Synthesis development towards FMN riboswitch ligands as novel antibacterial agents

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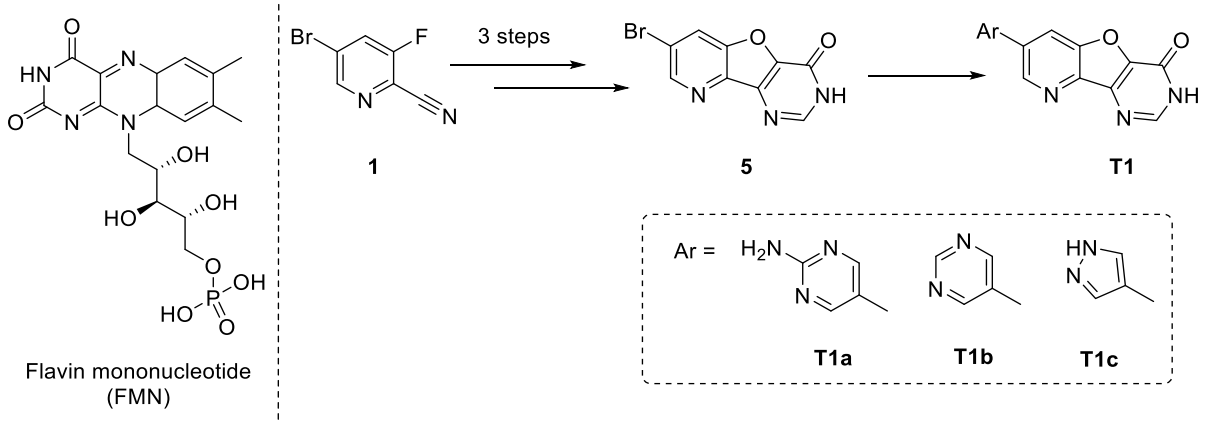
Abstract

Multidrug resistant (MDR) bacteria pose a serious threat to human health. Over the last decades more and more human pathogens have developed resistance against antibiotics and no new classes of antibiotics have been brought on the market since the 1980's. As such, development of classes of antibiotics with new mechanisms of action is a desperate necessity. Herein, the flavin mononucleotide (FMN) riboswitch is focused upon as novel target for antibiotics. Located in the 5' untranslated region of bacterial mRNA, the FMN riboswitch can be targeted with small molecule drugs to downregulate essential protein expression in bacteria and thus function as an effective antibiotic. Molecular modelling and SPR assays has revealed a target scaffold (**T1**) that shows strong binding affinity to the FMN riboswitch. The aim of this project was to prepare **T1** to allow for further assays to be performed. In addition, the synthetic pathway should be improved and allow for introduction of a variety of heteroaromatic substituents.

In this work, the synthesis route has been shortened from five steps to four by combining two steps as a one-pot reaction. While previous attempts have failed to prepare scaffold **5** and then perform a Suzuki-Miyaura coupling to prepare a variety of **T1** analogues, this research project has investigated experimental conditions to allow for the coupling reaction to be performed as the last step. Use of a large excess of base (10 equivalents) was found to be essential to achieve satisfactory conversion in the Suzuki-Miyaura coupling, as the pyrimidin-4(3*H*)-one scaffold itself contains a fairly acidic proton (pK_a ca. 11) which can be deprotonated under these reaction conditions. Additionally, the use of more polar solvents such as a dioxane-water mixture in a 5:1 ratio or DMSO are favourable for the Suzuki-Miyaura coupling reaction. **T1a** and **T1b** have been prepared and after further purification can be screened for their binding affinity to the FMN riboswitch and potentially function as a novel class of antibiotics.



List of abbreviations

ACN	- acetonitrile
ADDP	- 1,1'-(azodicarbonyl)dipiperidine
AMR	- antimicrobial resistance
DEAD	- diethyl azodicarboxylate
eq.	- equivalents
FAD	- flavin adenine dinucleotide
FMN	- flavin mononucleotide
FOR	- frequency of resistance
HPLC	- high-performance liquid chromatography
MDR	- multidrug resistant
MHz	- megahertz
MIC	- minimal inhibitory concentration
mRNA	- messenger ribonucleic acid
nM	- nanomolar
NMR	- nuclear magnetic resonance
PABA	- *p*-amino benzoic acid
PDB	- Protein Databank
PTPP	- pyrithiamine pyrophosphate
RBS	- ribosome binding site
RP	- reversed phase
RT	- room temperature
SEM	- 2-(trimethylsilyl)ethoxymethyl
SPR	- surface plasmon resonance
TFA	- trifluoroacetic acid
TIPA	- N,N,N',N'-tetraisopropylazodicarboxamide
TPP	- thiamine pyrophosphate
UTR	- untranslated region
WHO	- World Health Organization

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1. Introduction

After the discovery of penicillin by Alexander Fleming in 1928 [1], the development of antibiotic drugs evolved rapidly. Diseases that were previously incurable and lethal, such as syphilis, pneumonia and tuberculosis became treatable and the mortality rate of infections contracted during childbirth or routine surgery became vastly reduced [2]. However, over the last decade, an ever-increasing number of bacterial pathogens have developed a resistance against antibacterial drugs. Through continued overuse and misuse of antibiotics in human and veterinarian medicines bacteria have evolved antimicrobial resistance (AMR) [3]. Furthermore, research and development of new classes of antibiotics has been sluggish. A combination of these two issues has led the World Health Organization (WHO) to call out for an emerging healthcare crisis [4]. If current development of AMR continues at this pace, AMR is expected to cause over 10 million deaths annually by 2050 and damage the global economy with up to 100 trillion EUR [2].

