positions R\textsuperscript{2} and R\textsuperscript{3} of the pyrazole into a tetrahydro-pyridine moiety allowed to identify compound ARN21750 (Table 3.2). This new compound showed a better biological profile than ARN5562, with a considerable increase in both efficacy (\(E_{\text{max}}\): 2.8, where \(E_{\text{max}}\) is the maximum response achievable from an applied or dosed compound) and potency (\(EC_{50}\): 0.18 μM, where \(EC_{50}\) represents the concentration of a compound where 50% of its maximal effect is observed) in F508del-CFTR CFBE41o- cells (Table 3.2).
Table 3.2: Structure and biological activity ($E_{\text{max}}$ and $EC_{50}$) of ARN21750 in F508del-CFTR CFBE41o- cells. [compounds were tested in triplicate in independent experiments].

<table>
<thead>
<tr>
<th>Entry</th>
<th>$E_{\text{max}}$</th>
<th>$EC_{50}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARN5562</td>
<td>1.4</td>
<td>1.45</td>
</tr>
<tr>
<td>ARN21750</td>
<td>2.8</td>
<td>0.18</td>
</tr>
</tbody>
</table>

As a subsequent step, starting from the structure of ARN21750, aiming to further modify the tetrahydro-pyridine ring of the pyrazolopyridine moiety, new compounds were designed and tested (data not shown). These efforts led eventually to the discovery of compound 90, featuring an ethylene bridge to form a bridged aza-tricyclic scaffold with the substituted pyrazole ring (Figure 3.10).

The racemic tertiary amine 90 turned out to be quite active in rescuing F508del-CFTR in CFBE41o- cell line, showing a good efficacy and a potency circa 1,000-fold higher than the initial hit ARN5562 (Figure 3.12).

![Figure 3.10: Structure of racemic bridged aza-tricyclic tertiary amine 90.](image)

With these data in hand, further SAR studies on the functionalization of the tetrahydro-pyridyl ring of the pyrazolo[4,3-c]pyridine moiety allowed identifying different biologically active analogs (data not reported). Among them, the compound featuring a bridged aza-tricyclic group carrying a 3,5-dimethylisoxazole sulfonamide, compound 91 (Figure 3.11), showed a remarkable increase in biological activity in CFBE41o- cells ($E_{\text{max}}$: 1.89, $EC_{50}$: 0.081 $\mu$M), compared to previously identified analogs (Figure 3.12).
Notably, while the tertiary amine derivative 90 showed a comparable activity profile to the approved drug VX-809 (Lumacaftor®), the introduction of a sulphonamide residue, as in 91, led to an increase in potency, with only marginal decrease in efficacy at the highest concentration (Figure 3.12). Indeed, the maximal efficacy of compound 91 remained high in the concentration range 8 μM - 82 nM.

Given the high potency displayed by sulfonamide 91, in order to investigate other modifications and to possibly modulate drug-like properties, subsequent SAR studies were focused on exploring the heterocyclic moiety on the right-end side of the molecule, while keeping unmodified the substituted aza-tricyclic sulfonamide moiety (Figure 3.12).
Figure 3.12: Biological data of synthetized compounds 90, 91, 98a-c compared with the initial hit ARN5562 and ARN21750. [A = DMSO; all compounds were tested in triplicate in independent experiments].

The benzodioxole group in compound 91 was therefore substituted with mono- and bicyclic heteroaryl moieties (compounds 98a-c, Figure 3.12).

In general, the introduction of nitrogen-containing heterocycles on the amidic moiety resulted in a decrease in both efficacy and potency. Whereas the introduction of a 2-methylbenzoxazole group (98a) turned out to be fairly accepted in terms of potency at the lowest concentrations, the corresponding 2-methoxypyrimidine tertiary amide (98b) showed a marked drop in both potency and efficacy. Finally, the insertion of a pyrazolo-pyrimidine group (98c) resulted in a comparable activity in the high concentration range (20-3.2 µM).
with respect to VX-809, but led to a loss of rescuing effect at the lowest concentrations (Figure 3.12).

The newly synthesized correctors were further profiled for their *in-vitro* ADME properties. Compounds were tested for their kinetic solubility in buffer (PBS, pH 7.4), metabolic stability upon incubation with rat, dog and human liver microsomes (LMs) (phase I and II metabolism) and potential liver toxicity in the HepG2 cells (Table 3.3).

Initial hit ARN5562 was characterized by low metabolic stability in all tested species in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) co-factor, having half-life values lower than 7 minutes. The compound showed a moderate solubility (81 μM) in phosphate buffered saline (PBS) at pH 7.4 and a sign of cytotoxicity in HepG2 cells.

Table 3.3: Drug likeness profile (solubility, liver microsomal stability, cytotoxicity) of synthesized 90, 91, 98a-c and reference (ARN5562, ARN21750) compounds.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Solubility (µM)</th>
<th>Rat LM_NADPH $t_\text{50}$(min)$^{a,b,c}$</th>
<th>Dog LM_NADPH $t_\text{50}$(min)$^{b,c}$</th>
<th>Human LM_NADPH $t_\text{50}$(min)$^{b,c}$</th>
<th>Human LM_UDPGA $t_\text{50}$(min)$^{b,c}$</th>
<th>HepG2 (% Survival)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARN5562</td>
<td>81±9</td>
<td>&lt;5</td>
<td>7±1</td>
<td>&lt;5</td>
<td>&gt;60 (91%)$^e$</td>
<td>74% (CTG_gala)</td>
</tr>
<tr>
<td>ARN21750</td>
<td>235±16</td>
<td>6±1</td>
<td>7±1</td>
<td>12±3</td>
<td>&gt;60 (93%)$^e$</td>
<td>&gt;80%</td>
</tr>
<tr>
<td>90</td>
<td>242±6</td>
<td>8±2</td>
<td>13±4</td>
<td>16±5</td>
<td>&gt;60 (95%)$^e$</td>
<td>&gt;80%</td>
</tr>
<tr>
<td>91</td>
<td>&lt;1</td>
<td>10±2</td>
<td>11±1</td>
<td>8±3</td>
<td>&gt;60 (98%)$^e$</td>
<td>&gt;80%</td>
</tr>
<tr>
<td>98a</td>
<td>16±6</td>
<td>8±3</td>
<td>11±5</td>
<td>13±1</td>
<td>&gt;60 (97%)$^e$</td>
<td>&gt;80%</td>
</tr>
<tr>
<td>98b</td>
<td>34±7</td>
<td>16±4</td>
<td>10±2</td>
<td>16±4</td>
<td>&gt;60 (96%)$^e$</td>
<td>&gt;80%</td>
</tr>
<tr>
<td>98c</td>
<td>20±6</td>
<td>9±3</td>
<td>8±1</td>
<td>16±2</td>
<td>&gt;60 (98%)$^e$</td>
<td>&gt;80%</td>
</tr>
</tbody>
</table>

$^a$: kinetic solubility (PBS, pH7.4), n=3; $^b$: 4.6 µM in liver microsomes (LM) with NADPH or UDPGA as co-factors, 0.1%DMSO; $^c$: data collected as n≥3; $^d$: Percentage of survival of HepG2 cells at 20 µM determined by CTG and MTT assays. Viability of HepG2 cells is expressed as percent survival of the vehicle-treated controls (given as 100%). Values are from one experiment, performed in three technical replicates; $^e$: % compound remaining at 60min.

Although beneficial in terms of activity in biological assays in CFBE410- cells, the modification of the substitution pattern of the pyrazole ring and the insertion of a benzodioxole moiety on the right-end side of the molecule only marginally affected the ADME parameters. In particular, cyclization between the 4- and 5-position of the pyrazole
into a tetrahydro-pyridine ring, further functionalized with a methyl residue (ARN21750), resulted in only marginal beneficial effects on liver microsomal (LM) stability in the tested species. Not surprisingly, the tertiary amine ARN21750 showed an evident increase in solubility (235 μM) with respect to the initial hit. The insertion of a bridgehead ethyl group in the tetrahydro-pyridine ring to form a triaza-tricyclo-undecadiene moiety, while maintaining the benzodioxole ring on the right-end side of the molecule, as in racemic 90, positively affected the liver microsomal stability both in dog and human LMs (t½ = 13 min and 16 min, respectively). While improving the activity profile, the substitution of the nitrogen in the tetrahydro-pyridine ring with an isoxazole sulphonamide group (91) was detrimental for the metabolic stability (t½ around 10 min in the tested species) and kinetic solubility (<1 μM). The modifications of the right-end side, with the replacement of the benzodioxole ring with other heteroaryl groups (compounds 98a-c), along with an unfavourable effect on activity, gave unfortunately only minimal improvements in stability versus oxidative metabolism in the tested species.

The nitrogen-containing heterocyclic groups in the right-end part of the molecule partially helped in terms of solubility in PBS at physiological pH. Solubility levels were however still low/moderate (16-34 μM).

The possible effects of phase II metabolism in human LMs in the presence of uridine-5\'-diphospho-1-α-D-glucuronic acid (UDPGA), as co-factor, were also examined for this set of compounds (Table 3.3). For all tested analogues, LM stabilities were generally quite high (t½ >60 min, with >90% of starting compound remaining).

In order to rule out any preliminary liver toxicity (hepatotoxicity), the set of selected compounds was tested in an in-vitro assay, using immortalized human hepatocytes cell lines (HepG2) to identify drug-induced mitochondrial toxicity and possibly predict liver injury potential. The compounds were tested, along with a reference compound (rotenone), either in galactose or high glucose supplemented media, at two concentrations (2.0 μM and 20 μM) for 24 hours. Cellular viability was assessed by using the CellTiter-Glo® (CTG) Luminescent Cell Viability Assay and the Thiazolyl Blue Tetrazolium Blue (MTT) dye. A reduction of cell survival to less than 80% was set as the threshold for estimation of cytotoxicity. Except for initial hit ARN5562, which showed a decrease in cell viability in galactose-containing medium in the CTG assay, at the concentration of 20 μM, none of the other compounds induced any cytotoxic effect (cell viability > 80% of control) (Table 3.3).
3.4 Conclusions

With the aim to identify novel small-molecules as CFTR correctors, starting from HTS hit compounds ARN9364 and ARN5562, new derivatives of each chemo-type were synthetized and tested for their biological activity in rescuing F508del-CFTR function.

In the first part of the work, in order to investigate the Structure-Activity Relationships (SARs) around hit ARN9364, we explored the impact of different $\text{N}$-substitutions on the pyrrolidine moiety, the effect of an $\text{N}$-methyl substitution on the pyrazole, and the impact of diverse $\text{N}$-acyl modifications on the amino pyrimidine moiety in rescuing mutated CFTR function.

The biological activity of the newly synthesized racemic ARN9364 analogs was evaluated in F508del-CFTR CFBE41o- cells and resulted only in a low/moderate increase in rescuing effect. In particular, at the highest tested concentrations (20 $\mu$M – 5 $\mu$M) compounds 39f and 59c showed an increase in activity when compared with the initial hit, while only compounds 39c and 37 resulted in a moderate improvement in potency in the lower range of concentrations (2.5 $\mu$M -1.25 $\mu$M).

Considering these preliminary data, in order to possibly elicit a more significant effect in rescuing mutated CFTR function, a broader investigation in the substitutions of the three key-portions of ARN9364 (i.e., pyrrolidine alkylation, pyrazole $\text{N}1$-substitution and/or amino pyridine acylation) should be taken into consideration. Along with this further SAR expansion activity, the most promising correctors, in terms of both rescuing effect and in-vitro ADME profile, could also be tested as pure enantiomers. These combined studies will certainly help to identify few more promising correctors within this new chemotype with a better rescuing effect of mutant CFTR function.

In the second part of the PhD work, a different chemical class of correctors, deriving from hit ARN5562, was evaluated and studied. In order to mainly improve the activity and the drug-like properties of the identified novel analog ARN21750, we decided to study the effect of diverse modifications on the structure of this lead compound. SAR evolution efforts led to identify few analogs featuring a tricyclic triaza-tricyclo-undecadiene scaffold, substituted in its tetrahydropyridine ring with an isoxazole sulphonamide group. From the initially identified potent corrector 91, bearing a benzodioxole ring, the insertion of other
heteroaryls on the right-end side of the molecule unfortunately turned out to be not beneficial neither in terms of biological effects nor in the overall in-vitro ADME profile.

Although the data from these analogs, synthesized during this part of the PhD work, were confirmed to be unsatisfactory in terms of rescuing effect of F508del-CFTR activity in CFBE41o- cells, this contribution to the SAR studies allowed to eventually identify other potent drug-like analogs. Among them, one compound, characterized in vitro (CFBE41o- cells and primary human bronchial epithelial cells from F508del/F508del CF patients) by high potency in rescuing the activity of F508del-CFTR, was further investigated for its drug-like profile and selected as preclinical development candidate for the treatment of CF.

The synthesis and biological characterization of the compounds described in this PhD thesis certainly contributed to the expansion of SAR studies around this novel chemo-type of correctors, and will be included in a future manuscript, which will be submitted to a suited peer-reviewed journal in the field of drug discovery.

3.5 Experimental section

Halide-sensitive yellow fluorescent protein (HS-YFP) assay

Cell models and cell culture procedures

CFBE41o- cells stably expressing mutant F508del CFTR and the halide-sensitive yellow fluorescent protein (HS-YFP) YFP-H148Q/I152L, were previously generated and described. CFBE41o- cells were cultured using MEM medium, which was supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. For HS-YFP assays of CFTR activity, CFBE41o- cells were plated (50,000 cells/well) on clear-bottom 96-well black microplates (Corning Life Sciences, Acton, MA).