Since the discovery of the first antibiotics, hundreds of different types of antibiotic drugs have been marketed, though they can generally be classified into six categories: β-Lactams, fluoroquinolones, oxazolidinones, aminoglycosides, sulphonamides and lipopeptides [5]. Thus, most prescribed antibiotics combat bacterial infections following a mechanism of action of one of these six classes. Given the widespread nature of bacterial diseases, the options for curing an infection are rather limited. Moreover, all antibiotic classes were discovered and brought on the market in the golden era of antibiotics, which dates between 1928 and 1987 [6]. After the discovery of lipopeptides in 1987, the ''discovery void'' began [7]. A lack of innovation and motivation from both academic and commercial research institutes has led to a halt in the discovery of new antibiotic classes. Back then nearly all infections could be treated with the available drugs, as AMRs were not posing a severe threat and investigating new antibiotic classes was considered a waste of resources. Development of a new antibiotics *de novo* imposes a high economical risk. It can take a substantial amount of time before a target drug has been identified and taken through all the steps of preclinical and clinical testing. And after putting in all this effort, chances for successful clinical trials and getting on the market are slim. To date, the financially safer option has been to expand the antibiotic library within already existing classes by improving approved, marketed drugs in efficacy and safety, as well as implementing modifications to hit resistant pathogens [6].

However, the current approach is not sustainable and a class of antibiotics that works with a new mechanism of action and functions against multidrug resistant pathogens is a desperate necessity. In this work the FMN riboswitch, which is located in bacterial messenger RNA (mRNA), is focussed upon as a novel target for antibacterial drugs. Ligands are investigated that can bind to the riboswitch with high affinity and turn the switch ''off'' in order to downregulate essential protein expression.

1.1. Antibiotics to suppress bacterial growth

Antibiotics are a class of drugs that can both prevent and cure bacterial infections. They can be classified into two categories depending on the range of bacteria they work against, broad-spectrum and narrow-spectrum antibiotics [8]. The broad-spectrum antibiotics target unspecific bacterial infections. They are usually prescribed when an infection is suspected, but the exact bacteria that causes it or the group of bacteria it belongs to is not identified. They are also prescribed when the infection is caused by a multitude of bacteria. In theory the broad-spectrum antibiotics are ideal to be used in any infection, however they come with substantial downsides. Not only are they a very disruptive class of antibiotics, as they can also attack bacteria of the healthy human microbiome, but they are also prone to induce the formation of resistant pathogens [9]. Whenever possible, narrow-

spectrum antibiotics should be deployed. These antibiotics only targets a very specific bacteria or group of bacteria while being less disturbing for human microorganisms [10]. However, to employ narrow-spectrum antibiotics a clinical specimen of the infection must first be tested for drug sensitivity in a laboratory. Since this is a rather time-consuming process, it is not always feasible.

There are several mechanisms of action an antibiotic drug can follow to cure a bacterial infection. Generally, they either block vital processes needed for bacterial survival and duplication or the drugs destroy the bacterial cells directly by disrupting the integrity of the cell wall, see Figure 1 [5]. For instance, β-lactams, such as penicillin, prevent bacterial growth via binding of the β-lactam ring to DD-transpeptidase, a bacterial enzyme involved in bacterial wall synthesis. By targeting this enzyme, the cross-linking of peptidoglycan in bacterial cell walls is hindered [11]. Antibiotics belonging to the class of fluoroquinolones prevent bacterial growth by inhibiting two bacteria specific enzymes that are involved in unravelling duplex strands of bacterial DNA, preventing DNA duplication [12]. Both oxazolidinones and aminoglycosides target bacterial RNA to prevent the synthesis of essential proteins for bacterial survival [13] [14]. While oxazolidinones prevent translation by binding to the 50S ribosomal subunit of bacterial cells, aminoglycosides target the smaller 30S subunit. When an antibiotic is bound to either subunit, they cannot combine to form a 70S ribosome and protein expression is downregulated [15]. Sulphonamides function as a competitive inhibitor of *p*-amino benzoic acid (PABA) in the folic acid metabolism cycle, preventing the production of folic acid (vitamin B9). Without this essential vitamin bacteria cannot synthesise, repair and methylate DNA [16]. Finally, lipopeptides depolarize bacterial cell membranes, disrupting the wall integrity, which ultimately leads to cell death [5]. All currently prescribed antibiotics follow one of the six previously described mechanisms. A bacterial infection is a rather common ailment and to only have six classes of medicines to treat it is a rather small pool to select from. In addition, since bacteria can develop resistance against a whole class of antibiotics, the available options are rapidly reduced. Therefore, it is important to develop new classes of antibiotics that work with a fundamentally different principle.

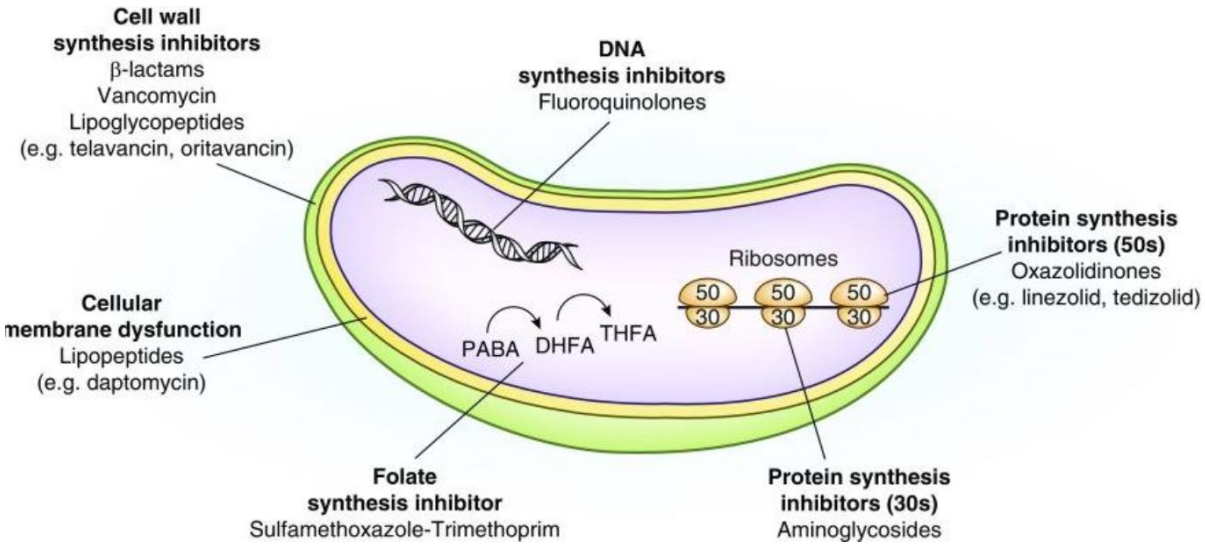


Figure 1. The targets of various antibacterial drug classes to block vital processes of bacteria. Reproduced from ref. [5]

1.2. Development of antimicrobial resistance by pathogens

Bacteria have a remarkable ability to adapt to their environment in order to survive and flourish under harsh conditions. Bacteria, such as the extremophiles, are known to have evolved and thrive under extreme harsh conditions such as in temperatures below –22 °C in frozen seawater or above 122 °C in hydrothermal vents, and even under extreme high pressure and extreme acidic or basic conditions [17]. However, this extraordinary ability to adapt to the environment also allows human pathogens to survive under harsh conditions that are aimed at eliminating the pathogen, i.e. during antibiotic treatment.

There are multiple ways in which a pathogen can become resistant to antibiotics. It can be either though intrinsic, adaptive or acquired principles [18]. Some pathogens are intrinsically more likely to develop resistance than others. For example, Gram-negative pathogens exhibit much higher intrinsic resistance than Gram-positive pathogens. This is due to the difference of the outer membrane of the cell. Passive diffusion of hydrophobic compounds is much slower through the outer membrane of Gram-negative bacteria and diffusion of larger hydrophilic compounds is essentially prevented. Thus, an antibiotic drug such as the large and hydrophilic glycopeptide vancomycin, which is commonly used against the Gram-positive methicillin-resistant *Staphylococcus aureus*, is completely ineffective against Gram-negative pathogens [19]. Furthermore, some Gram-negative pathogens lack non-specific porins in the outer membrane through which antibiotics can permeate, fully restricting access to the inner cell or they have even developed drug efflux pumps that actively pump out the unwelcome drugs. Intrinsic resistance can also be derived from an inherent lack of druggable binding sites for the macromolecular targets [18]. For a site to be considered druggable it needs to be able to bind to a small, drug-like molecule with high affinity and selectivity and subsequently lead to a therapeutic benefit. Many factors of the site play a role in order to obtain strong binding, such as the size of the pockets, the overall charge and hydrophobicity of the interaction surface [20]. A pathogen can inherently lack these proteins with druggable sites in its macromolecular structure.

Adaptive resistance is the ability of a pathogen to temporarily adapt to a change in environmental conditions by altering its intracellular activity, such as entering a dormant state. Furthermore, pathogens can temporarily halt their replication process. Antibacterial drugs that target DNA replication will have no effect on the pathogen if the replication process has paused. Once the drugs have been excluded from the body, the pathogen can switch to the active state again and restart the replication process [21]. Adaptive resistance also describes temporarily expression of essential genes and proteins that are required for survival. For instance, *P. aeruginosa* can survive in iron deficient environments by excreting high affinity iron chelating molecules that steal iron from host proteins [18].