Halide-sensitive yellow fluorescent protein (HS-YFP) assay

At the time of the assay, CFBE41o- cells were washed with PBS containing (in mM): 137 NaCl, 2.7 KCl, 8.1 Na2HPO4, 1.5 KH2PO4, 1 CaCl2, and 0.5 MgCl2. Cells were then incubated for 25 min with 60 µL of PBS plus forskolin (20 µM) and VX-770 (1 µM) to maximally stimulate F508del-CFTR. Cells were then transferred to microplate readers (FluoStar Optima; BMG Labtech, Offenburg, Germany) for CFTR activity determination. The plate readers were equipped with high-quality excitation (HQ500/20X: 500 ± 10 nm)
and emission (HQ535/30M: 535 ± 15 nm) filters for YFP (Chroma Technology). For the primary screening, the assay consisted of a continuous 12-s fluorescence reading, 1 s before and 11 s after injection of 165 µL of an iodide-containing solution (PBS with Cl− replaced by I−; final I− concentration 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine iodide influx rate, the final 10 s of the data for each well were fitted with a linear function to extrapolate initial slope (dF/dt). For the secondary evaluation of compounds, the assay had a duration of 14-s, with continuous fluorescence reading, 2 s before and 12 s after the injection of the iodide-containing solution. Iodide influx rate was determined by fitting the final 11 s of the data for each well with an exponential function.

**In vitro Microsomal Stability Study**

10 mM DMSO Stock solution of test compound was pre-incubated at 37 °C for 15 min with either *mouse, rat, dog, monkey, human* liver microsomes added 0.1 M Tris-HCl buffer (pH 7.4) with 0.1 % DMSO. The final concentration was 4.6 µM. After pre-incubation, the cofactors (NADPH or UDPGA, G6P, G6PDH and MgCl2 pre-dissolved in 0.1 M Tris-HCl) were added to the incubation mixture and the incubation was continued at 37 °C for 1 h. At each time point (0, 5, 15, 30, 60 min), 30 µL of incubation mixture was diluted with 200 µL cold CH3CN spiked with 200 nM Warfarin as internal standard, followed by centrifugation at 3750 rpm (3500g) for 30 min. The supernatant was further diluted with H2O (1:1) for analysis. [A reference incubation mixture (microsomes without co-factors) was prepared for each test compound and analyzed a t= 0 and t= 60 min in order to verify the compound stability in matrix]. The concentration of test compound was quantified by LC/MS-MS. The percentage of test compound remaining at each time point relative to t=0 was calculated. The half-lives (t½) were determined by a one-phase decay equation using a non-linear regression of compound concentration versus time and were reported as mean values along with their standard deviations (n = 3).

The analyses were performed on a Waters ACQUITY UPLC/MS TQD system consisting of a TQD (triple quadrupole detector) mass spectrometer equipped with an electrospray ionization interface and a photodiode array eλ detector. The analyses were run on an ACQUITY UPLC BEH C18 (50 x2.1 mmID, particle size 1.7 µm) with a VanGuard BEH C18 pre-column (5 x 2.1mmID, particle size 1.7 µm) at 40 °C, using 0.1 % HCOOH in H2O (A) and 0.1 % HCOOH in CH3CN (B) as mobile phase. Electrospray ionization (ESI) was
applied in positive/negative mode. Compound-dependent parameters as MRM transitions and collision energy were developed for each compound.

**Aqueous Kinetic Solubility Study**

The aqueous kinetic solubility was determined from a 10 mM DMSO stock solution of test compound in Phosphate Buffered Saline (PBS) at pH 7.4. The study was performed by incubation of an aliquot of 10 mM DMSO stock solution in PBS (pH 7.4) at a target concentration of 250 µM resulting in a final concentration of 2.5 % DMSO. The incubation was carried out under shaking at 25 °C for 24 h followed by centrifugation at 14,800 rpm (21100g) for 30 min. The supernatant was analyzed by UPLC/MS for the quantification of dissolved compound (in µM) by UV at a specific wavelength (215 nm).

The UPLC/MS analyses were performed on a Waters ACQUITY UPLC/MS system consisting of a SQD (single quadrupole detector) mass spectrometer equipped with an electrospray ionization interface and a photodiode array detector. The PDA range was 210-400 nm. Electrospray ionization in positive mode was used in the mass scan range 100-500 Da. The analyses were run on an ACQUITY UPLC BEH C\textsubscript{18} column (50 x2.1 mmID, particle size 1.7 µm) with a VanGuard BEH C\textsubscript{18} pre-column (5 x 2.1 mmID, particle size 1.7 µm), using 10 mM NH\textsubscript{4}OAc in H\textsubscript{2}O at pH 5.0 adjusted with AcOH (A) and 10 mM NH\textsubscript{4}OAc in MeCN-H\textsubscript{2}O (95:5) at pH 5.0 (B) as mobile phase.

**HepG2 cell toxicity assay**

**Cell culture conditions**

To increase the detection of drug induced mitochondrial effects in a preclinical cell-based assay, HepG2 hepatocellular carcinoma cells (ATCC HB-8065\textsuperscript{TM}) were forced to rely on mitochondrial oxidative phosphorylation rather than glycolysis by substituting galactose (10 mM) for glucose (25 mM) in the growth media (DMEM, Life Technologies).\textsuperscript{12}

**Cell viability assessment**

For the cytotoxicity assays cells were plated at 20,000 cells/well in 100 µL of cell culture media, in 96-well plates and allowed to grow overnight. Cells were then treated for 24 h with 2 µM or 20 µM of each compound.

All compounds were dissolved in DMSO with stock concentrations of 4 mM. The first dilution step of compounds was prepared in DMSO (200X stock solutions), while the second dilution step was carried out in complete cell culture medium (5% DMSO). 10 µL of this
dilution were added to the wells of the 96-well plate, with a final DMSO concentration of 0.5%. Rotenone, a well-known mitochondrial inhibitor, was used as reference compound. After treatment, cellular viability was assessed by using the CellTiter-Glo® (CTG) Luminescent Cell Viability Assay (Promega), which determines the number of viable cells based on quantitation of the ATP present, and the Thiazolyl Blue Tetrazolium Blue (MTT) dye (Aldrich), which is converted to water-insoluble MTT formazan crystals by mitochondrial dehydrogenases of living cells. The dual assessment of ATP content alongside cytotoxicity provides an enhanced understanding of the potential causes of toxic effects. Indeed, the different behaviour observed for these compounds may be associated with the target of mitochondrial dysfunction and may be used to direct further mechanistic studies.

**Media composition**

**High-glucose media**: high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen 11995-065) containing 25 mM glucose and 1 mM sodium pyruvate and supplemented with 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 10% FBS.

**Galactose media**: DMEM deprived of glucose (Invitrogen 11966-025) supplemented with 10 mM galactose, 2mM glutamine (6 mM final), 5 mM HEPES, 10% FBS and 1 mM sodium pyruvate.
3.6 Bibliography


4. Development of novel inhibitors of the anti-infective target DXS using Dynamic Combinatorial Chemistry (DCC)

4.1 Introduction

Discovery and development of new antibiotic agents with novel targets and mechanisms of action are urgently needed due to the increase of antibiotic resistance developed by pathogens or infectious agents.\(^1,2\)

The aim of this study was the development of selective and potent inhibitors of the important and underexplored anti-infective target 1-deoxy-D-xylulose-5-phosphate synthase (DXS).

DXS is the first enzyme of the \(2C\)-methyl-\(d\)-erythritol-4-phosphate (MEP) or non-mevalonate pathway, which is absent in humans but is essential for medically relevant pathogens (e.g., \(Plasmodium falciparum\), \(Mycobacterium tuberculosis\), \(Pseudomonas aeruginosa\), \(Acinetobacter baumannii\), \(Enterobacteriaceae\), and \(Klebsiella pneumoniae\)), which use this pathway for the biosynthesis of isoprenoid precursors, vitamins B\(^1\) and B\(^6\).\(^4\)

The absence of the MEP pathway in humans and the possibility to inhibit three crucial bacterial biosynthetic pathways at once, make it a promising target for the development of new anti-infective agents.

Despite substantial efforts dedicated to the discovery of inhibitors of DXS, to date, very few active compounds have been reported and none of them fulfil the requirements of an ideal candidate for further development.\(^5,6\) To address these issues, we decided to use a combination of structure-based drug design and protein-templated Dynamic Combinatorial Chemistry (ptDCC) as hit-identification strategy. To expand the structural diversity and obtain potent and selective inhibitors of DXS, we designed a dynamic combinatorial library (DCL) for acylhydrazone formation. Different heterocyclic hydrazides and aldehydes were chosen based on the calculated estimated affinity of all possible acylhydrazone products using LeadIT\(^7\) and \textit{SeeSAR}\(^8\) as software. Biochemical evaluation of several hit compounds
amplified in the ptDCC experiment against *M. tuberculosis* DXS and *D. radioduran* DXS afforded inhibitors with IC$_{50}$ in the range of 30 μM – 190 μM.

### 4.1.1 MEP pathway

Discovered in 1993, the MEP pathway is an alternative biosynthetic route to generate isoprenoid precursors, essential metabolites for cell survival.$^9$

Isoprenoids are a large and diverse class of natural products, which are involved a variety of vital cellular processes such as transcription and post-translational modifications, protein degradation, cell-wall biosynthesis, electron transport, apoptosis, photosynthesis, meiosis, intracellular signalling and secreted defense mechanisms.$^{10}$ All of these compounds are biosynthesized from the same precursors, isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP). Isoprenoid molecules are synthesized by two independent routes, the *mevalonate pathway*, which is used by most eukaryotes (all mammals and humans) fungi, plants, archaea and few bacteria, or the *MEP pathway*, used by most pathogenic bacteria, apicomplexa, algae and plants.$^{11}$

The absence of the MEP pathway in humans gives the possibility to have biological selectivity making it a promising target for the development of new anti-infective agents.$^{12}$

The MEP pathway consists of seven enzymatic steps (Scheme 4.1).
Scheme 4.1: Biosynthesis of IDP and DMADP via the MEP pathway. Thiamine pyrophosphate (ThDP), nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), nicotinamide adenine dinucleotide phosphate (oxidized) (NADP+), cytidine triphosphate (CTP), adenosine triphosphate (ATP), adenosine diphosphate (ADP), cytidine monophosphate (CMP). The scheme was adapted from Wang X., Dowd C. S., ACS Infect Dis., 2018, 4, 278-290.

DXS (1-deoxy-D-xylulose-5-phosphate synthase) catalyzes the first step of the biosynthesis, a condensation reaction between pyruvate and GAP (glyceraldehyde 3-phosphate) to form DXP (1-deoxy-D-xylulose-5-phosphate). The following reduction and isomerization of DXP, catalyzed by IspC (1-deoxy-D-xylulose-5-phosphate reductoisomerase) leads to MEP (2C-methyl-D-erythritol-4-phosphate), which is first coupled to cytidine triphosphate, phosphorylated by an ATP dependent kinase and finally cyclized into MEcPP (4-diphosphocytidyl-2C-methyl-D-erythritol 2, 4-cyclodiphosphate). The two last steps are catalyzed by IspG (1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase) and IspH (4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase) and consist in a two-electron reduction of MEcPP followed by conversion to IDP and DMADP.

Most of these enzymes play an essential role in the MEP pathway and can be considered a promising drug target for the development of antimicrobial agents; in particular, we focused our attention on DXS enzyme.
4.1.2 The DXS enzyme: a promising anti-infective target

DXS is the first enzyme of the MEP pathway, which catalyzes, with the release of carbon dioxide, the condensation of pyruvate and GAP to form DXP, with thiamin diphosphate (ThDP) as cofactor and Mg\(^{2+}\) ions required for the catalytic activity.\(^{13}\)

The DXP product is not only involved in the production of isoprenoid precursors, but also in the microbial vitamin B1 and B6 biosyntheses.\(^{5}\) Therefore, the inhibition of DXS gives the possibility to block multiple crucial biosynthetic pathways (Figure 4.1), which together with the biological selectivity, makes DXS a promising target for the synthesis of new inhibitors.

![Figure 4.1: Multiple crucial biosynthetic pathways blocked by the inhibition of DXS enzyme.](image)

Based on the two crystal structures reported for DXS from *E. coli* and *D. radiodurans* complexed with ThDP cofactor, DXS consists of a tightly associated dimer, where each monomer can be divided into three domains (I, II, and III), which have an α/β fold with the β-sheet that is sandwiched between α-helices. The active site is located at the interface of domains I and II within the same monomer; ThDP cofactor is also located in a defined pocket between these two domains.\(^{14}\)

The proposed mechanism for DXP formation is described in Scheme 4.2. In the first step, the nucleophile ThDP-ylide (derived from the deprotonation on the thiazolium ring) attacks the C-2 of pyruvate, affording an intermediate\(^{15}\), which is subjected to a decarboxylation reaction. A subsequent CO\(_2\) release produces a carbanion (in equilibrium with its enamine form), which then attacks the aldehyde carbon of d-GAP. The final deprotonation gives the ketone product (DXP) and the regenerated ThDP-ylide.\(^{5}\)
Different DXS inhibitors have been reported in the literature. Among them, 5-ketocloclomazone, a metabolite of the herbicide clomazone, has been demonstrated to inhibit DXS, forming a covalent adduct within the active site in a similar manner to pyruvate. Instead, Freil Meyers and co-workers have reported a series of alkylacetyl-phosphonates, such as butylacetylphosphonate, have been shown to inhibit different pathogens, included *Mycobacterium tuberculosis* (Mtb), as pyruvate mimic agents that are not able to undergo decarboxylation. Among DXS inhibitors, hydroxybenzaldoximes have been shown to act competitively against D-glyceraldehyde 3-phosphate D-GAP and, interestingly, trihydroxybenzaldoximes show an uncompetitive or noncompetitive inhibition mode with respect to pyruvate. Finally, thiamine derivatives, as Mtb DXS inhibitors, have been described by Hirsch and co-workers and are closely related to ThDP cofactor.

### 4.1.3 ptDCC as Hit-Identification strategy

The protein-templated Dynamic Combinatorial Chemistry (ptDCC) was selected as the hit-identification strategy for the development of new DXS’s inhibitors. DCC was used for...
the first time in the 1990’s and emerged as a powerful approach to identify new ligands for biological targets. Furthermore, it was shown that this methodology is also suited for drug discovery due to the possibility to combine the synthesis of inhibitors and screening for affinity for the target, in a method where the target selects its own inhibitors.

With this kind of technique, it is possible to generate compound libraries by reversible reaction of building block (Figure 4.2). These dynamic combinatorial libraries (DCLs) are under thermodynamic control and their composition is the result of the thermodynamic stability of each member. Because of the continuous interconversion of the constituents, these libraries are adaptive and external stimuli, such as a target protein, can influence their composition. The addition of a target protein indeed causes the shift of the DCL equilibrium composition, binding, stabilizing and amplifying library components with the strongest affinity for the target.

Two different kinds of methodology could be used for the ptDCC technique, a comparative and a non-comparative approach. In the first one, the composition of a library generated in the presence of a target (templated library) is compared to one generated in its absence (blank library). Instead, in the non-comparative approach, best ligands are identified without comparison with the blank for example, in the case the products are generated only in the presence of the target.

Different kind of reversible reactions can be used in ptDCC to form interchanging products from dynamic combinatorial libraries (DCL) in the presence of a target protein (Scheme 4.3).
In order to be biocompatible, the DCC experiments have been carried out in aqueous media and ideally at physiological pH. In addition to reversibility, these reactions should also be chemoselective to avoid cross-reactivity with different functional groups of the DCL or with the target. Moreover, all the building blocks and products need to be soluble to prevent precipitation and the equilibration of the DCL should be fast enough under the conditions of temperature and pH where the protein is stable.

For this study, we decided to use the acylhydrazone chemistry due to its compatibility under physiological condition. Considering the instability of DXS protein at low pH and the slow formation of acylhydrazone at neutral pH, the use of aniline as a nucleophilic catalyst was required. To see the template effect of protein in the DCC, a parallel blank DCC experiment (without protein) was ran with the same reaction conditions.

The analysis of the library composition could be performed using different types of techniques such as HPLC, MS, NMR or, as in this case, LC/MS.

Scheme 4.3: Reversible reactions used in tdDCC. 

Analysis of the data was conducted by comparing the protein-templated and blank DCC runs using the relative peak area (RPA). RPA is the fraction of each peak relative to the total peak area of the blank and pt-libraries. Starting from the relative peak area, % amplification and relative change were calculated (Figure 4.3).

\[
\text{% Amplification} = \left( \frac{\text{RPA}_{\text{protein templated}}}{\text{RPA}_{\text{blank}}} \right) \times 100
\]

\[
\text{Normalized change} = \left( \frac{\text{RPA}_{\text{protein templated}} - \text{RPA}_{\text{blank}}}{\text{RPA}_{\text{blank}}} \right)
\]

Figure 4.3: % Amplification and relative change.

Considering the normalized change of RPA, a positive bar indicates amplification of compounds in the protein templated sample; on the other hand, a negative one indicates depletion.\(^{21}\)

### 4.2 Selection and synthesis of Dynamic Combinatorial Libraries (DCLs)

The choice of the first DCL (dynamic combinatorial library) was based on selecting different building blocks with some structural similarity with the ThDP cofactor (Figure 4.4). In particular, different hydrazides, containing a pyrimidine scaffold and different aldehydes, featuring heterocyclic cores, were selected and their corresponding acylhydrazone products were used for docking studies.

Figure 4.4: ThDP cofactor structure.

The docking was performed using two distinct software, *LeadIT* for pose generation (Protein = 2O1X; Radius = 6.5 Å around ThDP; Poses = 30/molecule)\(^7\) and *SeeSAR* for the
calculation/affinity estimation. From this study, various acylhydrazones showed a promising estimated affinity ranging from nM to µM (Figure 4.5).

![Figure 4.5: Acylhydrazones used in the docking study.](image)

Considering that the different pyrimidine-containing hydrazides were not commercially available, the first key step in this project was the synthesis of these DCC hydrazide building blocks. In the beginning, we focused our attention on five different hydrazides, featuring simple scaffolds to test and optimize the synthetic route (Figure 4.6).

![Figure 4.6: Selected hydrazides building blocks.](image)

Regarding compound G1 and G8, we proposed two reasonable retrosynthetic disconnections (Scheme 4.4).

In the retrosynthetic path 1, we envisioned the functionalization of a preformed pyrimidine scaffold. Hydrazides, such as compound A, could be prepared by starting from the corresponding ethyl ester B, which could be obtained from commercially available building blocks, such as C and D through cross-coupling reactions. On the other hand, retrosynthetic path 2 required the formation of the pyrimidine core that could be accessed from the reaction of a 1,3-dicarbonyl compound E with a N–C–N fragment, such as acetimidamide (H). Intermediate E could be synthesized, starting from commercially available compounds F and G.
Based on the proposed retrosynthetic disconnections, three different synthetic routes were outlined (Scheme 4.5).

The first two approaches are based on cross-coupling reactions. In particular, in the synthetic route A (Scheme 4.5), we expected to get compound G1 using a Stille cross-coupling reaction. The stannyl ester G3 was obtained from the commercially available ethyl propionate G2 by a reaction of Sn(n-Bu)3Cl with n-BuLi and dicyclohexylamine in THF and used directly as crude in the following reaction. Unfortunately, reactions performed with different Pd-catalysts and following a range of conditions did not afford the desired ethyl ester (Table 4.1). One reason of this synthetic drawback could be ascribed to the low reactivity of the amino-pyrimidine G4 and the use of stannyl ester G3, which was used...
without any purification due to its low stability over silica columns and in work-up conditions.

Table 4.1: Stille coupling reaction conditions.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Pd Catalyst</th>
<th>Additive</th>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[Pd(PPh₃)₄]</td>
<td>---------</td>
<td>Toluene</td>
<td>110</td>
<td>18</td>
<td>No reaction</td>
</tr>
<tr>
<td>2</td>
<td>[Pd(dppf)Cl₂]-DCM</td>
<td>Zn(II)Br₂</td>
<td>DMF</td>
<td>80</td>
<td>18</td>
<td>No reaction</td>
</tr>
<tr>
<td>3</td>
<td>[Pd(dppf)Cl₂]-DCM</td>
<td>Zn(II)Br₂</td>
<td>DMF</td>
<td>160</td>
<td>18</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

Next, we tried to obtain product G5 by Suzuki cross-coupling and the Ni-catalyzed arylation (B, Scheme 4.5) using different catalysts, bases, and solvents (Table 4.2). As no reactions were described for this particular scaffold, we adapted and followed a similar, relevant reported protocol. Unfortunately, all attempts to obtaining the desired intermediate G5 were unsuccessful, probably due to the low reactivity of the two starting materials G6 and G7.

Table 4.2: Suzuki coupling and Ni-Catalyzed arylation conditions tested to synthesize compound G5.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Pd Catalyst</th>
<th>Base</th>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[Pd(dppf)Cl₂]-DCM</td>
<td>Cs₂CO₃</td>
<td>Dioxane</td>
<td>110</td>
<td>18</td>
<td>No reaction</td>
</tr>
<tr>
<td>2</td>
<td>[Ni(PPh₃)₄]</td>
<td>K₃PO₄</td>
<td>Toluene</td>
<td>60</td>
<td>18</td>
<td>No reaction</td>
</tr>
<tr>
<td>3</td>
<td>[Ni(PPh₃)₄]</td>
<td>K₃PO₄</td>
<td>Toluene</td>
<td>110</td>
<td>18</td>
<td>No reaction</td>
</tr>
<tr>
<td>4</td>
<td>[Ni(PPh₃)₄] + Naphthalene</td>
<td>K₃PO₄</td>
<td>Toluene</td>
<td>110</td>
<td>18</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

Having abandoned the cross-coupling strategy, we designed another synthetic route based on the retrosynthetic disconnections of path 2 (Scheme 4.4). As for route C in Scheme 4.5, the pyrimidine core could be accessed via a cyclization step between 1,3-dicarbonyl compound G11 and acetamidine G12. Compound G11 was synthesized starting from commercially available compound G9 and G10 in the presence of Na-methoxide in Et₂O. The intermediate was directly reacted with the N–C–N component G12 (the choice of the N–C–N component governs the substitution at C-2 in the product heterocycle), in presence
of Na-ethoxide in EtOH affording the desired pyrimidine derivative \( \text{G13} \) in 3 days. Compound \( \text{G13} \) was then used without any purification in the following chlorination step, performed with phosphoryl chloride at 80 °C for 2 hours, to provide compound \( \text{G14} \). The last step was the formation of the hydrazide, using hydrazine monohydrate and methanol as solvent (Scheme 4.6). Even using a stoichiometric or substoichiometric amount of hydrazide, inseparable mixtures of undesired side-products \( \text{G15} \) and \( \text{G16} \), along with the desired product \( \text{G8} \) were obtained. Therefore, we decided to use compound \( \text{G13} \) as a starting compound for this type of reaction (Scheme 4.7). This approach paid off leading to the desired compound \( \text{G17} \) in quantitative yield.

\[
\text{G14} \xrightarrow{\text{Hydrazine monohydrate, MeOH, reflux, 2 h}} \text{G17}
\]

Scheme 4.6: Synthesis of hydrazide \( \text{G8} \) and accompanying byproducts.

\[
\text{G13} \xrightarrow{\text{Hydrazine monohydrate, MeOH, reflux, 2 h}} \text{G17}
\]

Scheme 4.7: Synthesis of hydrazide \( \text{G17} \).

Similarly, compound \( \text{G19} \) (Figure 4.5) was obtained in quantitative yield, starting from the commercially available ethyl ester \( \text{G18} \), using hydrazine monohydrate and methanol as solvent (Scheme 4.8).

\[
\text{G18} \xrightarrow{\text{Hydrazine monohydrate, MeOH, 80°C, 2 h}} \text{G19}
\]

Scheme 4.8: Synthesis of hydrazide \( \text{G19} \).

A semicarbazide scaffold characterizes the two last selected compounds, \( \text{G20} \) and \( \text{G21} \) (Figure 4.5). The initial approach followed to synthesize them consisted in the condensation between the heterocyclic amine \( \text{G22a} \) or \( \text{G22b} \) with ethyl chloroformate (\( \text{G23} \)), followed by the addition of hydrazine monohydrate (Scheme 4.9).
Scheme 4.9: Synthetic route for the semicarbazide compounds.

The synthesis of the ethyl carbamates required initial optimization of the reaction conditions in order to obtain product $G_{24a}$ and $G_{24b}$ in almost pure form without purification by column chromatography (Table 4.3). The identification of pyridine, as solvent and base, allowed reaching full conversion of the amino-pyrimidine $G_{22}$ to the corresponding ethyl carbamate, which was used in the next step without further purification.

Table 4.3: Reaction condition for the synthesis of ethyl carbamate $G_{24a}$ and $G_{24b}$.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K$_2$CO$_3$</td>
<td>THF</td>
<td>r.t.</td>
<td>18</td>
<td>No reaction</td>
</tr>
<tr>
<td>2</td>
<td>Et$_3$N</td>
<td>THF</td>
<td>r.t.</td>
<td>18</td>
<td>Full conversion.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Purification problem.</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Pyridine</td>
<td>r.t.</td>
<td>48</td>
<td>Full conversion.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Used as crude.</td>
</tr>
</tbody>
</table>

Several attempts to obtain the final semicarbazide products, $G_{20}$ and $G_{21}$, scouting different reaction parameters (i.e, solvents, temperature, time), turned out to be unsuccessful, leading to only traces of desired products along with many unidentified impurities (Table 4).
Table 4.4: Reaction condition for the hydrazidation step.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (G22a)</td>
<td>Dioxane</td>
<td>110</td>
<td>48</td>
<td>Trace of G20, mix of G24 (a) and byproducts. Purification problem</td>
</tr>
<tr>
<td>2 (G22a)</td>
<td>EtOH</td>
<td>80</td>
<td>96</td>
<td>Trace of G20, mix of G24 (a) and byproducts. Purification problem</td>
</tr>
<tr>
<td>1 (G22b)</td>
<td>Dioxane</td>
<td>110</td>
<td>48</td>
<td>Trace of G21, mix of G24 (b) and byproducts. Purification problem</td>
</tr>
<tr>
<td>2 (G22b)</td>
<td>EtOH</td>
<td>80</td>
<td>96</td>
<td>Trace of G21, mix of G24 (b) and byproducts. Purification problem</td>
</tr>
</tbody>
</table>

Considering the difficulties in isolating the final compounds due to their instability under purification condition, and willing to get full conversion of the carbamate into the semicarbazide, the synthetic protocol was modified accordingly (Scheme 4.10). Therefore, taking into account that the reactivity of the carbamate is related to the pKₐ value of the released alcohol, the more reactive phenyl carbamate was used instead of the ethyl derivative (EtOH: pKₐ ~ 16; PhOH: pKₐ ~ 10).⁴¹

![Scheme 4.10: The redesigned synthetic route for the preparation of semicarbazide compounds.](image)