Acquired resistance describes evolution and genetic modification of bacterial DNA, allowing bacteria to gain immunity against antibacterial drugs following the survival of the fittest principle. Certain processes can be acquired allowing bacteria either to (i) chemically modify or deactivate the antibiotic drugs, (ii) genetically mutate the targeted site of the antibiotics, (iii) increase the efflux of antibiotics or (iv) reduce intracellular uptake of the drug. This genetic mutation usually leads to high level of resistance against a specific class of antibiotics drugs. An example of a pathogen that has developed a β-lactam resistance is *K. pneumoniae*. This pathogen has evolved to produce β-lactamase, an enzyme that is capable of hydrolysing the β-lactam functional group of β-lactam antibiotics, rendering it inactive in preventing bacterial cell wall synthesis. Through expression of this enzyme all drugs belonging to the β-lactam group, such as penicillins, cephalosporins and carbapenems, are ineffective against this pathogen [18]. Development of acquired resistance of pathogens is accelerated by continued exposure of pathogens to antibacterial drugs, which in turn is a factor of continued use

and misuse in both the human- and veterinary medical sector. Antibiotics are haphazardly prescribed for any ailment and loading livestock with antibiotics is a standard procedure in the animal agricultural industry [22].

In general, however, AMR is a result of a combination of all these factors. When developing a new class of antibiotics it is not only important to ensure an effective inhibition of bacterial growth at low concentrations, but also that this can sustain for an extended period of time. A drug that pathogens quickly get resistant to is impractical to develop. Thus, a new drug needs to be screened against the frequency of resistance (FOR) that pathogens exhibit.

1.3. Targeting riboswitches with small molecules as novel antibiotic drugs

Riboswitches are commonly found in the 5' untranslated region (UTR) of messenger RNA (mRNA). These non-coding parts of mRNA fold into 3D-structures that have defined binding pockets and can recognize specific ligands and regulate gene expression accordingly. They are almost exclusively found in prokaryotic cells, and as such are considered a promising target for antibacterial drugs [23]. The first example of bacterial mRNA being targeted was published in 2002, where expression of a vitamin B12 transporter protein was downregulated in *E. coli* by targeting a riboswitch with a small, drug-like molecule [24]. A riboswitch consists of two domains, an aptamer and an expression platform. The aptamer domain is the RNA element responsible for ligand recognition, which induces a conformational change of the 3D-structure of the RNA (see Figure 2). The Watson-Crick base pairs that stabilize the molecular structure of RNA get disrupted and reorganize to form a new stable conformation. In turn, this causes the section of RNA that expresses the production of essential metabolites, the expression platform, to undergo a conformational change as well. Depending on the type of switch, the conformational change can turn the expression platform into an anti-termination stem (ON-switch) or a termination stem (OFF-switches). The latter is the case for the FMN riboswitch where a terminator/sequester loop is formed, and the ribosome binding site (RBS) becomes inaccessible. The riboswitch is turned OFF and protein expression is stopped.

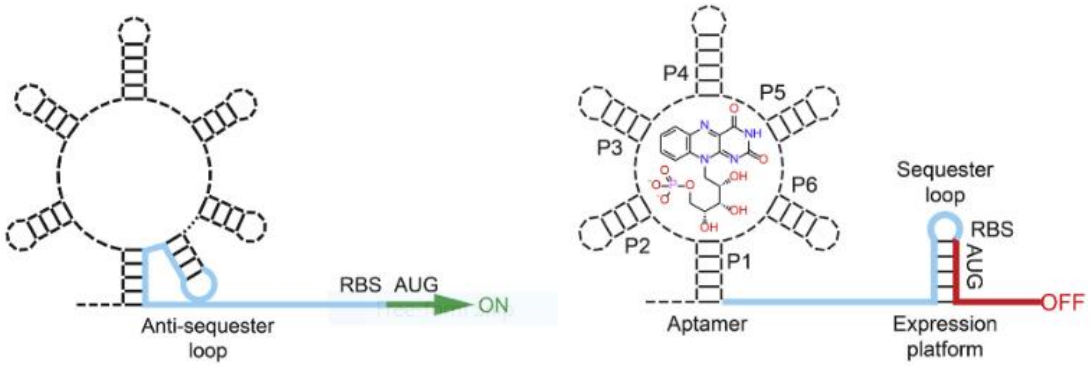


Figure 2. Conformational change of a riboswitch after binding of a cognate ligand (FMN depicted here), switching it OFF. Adapted from ref. [25]

Currently, 28 different riboswitches have been investigated and have experimentally proven to be effective targets for antibiotics [23]. One of the first examples that has been discovered, even before the identification of the principle of riboswitches, is pyrithiamine which targets the thiamine pyrophosphate (TPP) riboswitch. The TPP riboswitch is one of the most commonly found riboswitches in nature and is the only riboswitch present in eukaryotes. Thus, pyrithiamine can be used to prevent

growth of algae, fungi and plants. Pyrithiamine is intracellularly phosphorylated to the active form pyrithiamine pyrophosphate (PTPP). This analogue of TPP can then bind to the TPP riboswitch and prevent the production of essential metabolites [26].

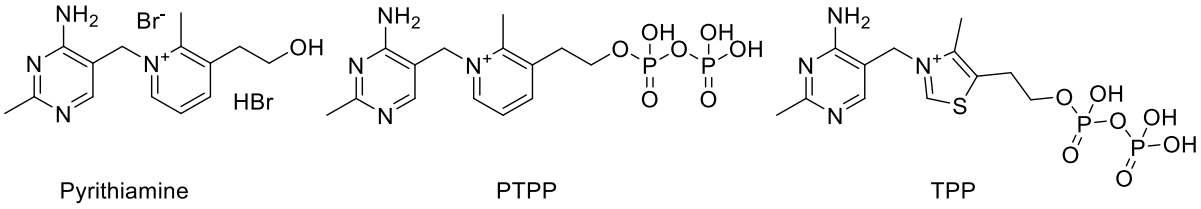


Figure 3. Pyrithiamine is intracellular phosphorylated to PTPP, a ligand of the TPP riboswitch

It is essential for a cognate ligand to bind to the aptamer domain of the riboswitch with both high selectivity and high affinity (K_D < 10 nM). Through rational design, endogenous ligands can inspire the development of new structures or functional analogues. Through emergence in research, more and more crystal structures of riboswitches are being reported. Currently, the Protein Databank (PDB) contains over 300 structures that can be used for rational design of new cognate ligands [23]. From a pharmacological point of view only riboswitches are of interest that are solely present in pathogenic bacterial strains and preferably in a wide range of strains, so that a cognate ligand can be designed that functions as an antibiotic drug for many different bacterial infections and classify as a broad-spectrum antibiotic. Furthermore, the riboswitch needs show high druggability, i.e. it needs to have a binding pocket of proper size for high affinity ligand binding, the pockets should not be too polar or too non-polar and most importantly, it needs to induce a conformational change and function as an OFF-switch once the ligand has bound.

High affinity binding to the aptamer domain can be achieved by multiple means. First, the cognate ligands must be of appropriate size and shape to fit seamlessly into the pocket of the aptamer. Second, as RNA mainly consists of nucleobases, interaction of the ligand with these structures is important, which is achieved through hydrogen bonding with the nucleobases and π-π stacking of the aromatic rings. Next to the aromatic nucleotides, the polar ribose and phosphate groups of the backbone of RNA can be utilized to create strong dipole-dipole interactions.

1.3.1. The FMN Riboswitch

The flavin mononucleotide (FMN) riboswitch is responsible for the expression regulation of genes involved in biosynthesis and transport of the vitamin B2, also known as riboflavin (see Figure 4). Transport of riboflavin is essential for the survival of bacteria as it is a key component in many biological processes, such as oxidative phosphorylation and energy transfer in the citric acid cycle. Furthermore, riboflavin is a precursor for FMN, which in turn is a precursor for flavin adenine dinucleotide (FAD). Both FMN and FAD play essential roles in a large variety of biological processes. Flavins have multiple oxidation states and as such play a vital role in catalysing difficult intracellular redox reactions, such as the oxidative dehydrogenation of a C-C bond to form an alkene [27]. The FMN riboswitch is one of the most studied riboswitches as a target for antibiotics and it is found in 41 human pathogens, including pathogens on the WHO-priority list such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophiles influenzae* [23]. Due to the high natural occurrence of the FMN riboswitch in pathogens and the absence in human RNA it is an excellent target for broad-spectrum antibiotics.

The naturally occurring ligand for the FMN riboswitch is the phosphorylated form of riboflavin, FMN, see Figure 4. Roseoflavin is another naturally occurring compound, extracted from *Streptomyces davawensis*, which showed to have antibacterial activity and especially in its phosphorylated form is showed to bind to FMN riboswitch with high affinity, of approximately 3 nM [23]. The adjoined aromatic rings of the structure ensure good hydrophobic interaction through π-π stacking with the pocket of the FMN riboswitch aptamer domain. The aromatic rings are sandwiched between the nucleobases A48 and A85 and further hydrogen bonding between the carbonyl moiety of FMN and the sugar backbone of A48 and exocyclic nitrogen of A99 further enhances the binding affinity [28], see Figure 5 A.