Although the phenyl carbamate proved to be more reactive, it also turned out to be more problematic to handle. Furthermore, due to purification and stability issues of the intermediate G26, the condensation reaction of the amino-pyridine and phenyl chloroformate required a careful optimization (Table 4.5). After different experiments, scouting different reaction parameters, compound G26 was obtained in 71% yield, using pyridine as a solvent and as a base at room temperature in two hours (entry 7, Table 4.5).
Table 4.5: Optimization of phenyl carbamate formation.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaHCO₃</td>
<td>THF/H₂O</td>
<td>r.t.</td>
<td>18</td>
<td>Full conversion of G22b in G26. Purification and stability problem</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>THF</td>
<td>r.t.</td>
<td>18</td>
<td>Full conversion G22b in G26. Purification and stability problem</td>
</tr>
<tr>
<td>3</td>
<td>..........</td>
<td>neat</td>
<td>80</td>
<td>0.17</td>
<td>Trace of G26</td>
</tr>
<tr>
<td></td>
<td>(microwave)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NaHCO₃</td>
<td>neat</td>
<td>80</td>
<td>0.17</td>
<td>Trace of G26</td>
</tr>
<tr>
<td></td>
<td>(microwave)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Et₃N</td>
<td>DCM</td>
<td>r.t.</td>
<td>18</td>
<td>Mix of G26, G22b and byproducts</td>
</tr>
<tr>
<td>6</td>
<td>K₂CO₃</td>
<td>DCM</td>
<td>r.t.</td>
<td>2</td>
<td>Full conversion of G22b in G26. Purification and stability problem</td>
</tr>
<tr>
<td>7</td>
<td>..........</td>
<td>Pyridine</td>
<td>r.t.</td>
<td>2</td>
<td>Yield = 71%</td>
</tr>
</tbody>
</table>

The final step was carried out with hydrazine monohydrate in EtOH, giving, after full conversion of the starting material G26 in 1h at room temperature, the desired product G21 in overall good yield. Due to its instability during work-up, purification and storage conditions, the semicarbazide G21 was discarded for these DCC experiments. Consequently, we decided to use it directly as starting material for the synthesis of two acylhydrazones were predicted to have a promising estimated affinity in the docking study (Scheme 4.11). The synthesis of the two final compounds was performed by treating the semicarbazide G21 with the corresponding aldehydes in methanol at 60 °C for 18 h to afford acylhydrazones G29 and G30 as a mixture of E and Z isomers.
Considering the instability of G30 in the purification steps, only compound G29 was subjected to biological evaluation.

### 4.3 DCC experiments

Due to possible stability problems of the semicarbazide scaffold in the DCC experiments, we decided to use hydrazides G8 and G19 as building blocks along with the other commercially available hydrazides (G31 – H35) and aldehydes (G36 – G38) (Figure 4.7).

#### Hydrazides

![Hydrazides](image)

#### Aldehydes

![Aldehydes](image)

Figure 4.7: Building blocks used in the DCC experiment 1 (DCC-1).
The first set of DCC experiments were performed in phosphate buffer (pH 7.04), using three equivalents of the acylhydrazides (stock solutions, 100 mM in DMSO), one equivalent of aldehyde (stock solutions, 100 mM in DMSO), excess of aniline (stock solution, 1 M in DMSO), and 40% of drDXS protein (we selected drDXS instead of mtDXS due to its stability in the DCC conditions). To achieve the final concentration of 5%, additional DMSO was added to the reaction mixture. To see the template effect of protein in the DCC, a parallel blank DCC experiment (without protein) was also run with the same reaction conditions.

The progress of both DCC runs was monitored via UPLC-MS after 0, 2, 4, 6 and 24 hours. Before running the UPLC-MS analysis, the reactions were treated with acetonitrile, to denature the protein, and the pH was raised to > 8 by adding NaOH (1 M) to freeze the reaction.

In the DCC experiment, it is important to know when an equilibrium state is reached. This can be easily identified by comparing the formation of different products over time. In our case, we observed very small or no differences in the formation of different products between 2 – 6 h (Figure 4.8). In the blank reaction, it is clear that the DCL equilibrated quite fast and the data after equilibration could be used to compare the blank reaction with the protein templated one.

In the current ptDCC experiment, the gradual amplification of the product was visible only after 6 h and it was quite prominent at 24 h. Therefore, we decided to use data at 24h for the comparison (Figure 4.9b).

Figure 4.8: Equilibrium state in the blank experiment calculated considering the peak area of the different products formed over time for DCC-1. Equilibrium was reached between 4 and 6 h.

The amplification of five acylhydrazones was confirmed by comparing UV-spectra and calculating percent amplification as well as a change in the RPA (Figure 4.9a–c).
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Figure 4.9a: Comparison of Uv-spectra at 24 h of blank and protein templated obtained from DCC-1. Five compound (G39–G43) resulted amplified.

Figure 4.9b: Percent amplification and normalized change data from the first DCC run.

Figure 4.9c: Hit compounds afforded from the first DCC run.

Once identified, all five hit compounds were synthesized by reacting the corresponding hydrazide and aldehyde in MeOH at reflux under N₂ atmosphere. Desired acylhydrazones were obtained as a mixture of E and Z isomers in moderate to good yield.
Considering the promising result from the DCC-1 (see 4.4 Biological Results), we decided to further optimize the initial hits. As all these compounds shared two common structural motifs (G33 and G36), all five hits were further exploited to improve their potency. We hypothesized that instead of traditional medicinal chemistry optimization, the use of these common scaffolds from the best hits of DCC-1 could allow the identification of better hits in a relatively fast manner if selected as building blocks in a second DCC experiment (DCC-2). To investigate our hypothesis, we took G33 and G36 as building blocks in a DCC-2 along with other new hydrazides and aldehydes (Figure 4.10).

![Building blocks used in the DCC-2.](image)

For this DCC-2, we used the same reaction conditions as for DCC-1. In the blank reaction from DCC-2 experiments, the equilibrium state was reached between 4 – 6 h (Figure 4.11). In contrast to the DCC-1, in DCC-2 the significant amplification of product in protein templated DCC was visible at 6 h, for this reason, we decided to use data at 6 h for the comparison of the two studies (Figure 4.12).
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Figure 4.11: Equilibrium state in the blank experiment calculated considering the peak area of the different products formed over time for DCC-2. Equilibrium was reached between 4 and 6 h.

From this DCC-2, five different hits were amplified to a different extent (Figure 4.11). To our delight, hits $\text{G52}$ and $\text{G53}$ were amplified more than the hits from DCC-1 (compare Figure 4.9b with Figure 4.12b), which supported our hypothesis about hit-optimization to some extent.

Figure 4.12a: Comparison of Uv-spectra at 6 h of blank and protein templated obtained from DCC-2. Five compounds ($\text{G43}$, $\text{G51}$–$\text{G54}$) resulted amplified.

Figure 4.12b: Percent amplification and normalized change data, DCC-2.
All the hit compounds were synthesized following the same synthetic procedure used for the DCC-1 hits.

At this point, the three controlled DCC runs were performed, one to evaluate the influence of the phosphate buffer and the two other to verify the binding mode of our hits (competitive DCC).

Regarding the influence of the buffer, it is important to verify whether the Mg$^{2+}$, present in the DXS protein, interacted with the phosphate buffer that we used in the DCC runs and eventually altered the results. To do this, a new DCC study was carried out (DCC-3) using the same library and conditions of DCC-2 experiment, but using HEPES buffer (pH 8) instead of phosphate buffer. In the current ptDCC experiment, the equilibrium point was reached after 6 h (Figure 4.13a) but even if the gradual amplification of the product was visible only after 6 h, it was quite prominent at 24 h. Therefore, we decided to use data at 24 h for the comparison (Figure 4.13b).

Figure 4.13a: Equilibrium state in the blank experiment calculated considering the peak area of the different products formed over time for DCC-3. Equilibrium was reached between 6 and 24 h.
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Figure 4.13b: Comparison of Uv-spectra at 24 h of blank (top) and protein templated (bottom) obtained from DCC-3. Four compound (G43, G51–G53) resulted amplified.

By comparing the percent amplification and the normalized change in RPA of the two DCC experiments, it was evident that the same five compounds from the DCL were similarly amplified in both buffers (Figure 4.14). No major differences in terms of amplification were noted except for compound G52, which was more amplified in the HEPES buffer (Figure 4.14). These data shown that no influence was exerted by the phosphate buffer on the overall activity of the enzyme.

**HEPES BUFFER**

![Graph showing amplification and normalized change in RPA for DCC-2 in HEPES buffer.]

**PHOSPHATE BUFFER**

![Graph showing amplification and normalized change in RPA for DCC-2 in phosphate buffer.]

Figure 4.14: Comparison of DCC-2 in HEPES and phosphate buffer.
Interesting results emerged from these experiments, carried out to verify the binding mode of our hits. Based on the crystal structure reported for drDXS enzyme complexed with ThDP cofactor (Figure 4.15), the big pocket containing the active site could be divided into the following four subpockets: the diphosphate, central, amino-pyrimidine, and substrate pocket. Using ThDP cofactor and ThDP in the presence of a covalent substrate inhibitor in two separate DCC experiments, we could verify whether our hits could either compete with the ThDP, occupy the substrate pocket or partially both.

In theory, if the protein pockets were filled with ThDP or BAP and our hits bound the same pocket, no amplification would be observed, meaning that our hits could be competitive co-factor and/or substrate-competitive binders. On the contrary, if the compounds filled other pockets, the same amplification of the normal template (without ThDP/BAP/Mg\(^{2+}\)) could be seen, indicating that hits are allosteric binders.

![Figure 4.15: Active site of drDXS enzyme complexed with ThDP.](image)

In order to verify the binding mode of the amplified hits, two different DCC runs were set up using the same library of the DCC-2.

DCC-4 was performed in phosphate buffer (pH 7.04), using three equivalents of the acylhydrazides (stock solutions, 100 mM in DMSO), one equivalent of aldehyde (stock solutions, 100 mM in DMSO), excess of aniline (stock solution, 1 M in DMSO), one equivalent of both ThDP cofactor (stock solutions, 100 mM in water), one equivalent of Mg\(^{2+}\) (stock solutions, 100 mM in water) and 40% of drDXS protein. To achieve the final concentration of 5%, additional DMSO was added in the reaction mixture. To see the templated effect of protein in the DCC, a parallel blank DCC experiment (without protein) was also set with the same reaction conditions. Equilibrium in the blank (Figure 4.16a) was reached after 24h and UV-spectra are reported in Figure 4.16b.
Regarding the reaction condition for DCC-5, that one was performed three equivalents of the acylhydrazides (stock solutions, 100 mM in DMSO), one equivalent of aldehyde (stock solutions, 100 mM in DMSO), excess of aniline (stock solution, 1 M in DMSO), one equivalent of both ThDP cofactor (stock solutions, 100 mM in water), one equivalent of Mg²⁺ (stock solutions, 100 mM in water), BAP (butylacetylphosphonate, a covalent substrate binder in stock solutions, 100 mM in water) and 40% of drDXS protein. To achieve the final concentration of 5%, additional DMSO was added in the reaction mixture. To see the templated effect of protein in the DCC, a parallel blank DCC experiment (without protein) was also set with the same reaction conditions. BAP (butylacetylphosphonate, a covalent substrate binder). Equilibrium in the blank (Figure 4.17a) was reached after 4h and UV-spectra are reported in Figure 4.17b.
Figure 4.17a: Equilibrium state in the blank experiment calculated considering the peak area of the different products formed over time for DCC-5. Equilibrium was reached between 2 and 4 h.

Figure 4.17b: Comparison of UV-spectra at 4 h of blank and protein templated obtained from DCC-5.

Comparing the results from DCC-2 (Figure 4.18a) and DCC-4 (Figure 4.18b), with and without ThDP/Mg\(^{2+}\), respectively, we observed that the same compounds were amplified in both cases and there were no marked differences in terms of percent amplification between them except for compound G52.

In this case, it was possible to hypothesize that product G52 might probably be a ThDP competitor. This hypothesis should be carefully verified by confirming the binding mode of this specific inhibitor. On the contrary, comparing the data from DCC-2 (Figure 4.18a) with DCC-5 (Figure 4.18c), with and without ThDP/Mg\(^{2+}\)/BAP, respectively, we could see a clear decrease in percent amplification for all the compounds in particular for compound G53.

Overall, the collected data pointed out that most of our hits from DCC-2 were occupying the substrate and parts of co-factor pocket. Given that and still seeing some amount of amplification of hits in DCC-5, other studies with a stronger covalent binder than BAP (which could occupy the whole substrate pocket, as BAP extends only to a part of the substrate pocket) should be carried out in the future.
4.4 Biological results

All the synthesized compounds were tested for their biological activity in enzymatic (IC$_{50}$: half-maximal inhibitory concentration on drDXS and mtDXS) as well as cell-based assays (MIC: minimal inhibitory concentration, on *E. coli* TolC with and without a permeabilizing agent).

*E. coli* TolC is an efflux pump mutant strain used to study the activity of small compounds inside the cells, which otherwise could be expelled by efflux pumps in the cell.
membrane. Moreover, testing hits with and without permeabilizing agents is important to verify if the compounds are able to penetrate the bacterial outer membrane and it is active inside the cell.

Regarding compound G29 (Scheme 4.11), from IC$_{50}$ assays it showed no inhibition on drDXS (Deinococcus radiodurans DXS) protein and did not present any activity in the MIC (minimal inhibitory concentration) test performed on E. coli TolC (E. coli mutant cells without efflux pump) and also with a permeabilizing agent Polymyxin B nonapeptide (PMBN).

Biological data for compounds deriving from DCC-1 (G39–G43) are reported in Table 4.6.

Table 4.6: Biological results of the hits from DCC-1.

<table>
<thead>
<tr>
<th>ENTRY</th>
<th>IC$_{50}$/ %inhibition (µM)</th>
<th>MIC (µM)</th>
<th>MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>drDXS</td>
<td>mtDXS</td>
<td>(E. coli TolC)</td>
</tr>
<tr>
<td>G29</td>
<td>no inhibition</td>
<td>no inhibition</td>
<td>not active</td>
</tr>
<tr>
<td>G39</td>
<td>32% at 50 µM</td>
<td>109</td>
<td>&gt; 25 (%) inh at 25 µM: 73%</td>
</tr>
<tr>
<td>G40</td>
<td>22% at 60 µM</td>
<td>30</td>
<td>&gt; 25 (%) inh at 25 µM: 33%</td>
</tr>
<tr>
<td>G41</td>
<td>67</td>
<td>no inhibition</td>
<td>&gt; 25 (%) inh at 25 µM: 32%</td>
</tr>
<tr>
<td>G42</td>
<td>76% at 200 µM</td>
<td>15% at 200 µM</td>
<td>22</td>
</tr>
<tr>
<td>G43</td>
<td>48</td>
<td>~ 200</td>
<td>not active</td>
</tr>
</tbody>
</table>

From these results, we could appreciate that the most amplified compound G43 in the DCC was the one that showed the best inhibition (IC$_{50}$ = 48 µM) against drDXS. Unfortunately, this compound seemed to lose its activity when tested against mtDXS. Moreover, it showed no activity against E. coli TolC with and without a permeabilizing agent. On the contrary, compound G40, the second most amplified hit in the DCC-1, resulted in only 22% inhibition of activity when tested against drDXS at 60 µM, but gave a really promising IC$_{50}$ (30 µM) against mtDXS. Unfortunately, the same compound showed only 33% inhibition at 25 µM when tested against E. coli TolC. Interestingly, the activity was restored back to 85% inhibition at 25 µM when the compound was tested against E. coli.
CHAPTER 4

TolC in the presence of PMBN (polymyxin B nonapeptide), indicating the possibility of permeability issues for this derivative.

Compounds G39 and G42, which were less amplified as compared to compound G43 and G40, did not show promising enzymatic activity against drDXS and mtDXS, confirming the correlation between amplification and enzymatic activity. On the other hand, these compounds are active against E. coli TolC and E. coli TolC in the presence of PMBN (compare entry G39 and G42 with G43 and G40, Table 4.6). Interestingly, these findings could suggest that at least for compound, G39 and G42 along with DXS there might be other possible biological targets. In order to confirm this hypothesis, specific target-identification studies should be carefully performed. Finally, compound G41, which was amplified the least as compared to other hits, showed moderate activity against drDXS (IC₅₀ = 67 µM) and no activity against mtDXS and E. coli TolC.

Regarding the biological evaluation to determine the activity of hit compounds deriving from DCC-2 (G51–G54, Table 4.7), unfortunately, due to the poor solubility of these derivatives, their biological activity was not established, only compound G52 showed a promising MIC value of 20 µM. In order to possibly solve the solubility issue, new DCC experiment using more polar building blocks are currently ongoing.

Table 4.7: Biological result of DCC-2 hits.

<table>
<thead>
<tr>
<th>ENTRY</th>
<th>IC₅₀/ %inh (µM)</th>
<th>IC₅₀/ %inh (µM)</th>
<th>MIC (µM) (E. coli TolC):</th>
<th>MIC (µM) (E. coli TolC + pmbn):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>drDXS</td>
<td>mtDXS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G51</td>
<td>no inhibition</td>
<td>no inhibition</td>
<td>not active</td>
<td>not active</td>
</tr>
<tr>
<td>G52</td>
<td>No data</td>
<td>50% at 100 µM</td>
<td>No data</td>
<td>20</td>
</tr>
<tr>
<td>G53</td>
<td>solubility problem</td>
<td>solubility problem</td>
<td>solubility problem</td>
<td>solubility problem</td>
</tr>
<tr>
<td>G54</td>
<td>solubility problem</td>
<td>solubility problem</td>
<td>&gt; 25 µM (% inh at 25 µM: 40%)</td>
<td>&gt; 25 µM (% inh at 25 µM: 15%)</td>
</tr>
<tr>
<td>G43</td>
<td>48</td>
<td>~ 200</td>
<td>not active</td>
<td>not active</td>
</tr>
</tbody>
</table>
4.5 Experimental section

4.5.1 Materials and methods

Chemicals were purchased from commercial suppliers and used without pretreatment. Solvents used for the experiments were reagent-grade and dried, if necessary, according to standard procedures. The yields were calculated for the analytically pure compounds and were not optimized for. The purifications were performed using column chromatography with Macherey-Nagel Silica 60 M 0.04–0.063 mm. Preparative high-pressure liquid chromatography (HPLC, Ultimate 3000 UHPLC+ focused, Thermo Scientific) purification was performed on reverse phase column (C18 column, 5 µm, Macherey-Nagel, Germany). The solvents used for the chromatography were water (0.1% formic acid) and MeCN (0.1% formic acid), or EtOAc and DCM. \(^1\)H, \(^{13}\)C and \(^{31}\)P-NMR spectra were measured on a Bruker Avance Neo 500 MHz with prodigy cryoprobes system (500, 126 or 202 MHz), respectively. The chemical shifts were reported in parts per million (ppm) relative to the corresponding solvent peak. The coupling constants of the splitting patterns were reported in Hz and were indicated as singlets (s), doublets (d), triplets (t) and multiplets (m). UPLC-MS and HRMS measurements were performed using Thermo Scientific systems.

4.5.2 DCC Conditions

DCC-1 and DCC-2: The corresponding hydrazides (each 3 µL, stock solutions 100 mM in DMSO) and the aldehydes (each 1 µL, stock solutions 100 mM in DMSO) were added to a phosphate buffer (pH 7.04). Aniline (10 µL, stock solution 1 M) and drDXS (425.5 µL, stock solution 94 µM final concentration of protein 40%) were added accordingly. DMSO was added to reach a final concentration of DMSO (524.5 µL) in the DCL of 5%. The end-volume was 1 mL. Final concentrations in the DCLs are shown in Table 4.8. The DCL was left shaking at room temperature and was frequently monitored via UPLC-MS.
Table 4.8: Final concentrations in the DCLs.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Protein template</th>
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</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Aniline</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>100 µM (each)</td>
<td>100 µM (each)</td>
</tr>
<tr>
<td>Hydrazide</td>
<td>300 µM (each)</td>
<td>300 µM (each)</td>
</tr>
<tr>
<td>Protein</td>
<td>40%</td>
<td>-------</td>
</tr>
</tbody>
</table>

DCC-3: The corresponding hydrazides (each 3 µL, stock solutions 100 mM in DMSO) and the aldehydes (each 1 µL, stock solutions 100 mM in DMSO) were added to a HEPES buffer (pH 8). Aniline (10 µL, stock solution 1 M) and drDXS (559.67 µL, stock solution 71.47 µM final concentration of protein 40%) were added accordingly. DMSO (524.5 µL) was added to reach a final concentration of DMSO in the DCL of 5%. The end-volume was 1 mL. Final concentrations in the DCLs were shown in Table 4.9. The DCL was left shaking at room temperature and was frequently monitored via UPLC-MS.

Table 4.9: Final concentrations in the DCLs.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Protein template</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Aniline</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>100 µM (each)</td>
<td>100 µM (each)</td>
</tr>
<tr>
<td>Hydrazide</td>
<td>300 µM (each)</td>
<td>300 µM (each)</td>
</tr>
<tr>
<td>Protein</td>
<td>40%</td>
<td>-------</td>
</tr>
</tbody>
</table>

DCC-4: The corresponding hydrazides (each 3 µL, stock solutions 100 mM in DMSO) and the aldehydes (each 1 µL, stock solutions 100 mM in DMSO) were added to a phosphate buffer (pH 7.04). Aniline (10 µL, stock solution 1 M), drDXS (360.39 µL, stock solution 111 µM final concentration of protein 40%), ThDP (1 µL, stock solution 100 mM in H2O) and Mg²⁺ (1 µL, stock solution 100 mM in H2O) were added accordingly. DMSO (587.61 µL) was added to reach a final concentration of DMSO in the DCL of 5%. The end-volume
was 1 mL. Final concentrations in the DCLs are shown in Table 4.10. The DCL was left shaking at room temperature and was frequently monitored via UPLC-MS.

Table 4.10: Final concentrations in the DCLs.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Protein templated</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Aniline</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>100 μM (each)</td>
<td>100 μM (each)</td>
</tr>
<tr>
<td>Hydrazide</td>
<td>300 μM (each)</td>
<td>300 μM (each)</td>
</tr>
<tr>
<td>Protein</td>
<td>40%</td>
<td>-----------</td>
</tr>
<tr>
<td>THDP</td>
<td>100 μM (each)</td>
<td>100 μM (each)</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>100 μM (each)</td>
<td>100 μM (each)</td>
</tr>
</tbody>
</table>

DCC-5: The corresponding hydrazides (each 3 μL, stock solutions 100 mM in DMSO) and the aldehydes (each 1 μL, stock solutions 100 mM in DMSO) were added to a phosphate buffer (pH 7.04). Aniline (10 μL, stock solution 1 mM), drDXS (360.39 μL, stock solution 111 μM final concentration of protein 40%), ThDP (1 μL, stock solution 100 mM in H₂O), Mg^{2+} (1 μL, stock solution 100 mM in H₂O) and BAP (1 μL, stock solution 100 mM in H₂O) were added accordingly. DMSO (586.61) was added to reach a final concentration of DMSO in the DCL of 5%. The end-volume was 1 mL. Final concentrations in the DCLs are shown in Table 4.11. The DCL was left shaking at room temperature and was frequently monitored via UPLC-MS.
Table 4.11: Final concentrations in the DCLs.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Protein template</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Aniline</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>100 µM (each)</td>
<td>100 µM (each)</td>
</tr>
<tr>
<td>Hydrazide</td>
<td>300 µM (each)</td>
<td>300 µM (each)</td>
</tr>
<tr>
<td>Protein</td>
<td>40%</td>
<td>-------</td>
</tr>
<tr>
<td>THDP</td>
<td>100 µM (each)</td>
<td>100 µM (each)</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>100 µM (each)</td>
<td>100 µM (each)</td>
</tr>
<tr>
<td>BAP</td>
<td>100 µM (each)</td>
<td>100 µM (each)</td>
</tr>
</tbody>
</table>

For monitoring via UPLC-MS, 10 µL of the corresponding library were diluted in 90 µL acetonitrile, the pH was raised to pH > 8 by adding 2 µL NaOH (1 M) to freeze the reaction. The mixture was centrifuged at 10,000 rpm for 2 minutes, and the supernatant was analyzed via UPLC-MS.

Analysis of the data was conducted by comparing the protein templated and blank DCC runs using the relative peak area (RPA). Starting from the relative peak area, % amplification and relative change were calculated (Figure 4.19).

\[
\%\text{Amplification} = \left( \frac{\text{RPA}_{\text{protein templated}}}{\text{RPA}_{\text{blank}}} \right) \times 100
\]

\[
\text{Normalized change} = \left( \frac{\text{RPA}_{\text{protein templated}} - \text{RPA}_{\text{blank}}}{\text{RPA}_{\text{blank}}} \right)
\]

Figure 4.19: % Amplification and relative change.

### 4.5.3 Characterization of compounds (G11–G54):

**Diethyl 2-formylbutanedioate (G11):** In a heat-dried Schlenk tube under N₂ at 0 °C, sodium methoxide (1.61 g, 30 mmol) was dissolved in dry Et₂O (20 mL). A solution of dimethylsuccinamide (3.35 mL, 20 mmol) and methyl formate (4.93 mL, 80 mmol) in Et₂O (20 mL) was added dropwise under N₂ at 0 °C for 30 min. The solution was stirred at
room temperature. After 18 h, a solution of HCl 1% was added, and the aqueous phase was extracted with Et₂O. The combined organic layers were dried over Na₂SO₄, filtered and the solvent was removed under vacuum. The resulting residue (1.4 g) was used directly in the next step.

**Ethyl 2-(4-hydroxy-2-methyl-pyrimidin-5-yl)acetate (G13):** In a heat-dried Schlenk tube under N₂ at 0 °C, a solution of G11 (1.4 g, 6.04 mmol) in EtOH (5 mL) was added to a solution of Na (0.15, 6.93 mmol) in cold EtOH (20 mL). After 30 min, G12 (1.11 g, 11.8 mmol) was added at 0 °C. After 3 days, HCl 5% (2 mL) and water (1 mL) were added, and the solution was stirred for 30 min, after which solvent was removed under vacuum and the precipitate obtained was filtered. The white residue obtained (250 mg) was directly used in the following step.

**2-(4-Hydroxy-2-methyl-pyrimidin-5-yl)acetohydrazide (G17):** In a round-bottomed flask, compound G13 (0.25 g, 1.27 mmol) was dissolved in EtOH (2 mL) and the resulting solution was treated with hydrazine (65%) monohydrate (183 µL, 3.81 mmol). After 18 h the reaction, the solvent was removed under reduced pressure and poured into a NH₄Cl solution, the aqueous phase was extracted with Et₂O, and the combined organic layers were washed with saturated aqueous NaCl solution and dried over Na₂SO₄. The filtrated solvent was removed under vacuum, and the resulting residue was mixed with Et₂O and Hexane. The white precipitate was then filtered and washed with cold Et₂O and Hexane and dried under vacuum. (230 mg, 5% yield over three steps).

**UPLC-MS:** $t_R = 0.54$ min; MS (ESI) m/z calcd for C₇H₉N₄O₂: (M–H)⁻: 181.0804; found: 181.1092.

**2-(3-Pyridyl)acetohydrazide (G19):** In a round-bottomed flask, compound G18 (1.00 g, 6.05 mmol) was dissolved in EtOH (10 mL) and the resulting solution was treated with hydrazine (65%) monohydrate (752 µL, 15.12 mmol). After 2 h, the solvent was removed under reduced pressure and poured into a NH₄Cl solution, the aqueous phase was extracted with Et₂O and the combined organic layers were washed with brine and dried over Na₂SO₄ and filtered.
The solvent was removed under vacuum and the resulting residue was mixed with Et₂O and Hexane. The white precipitate was then filtered and washed with cold Et₂O and Hex and dried under vacuum. (914mg, quantitative yield). 