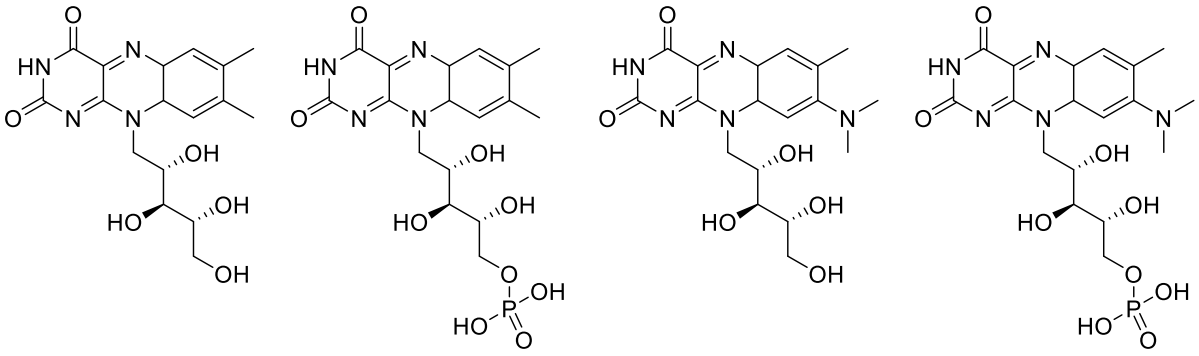


Figure 4. Structures of ligands of the FMN riboswitch and their binding affinity [23]

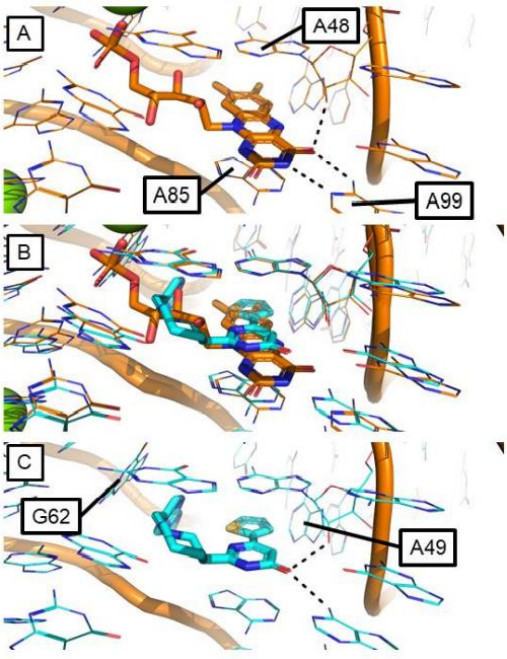


Figure 5. X-ray crystal structure of the FMN riboswitch shows interactions with A) FMN, B) FMN and Ribocil-C superimposed and C) Ribocil-C. Adapted from ref. [28]

Through phenotypic screening, Merck has investigated a library of over 57,000 compounds for activity against an *E. coli* strain. They successfully identified and disclosed ribocil (see Figure 6), a hit showing promising results in binding to the FMN riboswitch [29]. The *R*-enantiomer (ribocil-A) showed significantly less activity over the *S*-enantiomer (ribocil-B), of K_D > 10,000 over 6.6 nM respectively. Through further research and modification ribocil-C was developed, which showed an approximately 8-fold greater potency against *E. coli* strains [30]. However, pathogens show a high frequency of resistance (FOR) against ribocil-C [23], causing it to lose appeal to be used as a commercial antibiotic drug.

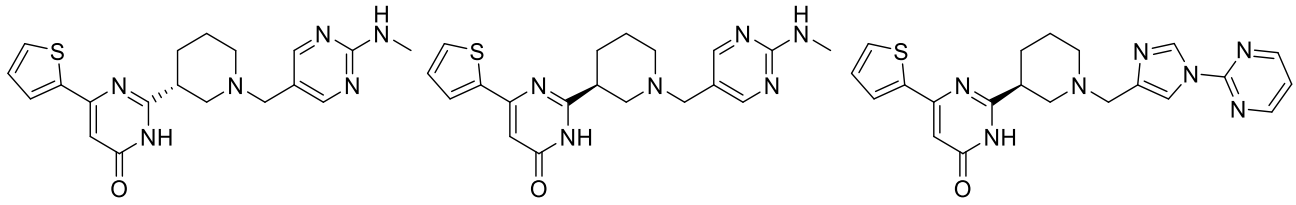


Figure 6. FMN riboswitch ligands developed by Merck

Previous research by our group of screening commercially available compounds against the FMN riboswitch has led to some promising results. Compound **RSL-0035**, see Figure 7 C, was found as a hit and showed activity of binding to the FMN riboswitch with a K_D of 240 µM [31]. **RSL-0035** has a similar core structure and proposed binding mode as FMN, with a tricyclic aromatic structure that can interact through π-π stacking with A48 and A85, see the cyan dashed lines in Figure 7 D. Furthermore, the pyrimidone ring (A) of **RSL-0035** displays the same crucial hydrogen bonding interactions with A99, as indicated by the black dashed lines.