\(^1\)H NMR (500 MHz, DMSO-d₆) δ 9.29 (bs, 1H), 8.44 (d, J = 2.3 Hz, 1H), 8.42 (dd, J = 4.8, 1.7 Hz, 1H), 7.66 (dt, J = 7.8, 2.0 Hz, 1H), 7.37 – 7.27 (m, 1H), 4.24 (bs, 2H), 3.38 (s, 2H).

\(^{13}\)C NMR (126 MHz, DMSO-d₆) δ 169.1, 149.9, 147.7, 136.5, 132.0, 123.4, 37.5.

UPLC-MS: \( t_R = 0.53 \) min; MS (ESI) m/z calcld for C\(_7\)H\(_{10}\)N\(_3\)O: (M+H)\(^+\): 152.0746; found: 152.1096.

Phenyl N-(3-pyridyl)carbamate (G26): In a two neck flask, at 0 °C under N\(_2\), compound G22b (0.100 g, 1.00 mmol) was dissolved in pyridine (1 mL) and G25 (66 µL, 0.5 mmol) was added dropwise. After 2 h, the reaction was diluted with EtOAc and washed with water, NH₄Cl, brine and dried over Na\(_2\)SO\(_4\) and filtered. Removal of the organics gave a white solid (0.153 g), which was not subjected to any purification and directly used in the next step of reaction.

\(^1\)H NMR (500 MHz, DMSO-d₆) δ 10.47 (bs, 1H), 8.69 (d, J = 2.6 Hz, 1H), 8.27 (dd, J = 4.7, 1.5 Hz, 1H), 7.98 – 7.86 (m, 1H), 7.46 – 7.40 (m, 2H), 7.42 – 7.33 (m, 1H), 7.32 – 7.19 (m, 3H). 

\(^{13}\)C NMR (126 MHz, DMSO-d₆) δ 151.9, 150.4, 144.0, 140.6 – 139.9 (m), 135.4, 129.5 (x2), 125.6, 123.7, 121.9 (x2), 115.2. UPLC-MS: \( t_R = 1.83 \) min; MS (ESI) m/z calcld for C\(_{12}\)H\(_{11}\)N\(_2\)O\(_2\)S: (M+H)\(^+\): 215.0742; found: 215.1088.

1-amino-3-(3-pyridyl)urea (G21): In a round-bottomed flask, compound G26 (0.153 g, 0.71 mmol) was dissolved in EtOH (10 mL) and the resulting solution was treated with hydrazine (65%) monohydrate (65 µL, 1.3 mmol). After 1 h, the solvent was removed under reduced pressure and poured into a NH₄Cl solution, the aqueous phase was extracted with Et₂O and the combined organic layers were washed with brine and dried over Na\(_2\)SO\(_4\) and filtered. The solvent was removed under vacuum, and the resulting residue was mixed with Et₂O and Hexane. The white precipitate was then filtered and washed with cold Et₂O and Hexane and dried under vacuum. The white solid obtained (68 mg) was not subjected to any purification and directly used in the next step of the reaction.

\(^1\)H NMR (500 MHz, DMSO-d₆) δ 8.85 (bs, 1H), 8.70 (app, 1H), 8.13 (dd, J = 4.6, 1.5 Hz, 1H), 8.00 (d, J = 8.3 Hz, 1H), 7.59 (bs, 1H), 7.25 (dd, J = 8.3, 4.6 Hz, 1H), 4.41 (bs, 2H).
1-[(3,4-Dihydroxyphenyl)methyleneamino]-3-(3-pyridyl)urea (G29): In a two neck flask under N₂, compound G21 (0.034 g, 0.2 mmol) was dissolved in MeOH (1 mL) and G27 (0.032 mg, 0.2 mmol) was added. The resulting solution was stirred at 60 °C. After 18 h, the reaction was cooling to room temperature, the and concentrated in vacuo. The residue obtained was washed with cold Et₂O, Hexane and MeOH to remove the impurities. The resulting suspension was filtered and the filtrate was concentrated in vacuo to afford a white solid (0.010 g, 18%). m. p. 208–209. ¹H NMR (500 MHz, DMSO-d₆) δ 10.76 (s, 1H), 9.43 (s, 2H), 9.08 (s, 1H), 8.80 (d, J = 2.5 Hz, 1H), 8.28 (s, 1H), 8.21 (dd, J = 4.7, 1.5 Hz, 1H), 8.09 – 8.00 (m, 1H), 7.40 (d, J = 7.8 Hz, 1H), 7.32 (dd, J = 8.3, 4.6 Hz, 1H), 6.80 (dd, J = 7.8, 1.6 Hz, 1H), 6.68 (t, J = 7.8 Hz, 1H). ¹³C NMR (126 MHz, DMSO-d₆) δ 153.1, 145.5, 144.9, 143.3, 141.7, 135.9, 126.7, 123.3, 120.9, 119.0, 117.4, 116.2. UPLC-MS: tᵣ = 1.80 min; MS (ESI) m/z calc'd for C₁₃H₁₃N₄O₃: (M+H)⁺: 273.0909; found: 272.9879 HRMS (ESI) calc'd for C₁₃H₁₁N₄O₃ [M-H]⁻: 271.0909, found 271.0835.

General procedure for acylhydrazone formation:
To the hydrazide (1 eq.) dissolved in MeOH, the corresponding aldehyde (1.2 eq.) was added. The reaction mixture was stirred at reflux until completion. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The residue obtained was washed with cold methanol to remove the impurities or was taken up in cold methanol. The resulting suspension was filtered and the filtrate was concentrated in vacuo to afford the corresponding acylhydrazone (as a mixture of E and Z isomers) as solid in 23-85% yield.

(E/Z)-2-(1H-benzimidazol-2-ylsulfanyl)-N-[4,5,6,7-tetrahydrobenzothiophen-2-ylmethyleneamino]acetamide (G39): The acylhydrazone was synthesized under N₂ atmosphere at reflux for 18 h, following the general procedure, using acetic acid 2-(1H-benzimidazol-2-ylthio)-hydrazide (30 mg, 0.13 mmol) in MeOH (1 mL) and 4,5,6,7-Tetrahydrobenzo[bl]thiophene-2-carboxaldehyde (26 mg, 0.16 mmol). The acylhydrazone (G39) was obtained as a mixture of E and Z isomers as a white solid (16 mg, 33%). m. p. 198–199. ¹H-NMR (500 MHz, DMSO-d₆) (E:Z=0.85:1) ¹H NMR (500 MHz, DMSO-d₆) δ 12.6 (bs, 0.85H, E), 12.6 (bs, 1H, Z), 11.7 (s, 0.86H, E), 11.5 (s, 1H, Z), 8.3 (s, 0.85H, E), 8.1 (s, 1H, Z), 7.6 – 7.3 (m, 4H, mixture E/Z), 7.3 – 6.9 (m, 6H, mixture E/Z), 4.5 (s, 2H, E).
(E/Z)-N-[4,5,6,7-tetrahydrobenzo[2,4]diazepin-2-ylmethyleneamino]thiophene-2-carboxamide (G40): The acylhydrazone was synthesized under N₂ atmosphere at reflux for 18 h, following the general procedure, thiophene-2-carboxyhydrazide (30 mg, 0.21 mmol) in MeOH (1 mL) and 4,5,6,7-Tetrahydrobenzo[b]thiophene-2-carboxaldehyde (42 mg, 0.25 mmol). The acylhydrazone (G40) was obtained as a mixture of E and Z isomers as a yellow solid (25 mg, 41%). m. p. 186–187. ¹H-NMR (500 MHz, DMSO-d₆) (E:Z = 1:1) δ 11.8 – 11.7 (m, 2H, mixture E/Z), 8.5 (bs, 1H, E), 8.2 (s, 1H, Z), 8.1 – 8.0 (m, 1H, mixture E/Z), 8.0 – 7.9 (m, 1H, mixture E/Z), 7.9 – 7.8 (m, 2H, E), 7.2 (s, 2H, Z), 7.2 – 7.2 (m, 5H, mixture E/Z). 7.2 – 7.1 (m, 2H, mixture E/Z), 2.8 – 2.7 (m, 4H, mixture E/Z), 2.6 – 2.5 (m, 4H, mixture E/Z), 1.8 – 1.7 (m, 8H, mixture E/Z).

¹³C NMR (126 MHz, DMSO-d₆) (combined peaks of E/Z) δ 160.9, 157.5, 143.1, 138.8, 138.4, 135.8, 135.0, 134.5, 133.4, 132.0, 131.7, 128.7, 128.1, 126.7, 24.9, 24.8, 22.9, 22.2. UPLC-MS: tᵣ = 3.58 min; MS (ESI) m/z calcd for C₁₅H₁₉N₄O₃S₂: (M+H)+ : 371.0547; found: 371.0547; found 291.0602.

(E/Z)-N-[5-Bromo-2-pyridyl)methyleneamino]-2-(2,4-dichlorophenoxy)acetamide (G41): The acylhydrazone was synthesized under N₂ atmosphere at reflux for 18 h, following the general procedure, acetic acid 2-(4-dichlorophenoxy)-hydrazide (30 mg, 0.13 mmol) in MeOH (1 mL) and 5-bromofomylpyridine (28 mg, 0.15 mmol). The acylhydrazone (G41) was obtained as a mixture of E and Z isomers as a gray solid (33 mg, 63%). m. p. 190–191. ¹H-NMR (500 MHz, DMSO-d₆) (E:Z = 0.31:1) δ 12.0 (bs, 0.34H, E), 11.9 (bs, 1H, Z), 8.8 – 8.7 (m, 0.28H, E), 8.7 – 8.7 (m, 1H, Z), 8.2 (s, 0.34H, E), 8.2 – 8.1 (m, 2H, mixture E/Z), 8.0 (s, 1H, Z), 8.0 (d, J = 8.5 Hz, 1H, Z), 7.9 (d, J = 8.6 Hz, 0.34H, E), 7.6 (d, J = 2.6 Hz, 0.31H, E), 7.6 (d, J = 2.6 Hz, 1H, Z), 7.4 (dd, J = 8.9, 2.6 Hz, 0.36H, E), 7.3 (dd, J = 8.9, 2.6 Hz, 1H, Z), 7.1 – 7.1 (m, 1H, mixture E/Z), 5.3 (s, 2H, Z), 4.8 (s, 0.68H,
E). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 168.6, 164.0, 152.8, 152.6, 151.8, 151.6, 150.3, 150.3, 146.8, 143.2, 139.7, 139.5, 129.5, 129.3, 128.1, 127.9, 125.2, 124.6, 122.5, 122.2, 121.6, 121.5, 121.0, 120.8, 115.5, 115.2, 67.1, 65.7.

UPLC-MS: $t_R = 3.64$ min; MS (ESI) m/z calcd for $C_{14}H_{11}BrCl_2N_3O_2$: (M+H)$^+$: 401.9333; found: 401.8491. HRMS (ESI) calcd for $C_{14}H_{11}BrCl_2N_3O_2$ [M+H]$^+$: 401.9333, found 401.9388.

$(E/Z)$-2-(2,4-Dichlorophenoxy)-N-[1H-indol-3-ylmethyleneamino]acetamide (G41):

The acylhydrazone was synthesized under N$_2$ atmosphere at reflux for 18 h, following the general procedure, acetic acid 2-(2,4 dichlorophenoxy)-hydrazide (30 mg, 0.13 mmol) in MeOH (1 mL) and indole-3-carboxaldehyde (22 mg, 0.15 mmol). The acylhydrazone (G41) was obtained as a mixture of $E$ and $Z$ isomers as a white solid (20 mg, 42%). m. p. 216–217. $^1$H-NMR ($E:Z = 0.30:1$) $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 11.6 (s, 1H, mixture $E/Z$), 11.4 (s, 1H, Z), 11.3 (s, 0.30H, E), 8.4 (s, 0.30H, E), 8.2 (s, 1H, Z), 8.1–8.1 (m, 1H, mixture $E/Z$), 7.8 (s, 1H, mixture $E/Z$), 7.6 (d, $J = 2.6$ Hz, 0.30H, E), 7.6 (d, $J = 2.6$ Hz, 1H, Z), 7.5–7.4 (m, 1H, mixture $E/Z$), 7.4 (dd, $J = 8.9, 2.6$ Hz, 0.30H, E), 7.3 (dd, $J = 8.9, 2.6$ Hz, 1H, Z), 7.2–7.2 (m, 1H, mixture $E/Z$), 7.2–7.1 (m, 2H, mixture $E/Z$), 7.1 (d, $J = 9.0$ Hz, 1H, mixture $E/Z$), 5.3 (s, 2H, Z), 4.8 (s, 1H, E).

$^{13}$C NMR (126 MHz, DMSO-$d_6$) (combined peaks of $E/Z$) $\delta$ 167.4, 162.5, 153.0, 152.7, 145.0, 141.5, 137.1, 130.7, 129.4, 129.2, 128.1, 127.9, 125.0, 124.4, 124.3, 124.1, 122.7, 122.6, 122.5, 122.1, 121.9, 121.8, 120.7, 120.4, 115.4, 115.0, 111.9, 111.4, 111.3, 67.3, 65.8.

UPLC-MS: $t_R = 3.57$ min; MS (ESI) m/z calcd for $C_{17}H_{14}Cl_2N_3O_2$: (M+H)$^+$: 362.0385; found: 362.1011. HRMS (ESI) calcd for $C_{17}H_{14}Cl_2N_3O_2$ [M+H]$^+$: 362.0385, found 362.0440.