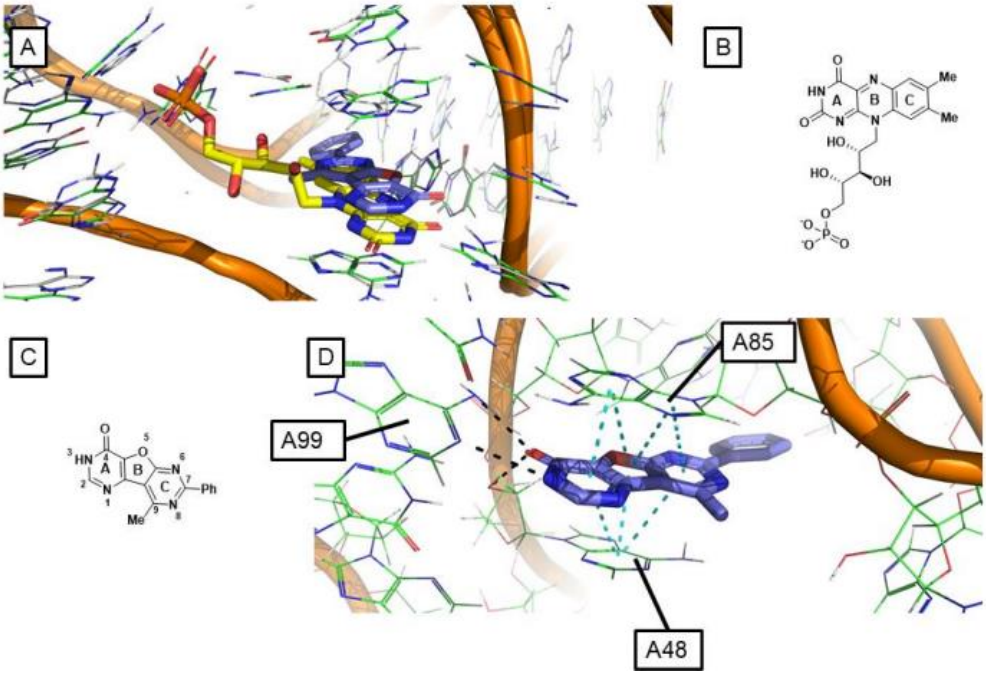


*Figure 7. A) X-ray diffraction structure of the FMN riboswitch bound with FMN (yellow) and superimposed with **RSL-0035** (blue), B) the structure of FMN, C) structure of **RSL-0035** and D) binding of **RSL-0035** to the FMN riboswitch. Reproduced from ref. [28]*

Through further docking studies a scaffold hit (**T1**) was identified. The C-7 phenyl substitution of **RSL-0035** showed no contribution to any binding modes, however it was proposed that heteroaromatic substitutions in the scaffold allow additional interactions, see Figure 8. Affinity of **T1a**, **T1b** and **T1c** to the FMN riboswitch was determined in a surface plasmon resonance (SPR) assay and was measured to have a K_D value of 2.2 ± 0.7 and 5.9 ± 0.3 µM for **T1a** and **T1b** respectively. Data of sufficient quality could not be obtained for **T1c** due to poor solubility of the compound in DMSO [28]. Based on other SPR experiments **T2** was devised, which does not contain the signature tricyclic aromatic structure that FMN and **T1** share. Though, promising results have been obtained and further modification can be made to increase binding affinity.