$(E/Z)$-2-(2,4-Dichlorophenoxy)-N-[4,5,6,7-tetrahydrobenzothiophen-2-ylmethyleneamino]acetamide (G42): The acylhydrazone was synthesized under N$_2$ atmosphere at reflux for 18 h, following the general procedure, acetic acid 2-(2,4 dichlorophenoxy)-hydrazide (30 mg, 0.13 mmol) in MeOH (1 mL) and 4,5,6,7-Tetrahydrobenzo[b]thiophene-2-carboxaldehyde (25 mg, 0.15 mmol). The acylhydrazone (G42) was obtained as a mixture of $E$ and $Z$ isomers as a yellow solid (40 mg, 80%). m. p. 224–225. $^1$H-NMR (500 MHz, DMSO-$d_6$) ($E:Z = 0.60:1$) $\delta$ 11.6 (bs, 1H, Z), 11.5 (bs, 0.60H,


CHAPTER 4

\(E\), 8.3 \((s, 0.60H, E)\), 8.1 \((s, 1H, Z)\), 7.6 \((d, J = 2.6 \text{ Hz}, 0.60H, E)\), 7.6 \((d, J = 2.6 \text{ Hz}, 1H, Z)\), 7.4 \((dd, J = 8.9, 2.6 \text{ Hz}, 0.60H, E)\), 7.3 \((dd, J = 9.0, 2.6 \text{ Hz}, 1H, Z)\), 7.2 – 7.1 \((m, 2H, \text{ mixture } E/Z)\), 7.0 \((d, J = 8.9 \text{ Hz}, 1H, \text{ mixture } E/Z)\), 5.1 \((s, 2H, Z)\), 4.8 \((s, 1.37H, E)\), 2.8 – 2.7 \((m, 4H, \text{ mixture } E/Z)\), 2.6 – 2.5 \((m, 4H, \text{ mixture } E/Z)\), 1.8 – 1.6 \((m, 8H, \text{ mixture } E/Z)\).

\(^{13}\text{C} \text{ NMR} \ (126 \text{ MHz, DMSO-}d_6) \text{ (combined peaks of } E/Z \text{)} \delta 167.7, 163.1, 152.8, 152.6, 143.2, 139.5, 139.0, 138.4, 135.7, 134.7, 134.6, 132.2, 131.5, 129.4, 129.3, 128.1, 127.9, 125.1, 124.5, 122.5, 122.2, 115.4, 115.1, 67.1, 65.4, 24.8, 24.8, 24.7, 22.8, 22.2, 22.2.

UPLC-MS: \(t_R = 4.16 \text{ min}; \text{ MS (ESI) } m/z \text{ calcd for } C_{17}H_{17}Cl_2N_2O_2S: (M+H)⁺ : 383.0309; \text{ found: } 382.9785. \text{ HRMS (ESI) calcd for } C_{17}H_{17}Cl_2N_2O_2S [M+H]⁺: 383.0309, \text{ found } 383.0365.

\((E/Z)-2-(2,4-\text{Dichlorophenoxy})-N-[(2-\text{methylpyrimidin-5-yl)methyleneamino}]acetamide \ (G51)\): The acylhydrazone was synthesized under \(N_2\) atmosphere at reflux for 18 h, following the general procedure, acetic acid 2- (2,4 dichlorophenoxy)-hydrazide (30 mg, 0.13 mmol) in MeOH (1 mL) and 2-methyl-5-pyrimidinidne (18 mg, 0.15 mmol). The acylhydrazone (G51) was obtained as a mixture of \(E\) and \(Z\) isomers as a pink solid (10 mg, 23\%). m. p. 150–151. \(^{1}H\)-NMR (500 MHz, DMSO-\(d_6\)) \((E:Z = 0.37:1) \delta 11.9 \text{ (bs, 1H, mixture } E/Z)\), 9.0 \((s, 2H, Z)\), 9.0 \((s, 0.60H, E)\), 8.3 \((s, 0.33H, E)\), 8.0 \((s, 1H, Z)\), 7.6 \((d, J = 2.6 \text{ Hz, 0.36H, } E)\), 7.6 \((d, J = 2.6 \text{ Hz, 1H, } Z)\), 7.4 \((dd, J = 8.9, 2.6 \text{ Hz, 0.37H, } E)\), 7.3 \((dd, J = 9.0, 2.6 \text{ Hz, 1H, } Z)\), 7.1 \((d, J = 8.9 \text{ Hz, 1H, mixture } E/Z)\), 5.3 \((s, 2H, Z)\), 4.8 \((s, 1H, E)\), 2.7 – 2.6 \((\text{appm, 3H, mixture } E/Z)\).

\(^{13}\text{C} \text{ NMR} \ (126 \text{ MHz, DMSO-}d_6) \text{ (combined peaks of } E/Z \text{)} \delta 168.7, 167.9, 155.2, 152.8, 142.8, 138.7, 129.3, 127.9, 125.1, 124.5, 122.1, 115.2, 65.8, 25.7, 25.7. \text{ UPLC-MS: } t_R = 3.00 \text{ min; MS (ESI) } m/z \text{ calcd for } C_{14}H_{13}Cl_2N_4O_2: (M+H)⁺ : 339.0337; \text{ found: } 339.0881. \text{ HRMS (ESI) calcd for } C_{14}H_{13}Cl_2N_4O_2 [M+H]⁺: 339.0337, \text{ found } 339.0403.

\((E/Z)-2-(3,4-\text{Dimethoxyphenyl})-N-[4,5,6,7-\text{tetrahydrobenzothiophen-2-ylmethyleneamino}]acetamide \ (G52)\): The acylhydrazone was synthesized under \(N_2\) atmosphere at reflux for 18 h, following the general procedure, benzene acetic acid 3,4 dimethoxy-hydrazide (30 mg, 0.14 mmol) in MeOH (1 mL) and 4,5,6,7-Tetrahydrobenzo[b]thiophene-2-carboxaldehyde (28 mg, 0.17 mmol). The acylhydrazone (G52) was obtained as a mixture of \(E\) and \(Z\) isomers as a yellow solid (39 mg, 77\%). m. p.
164–165. $^1$H-NMR (500 MHz, DMSO-d$_6$) ($E$:Z = 0.88:1) $\delta$ 11.4 (s, 1H), 11.2 (s, 1H), 8.3 (s, 1H, $E$), 8.0 (s, 1H, Z), 7.1 (s, 1H, $E$), 7.0 (s, 1H, Z), 6.9 – 6.9 (m, 2H, mixture $E$/Z), 6.9 – 6.9 (m, 1H, mixture $E$/Z), 6.8 – 6.8 (m, 1H, mixture $E$/Z), 3.7 (s, 3H, $E$), 3.7 (s, 6H, Z), 2.7 – 2.7 (m, 4H, mixture $E$/Z), 2.5 – 2.5 (m, 4H, mixture $E$/Z), 1.8 – 1.6 (m, 8H, mixture $E$/Z).

$^{13}$C NMR (126 MHz, DMSO-d$_6$) (combined peaks of $E$/Z) $\delta$ 172.0, 166.5, 155.4, 148.5, 148.4, 147.6, 147.5, 141.9, 140.9, 138.4, 138.0, 137.8, 136.2, 135.7, 135.6, 135.2, 135.0, 134.7, 134.5, 131.7, 130.9, 128.0, 127.9, 121.4, 121.0, 113.2, 111.8, 111.8, 55.5, 55.5, 55.4, 55.4, 40.8, 38.5, 24.9, 24.9, 24.8, 24.8, 24.7, 22.9, 22.2, 22.2, 22.2.

UPLC-MS: $t_R$ = 3.50 min; MS (ESI) m/z calcd for C$_{19}$H$_{23}$N$_2$O$_3$S: (M+H)$^+$: 359.1351; found: 359.1717. HRMS (ESI) calcd for C$_{19}$H$_{23}$N$_2$O$_3$S [M+H]$^+$: 359.1351, found: 359.1415.

$(E$/Z)-2-(2,4-Dichlorophenoxy)-N-[1H-indol-5-ylmethyleneamino]acetamide (G53):

The acylhydrazone was synthesized under N$_2$ atmosphere at reflux for 18 h, following the general procedure, acetic acid 2- (2,4 dichlorophenoxy)-hydrazide (30 mg, 0.13 mmol) in MeOH (1 mL) and indole-5-carboxaldehyde (22 mg, 0.15 mmol). The acylhydrazone (G53) was obtained as a mixture of $E$ and $Z$ isomers as a pink solid (40 mg, 85%).

m. p. 224–225. $^1$H-NMR (500 MHz, DMSO-d$_6$) ($E$:Z = 0.40:1) $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 11.5 (bs, 1H, Z), 11.4 (bs, 0.40H, $E$), 11.3 (bs, 0.40H, $E$), 11.3 (bs, 1H, Z), 8.3 (s, 0.40H, $E$), 8.1 (s, 1H, Z), 7.8 – 7.8 (m, 1H, mixture $E$/Z), 7.6 – 7.6 (m, 0.36H, $E$), 7.6 – 7.6 (m, 1H, Z), 7.6 – 7.5 (m, 1H, mixture $E$/Z), 7.5 – 7.4 (m, 3H, mixture $E$/Z), 7.3 – 7.3 (m, 1H, mixture $E$/Z), 7.1 (d, $J = 8.9$ Hz, 0.42H, $E$), 7.1 (d, $J = 9.0$ Hz, 1H, Z), 6.5 – 6.5 (m, 1H, mixture $E$/Z), 5.3 (s, 2H, Z), 4.8 (s, 0.80H, $E$).

$^{13}$C NMR (126 MHz, DMSO-d$_6$) (combined peaks of $E$/Z) $\delta$ 167.9, 163.0, 152.9, 149.8, 146.1, 137.1, 136.9, 129.4, 129.3, 128.1, 127.9, 127.6, 126.5, 125.1, 125.1, 124.4, 122.5, 122.2, 121.4, 120.9, 119.3, 115.4, 115.1, 112.0, 112.0, 102.0, 101.9, 67.2, 65.7.

UPLC-MS: $t_R$ = 3.50 min; MS (ESI) m/z calcd for C$_{17}$H$_{14}$Cl$_2$N$_3$O$_2$: (M+H)$^+$: 362.0385; found: 362.0170. HRMS (ESI) calcd for C$_{17}$H$_{14}$Cl$_2$N$_3$O$_2$ [M+H]$^+$: 362.0385, found: 362.0448.
(E/Z)-2-(3-Methoxyphenyl)-N-[4,5,6,7-tetrahydrobenzothiophen-2-ylmethyleneamino]acetamide (G54): The acylhydrazone was synthesized under N\textsubscript{2} atmosphere at reflux for 18 h, following the general procedure, benzenacetic acid 3 methoxy-hydrazide (30 mg, 0.16 mmol) in MeOH (1 mL) and 4,5,6,7-Tetrahydrobenzo[b]thiophene-2-carboxaldehyde (33 mg, 0.2 mmol). The acylhydrazone (G54) was obtained as a mixture of E and Z isomers as a white solid (31 mg, 59\%). m. p. 160–161. ¹H-NMR (500 MHz, DMSO-d\textsubscript{6}) (E:Z =0.86:1) δ 11.4 (s, 1H, E), 11.2 (s, 1H, Z), 8.3 (s, 1H, E), 8.0 (s, 1H, Z), 7.3 – 7.2 (m, 2H, mixture E/Z), 7.1 (s, 1H, E), 7.1 (s, 1H, Z), 6.9 – 6.7 (m, 6H, mixture E/Z), 3.8 (s, 2H, Z), 3.7 (s, 3H, E), 3.7 (s, 3H, Z), 3.5 (s, 2H, E), 2.7 – 2.7 (m, 4H, mixture E/Z), 2.6 – 2.5 (m, 4H, mixture E/Z), 1.8 – 1.6 (m, 8H, mixture E/Z).

¹³C NMR (126 MHz, DMSO-d\textsubscript{6}) (combined peaks of E/Z) δ 171.7, 166.1, 159.2, 159.1, 142.0, 138.5, 138.2, 137.9, 137.1, 137.1, 135.7, 135.6, 135.1, 135.0, 131.8, 130.9, 129.4, 129.2, 121.6, 121.3, 115.1, 114.8, 112.0, 111.9, 55.0, 54.9, 41.3, 24.9, 24.8, 24.4, 24.7, 22.9, 22.2, 22.2.

UPLC-MS: \( t_R = 3.70 \) min; MS (ESI) m/z calcd for C\textsubscript{18}H\textsubscript{21}N\textsubscript{2}O\textsubscript{2}S: (M+H)\(^+\) : 329.1245; found: 329.1303. HRMS (ESI) calcd for C\textsubscript{18}H\textsubscript{21}N\textsubscript{2}O\textsubscript{2}S [M+H]\(^+\) : 329.1245, found.329.1309.

### 4.5.4 Biological experimental section

Half maximal inhibitory concentration (IC\textsubscript{50}) determinations (DXS-IspC coupled assay):

The efficacy of our inhibitor was validated by coupling the formation of DXP which generated due to DXS activity to the oxidation of NADPH in presence of IspC as an auxiliary enzyme. By monitoring the fluorescence signal of NADPH disappearance, we measured the velocity of the enzyme. The IC\textsubscript{50} experiment was performed by titrating the compound in DMSO using two-fold dilution then pipetting 3 \( \mu \)L of compound to 30 \( \mu \)L of buffer B (200 Mm HEPES pH 8, 0.01% TritonX, 0.3 mM D-GAP, 0.1 mM pyruvate) in 384-well plate (Greiner). Afterwards, we added 30 \( \mu \)L of buffer A (200 mM HEPES pH 8, 4 mM DTT, 0.0006 mM ThDP, 1mM MgCl\textsubscript{2}, 0.05 mM NADPH, 0.0015 mM IspC, 0.00005 mM mtDXS) to buffers B which already has the compound. Fluorescence signal was measured for 45 min
with a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 340-15 nm and emission at 520-20 nm at room temperature. IC_{50} was calculated by dividing the slope of samples containing inhibitor by the slope of simultaneously started uninhibited enzymatic velocity. IC_{50} value was determined with nonlinear regression fitting using origin 2019 software. The IC_{50} value was calculated from two independent experiments.

**Minimal inhibitory concentration (MIC) determinations**:44

MIC values were determined in 96-well plates (Sarstedt, N"umbrecht, Germany) against *Staphylococcus aureus subsp. aureus* (Newman strain), *Bacillus subtilis subsp. subtilis*, *Pseudomonas aeruginosa* PAO1, *E. coli* K12, *E. coli* TolC, and the Rif-resistant *E. coli* TolC mutants: *E. coli* TolC b Q513L and *E. coli* TolC b H526Y. As bacteria start OD_{600} 0.03 was used in a total volume of 200 µL in lysogeny broth (LB) medium containing the compounds dissolved in DMSO (maximal DMSO concentration in the experiment: 1%). Final compound concentrations (in duplicates) were prepared by serial dilution ranging from 0.02–100 µM depending on their antibacterial activity and solubility in growth medium. The ODs were measured using a CLARIOstar (BMG labtech, Offenburg, Germany) after inoculation and after incubation for 18 h at 37 °C with 50 rpm (200 rpm for *P. aeruginosa* PAO1). Given MIC values are means of two independent determinations (three if MIC <10 µg mL^{-1}) and defined as the lowest concentration of compound that reduced OD_{600} by ≥ 95%.

**MIC determinations in presence of polymyxin B nonapeptide (PMBN) or phenylarginine-b-naphthylamide (PAbN)**44:

The same procedures followed as mentioned above with minor modifications: before inoculation, bacteria were cultured in LB medium containing PMBN (1 µg mL^{-1}) or PAbN (20 µg mL^{-1}) (10 mg mL^{-1} in case of *E. coli* TolC) for 2 h and subsequently diluted with the same medium to OD_{600} 0.06. Inocula of 100 µL were added to the wells containing 100 µL of the specific concentrations of the compounds in PMBN/PAbN containing medium. MIC values were determined for *E. coli* K12, and *E. coli* D22.
4.6 Conclusions

DCC, used as hit-identification technique on DXS protein for the first time, afforded nine promising hit compounds, which showed a good amplification. Due to solubility problems of some of these compounds, only six hits were tested to evaluate their biological activity (Figure 4.20). Two of them (G40 and G43) showed good inhibition of DXS, with IC₅₀ values of 30 µM on mtDXS, and 48 µM on drDXS, respectively. Compound G39, G42 and G52 showed comparatively lower enzymatic inhibitory activity, along with really promising activity in cell-based assays, with MIC of 22 µM and 11.25 µM (E. coli TolC and E. coli TolC + PMBN), respectively.

To better elucidate their biological activity, other experiments have to be done, in particular, activity against native E. coli as well as off-target studies. Furthermore, to further understand the binding mode of these inhibitors, new competitive DCC with a bigger covalent binder, which fills completely the substrate pocket, and co-crystallization experiments are currently underway. Finally, new DCC experiments are already on-going trying to optimize the current identified hits and to solve the solubility issues.

Figure 4.20: Promising hit compounds obtained by DCC.
4.7 Bibliography


41. Bogolubsky, A. V.; Moroz, Y. S.; Mykhailiuk, P. K.; Dmytriv, Y. V.; Pipko, S. E.; Babichenko, L. N.; Konovets, A. I.; Tolmachev, A., Facile one-pot synthesis of 4-substituted semicarbazides. *RSC Advances* 2015, **5** (2), 1063-1069.


Summary

Cystic Fibrosis (CF) is a lethal, autosomal recessive genetic disease caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein. CFTR is a cAMP-regulated anion channel expressed at the apical surface of epithelial cells of multiple organs, which plays a critical role in fluid and electrolyte transport (in particular in the transport of chloride and bicarbonate ions) and in mucociliary clearance in the airways. The clinical manifestations of this disorder are characterized by chronic obstruction of the airways and inflammation of multiple organs including lungs, pancreas, intestine, reproductive tract and sweat ducts.

The nearly 2000 different mutations identified in the CFTR gene have been categorized into six different classes (I-VI) according to the resulting molecular defect. The most prevalent class II mutation, the deletion of phenylalanine 508 (F508del), results in misfolded CFTR protein, which is recognized as defective and targeted for degradation in the proteasome. The few mutant proteins that manage to pass the internal cellular quality control are eventually incorporated into the plasma membrane, but they are not correctly or completely folded, so their activity is low.

Nowadays, in addition to the traditional symptomatic therapy, a different pharmacological treatment based on the development of small-molecule drugs, called CFTR modulators, is being pursued to address the primary cause of CF. According to the underlying defect they are intended to address, CFTR modulators can be classified in potentiators and correctors.

Potentiators (e.g., Ivacaftor®) are small-molecule compounds that interact with the mutated CFTR channel at the cell membrane, increasing the flow of chloride ions. In contrast, correctors (e.g., Lumacaftor® and Tezacaftor®) modulates the quantity of CFTR present at the plasma membrane, increasing the processing and trafficking of mutated CFTR to the cell surface.

So far, Orkambi®, Symdeco® and Trikafta® are the combinations of small molecule drugs approved for the treatment of CF patients, carrying at least one copy of F508del mutation.

Aiming to expand the portfolio of novel modulators available to CF patients, also considering the significant but limited pharmacological efficacy elicited in particular by treatments with first generation’s correctors (e.g., Lumacaftor® and Tezacaftor®), there is
still the need to develop other CFTR small molecule modulators, primarily correctors, which may address the primary cause of CF by rescuing the activity of defective CFTR proteins. (Chapter 1)

The aim of this PhD project was the design, synthesis and biological characterization of novel CFTR modulators. In particular, the main topic of this work was oriented towards the identification of novel small-molecules as CFTR correctors.

The first part of project was mainly dedicated to the (re)synthesis of the primary hit ARN9364 (Figure s.1) (Chapter 2). The compound was identified by a high-throughput screening (HTS) of the IIT compound collection consisting of 11,334 commercial small molecules. The screening was conducted at the Istituto Giannina Gaslini (IGG, Genoa), using a phenotypic cell assay, on two different cell lines, Fischer Rat Thyroid [FRT] and Cystic Fibrosis Bronchial Epitelial-41o- [CFBE41o-] cells, both stably expressing the F508del-CFTR protein and transfected with the Halide Sensitive Yellow Fluorescent Protein (HS-YFP). ARN9364 was selected as a starting point for this PhD project due to its initial promising biological activity.

Unexpectedly, the careful UPLC/MS and NMR characterizations first, and biological data afterwards, proved that the chemical structures of the library hit and of the in-house re-synthesized compound were not in agreement. After an accurate analysis of NMR data (both $^1$H and $^{13}$C mono- and bi-dimensional studies), a new structure of the library hit was assigned (Figure s.1).

![Figure s.1: Wrong (left) and correct (right) chemical structure of ARN9364.](image)

The re-synthesis of ARN9364 was then planned following the synthetic approach reported in Scheme s.1.
Scheme s.1: Synthetic route for the preparation of compound 38 (ARN9364 with the correct structure).

Aiming to possibly elucidate the Structure-Activity Relationship (SAR) patterns around this novel chemo-type, new derivatives (Table s.1) were designed and synthesized, in particular focusing our attention on the pyrrolidine (R¹), pyrazole (R²) and amino-pyridine (R³ and R⁴) moieties (Chapter 2).
Table s.1: Synthesized ARN9364 analogs.

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</table>
All the synthetized racemic analogs were characterized by UPLC/MS and NMR analysis and subjected to *in vitro* biological assays to assess their activity in rescuing mutated CFTR function. The biological assays were performed at the Istituto Giannina Gaslini (IGG, Genoa) using a phenotypic cell-based assay (HS-YFP) on CFBE41o- cells, co-expressing F508del-CFTR and the halide-sensitive yellow fluorescent protein.\(^5\) The synthetized racemic compounds were tested in the concentrations range 20 \(\mu\)M – 1.25 \(\mu\)M (Chapter 3).

The biological activity of the newly synthesized racemic \textbf{ARN9364} analogs was evaluated in F508del-CFTR CFBE41o- cells and resulted only in a low/moderate increase in rescuing effect. In particular, at the highest tested concentrations (20 \(\mu\)M – 5 \(\mu\)M) compounds \textbf{39f} and \textbf{59c} showed an increase in activity when compared with the initial hit, while only compounds \textbf{39c} and \textbf{37} resulted in a moderate improvement in potency in the lower range of concentrations (2.5 \(\mu\)M -1.25 \(\mu\)M) (Chapter 3).

A second part of this project, aimed at the discovery of novel potent CFTR correctors, was focused on the design and synthesis of close analogs of a compound deriving from another hit identified in the same HTS. In this case, the initial hit \textbf{ARN5562} (Figure s.2) showed a chemically distinct structure from the previously described \textbf{ARN9364}, and an initial promising biological activity. Compound \textbf{ARN5562}, with its encouraging efficacy and potency, represented an important starting point for the search of novel correctors (Chapter 2).

![Figure s.2: Structure of new corrector hit ARN5562.](image)

Rounds of chemical modifications, exploring primarily the heterocyclic moiety on the right-end side and the substitutions on the pyrazole ring of \textbf{ARN5562}, provided the information to build Structure-Activity Relationships (SARs) and to identify two distinct structural classes of correctors. In particular, the replacement of the 1,4-dioxane ring with an unsubstituted 5-membered dioxole group, along with the cyclization of positions 4 and 5 of the pyrazole into a tetrahydro-pyridine moiety, allowed identifying lead compound \textbf{ARN21750} (Figure s.3), which showed a better biological profile in CFBE41o- cells.
Further modifications on the structure of this lead structure led to identify few analogs featuring a triaza-tricyclic-undecadiene scaffold, substituted in the tetrahydro-pyridine ring with an isoxazole sulphonamide group. From the initially identified potent corrector 91 (Figure s.3), in order to additionally investigate other modifications and to possibly modulate drug-like properties, subsequent SAR studies were focused on exploring the heterocyclic moiety on the right-end side of the molecule, while keeping unmodified the substituted azatricyclic sulfonamide moiety (Chapter 3).

![ARN21750 and 91](Figure s.3: Substituted azatricyclic compounds ARN21750 and 91.)

Although the biological data from these analogs were confirmed to be unsatisfactory in terms of rescuing effect of F508del-CFTR activity in CFBE41o- cells, this contribution to the SAR studies allowed to eventually discover other potent drug-like analogs. Among them, one compound, characterized in vitro (CFBE41o- cells and primary human bronchial epithelial cells from F508del/F508del CF patients) by high potency in rescuing the activity of F508del-CFTR, was further investigated for its drug-like profile and selected as preclinical development candidate for the treatment of CF.

The third part of this project (Chapter 4) was conducted at Helmholtz Institute for Pharmaceutical Research Saarland (HIPS, Saarbrücken-Germany) and was focused on the development of novel inhibitors of the anti-infective target DXS (1-Deoxy-d-Xylulose-5-phosphate Synthase) using a Dynamic Combinatorial Chemistry (DCC) approach.

Discovery and development of new antibiotic agents with novel targets and mechanisms of action are urgently needed due to the increase of antibiotic resistance developed by pathogen or infectious agents.

DXS is the first enzyme of the 2C-methyl-D-erythritol-4-phosphate (MEP) or nonmevalonate pathway, which is absent in humans but is essential for medically relevant pathogens (e.g., *Plasmodium falciparum*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *methicillin-resistant Staphylococcus aureus*), which use this pathway for
the biosynthesis of isoprenoid precursors, vitamins B1\(^6\) and B6\(^7\). The absence of the MEP pathway in humans and the possibility to inhibit three crucial bacterial biosynthetic pathways at once, make it a promising target for the development of new antimicrobial chemotherapeutics.

As hit-identification strategy for the development of new DXS inhibitors the target-directed Dynamic Combinatorial Chemistry (tdDCC) was used. With this kind of technique, it is possible to generate compound libraries by reversible reaction of building block fragments (Figure s.4). These Dynamic combinatorial libraries (DCL) are under thermodynamic control and their composition is the result of the thermodynamic stability of each member. Because of the continuous interconversion of the constituents, these libraries are adaptive and external stimuli, such as a target protein, can influence their composition. The addition of a target protein indeed causes the shift of the DCL equilibrium composition, binding, stabilizing and amplifying library components with the strongest affinity for the target.\(^8\)

![Figure s.4: Schematic illustration of tdDCC.](image)

Different kind of reversible reactions could be used in tdDCC to form interchanging products from dynamic combinatorial libraries (DCL) in the presence of a target protein. For this study, we decided to use the acylhydrazone chemistry due to its compatibility in the physiological condition. To expand the structural diversity and obtain potent and selective inhibitors of DXS, we designed a dynamic combinatorial library (DCL) for acyl hydrazone formation. Different heterocyclic hydrazides and aldehydes were chosen based on the calculated estimated affinity of all possible acyl hydrazone products, using LeadIt and SeeSAR as software.

Biochemical evaluation of several hit compounds amplified in the tdDCC experiment against *M. tuberculosis* DXS and *D. radiodurans* DXS afforded inhibitors with IC\(_{50}\) in the range of 30 \(\mu\)M – 190 \(\mu\)M.
Bibliography