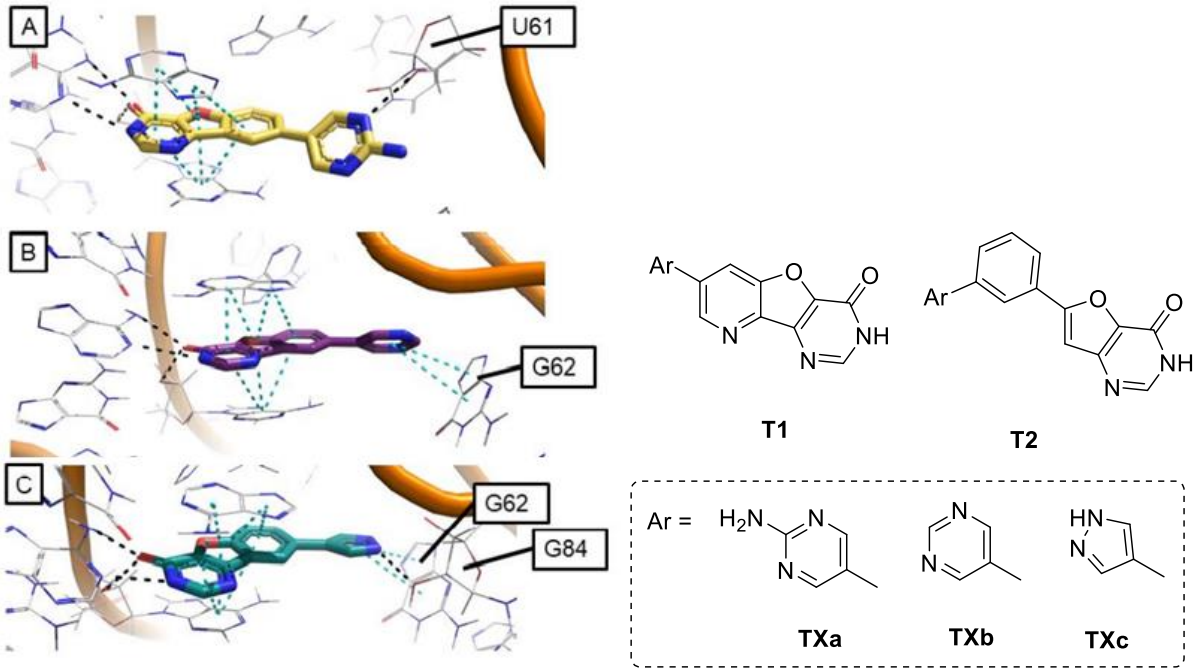


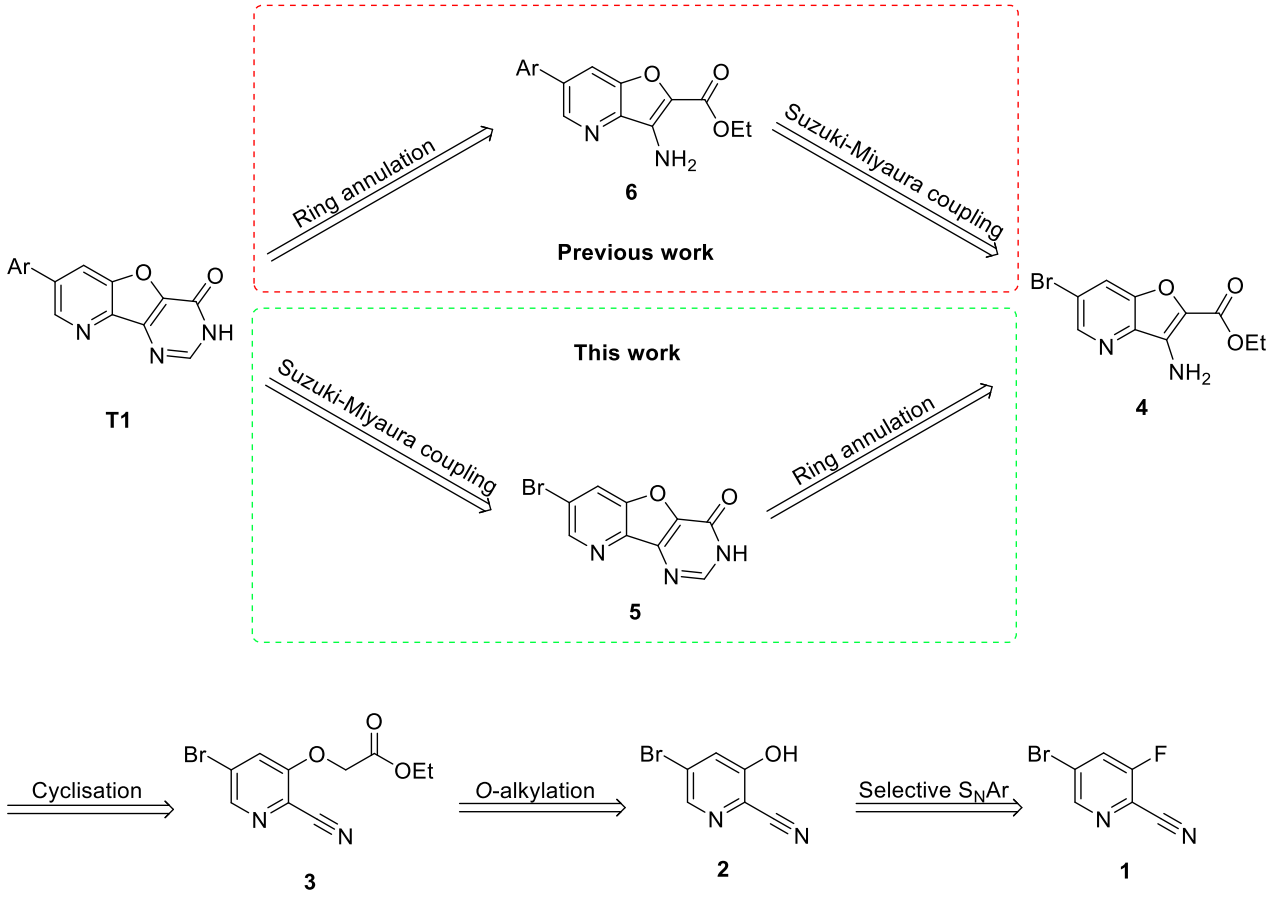
*Figure 8. X-ray diffraction structure of the FMN riboswitch with docking hits A) **T1a**, B) **T1b** and C) **T1c**. Adapted from ref. [28]*

1.4. Aim of this study

Previous research in the Brenk and Haug research groups has led to target scaffold (**T1**) that shows high binding affinity to the FMN riboswitch in SPR assay. However, later determination of the purity of the ligands showed the presence of an unknown purity, which questions the reliability of the measurements. The goal of this work is to prepare the scaffold and perform a final step modification to generate a series of analogues (**T1a-c**) that have potential to be used as starting points for the discovery of novel antibiotics.

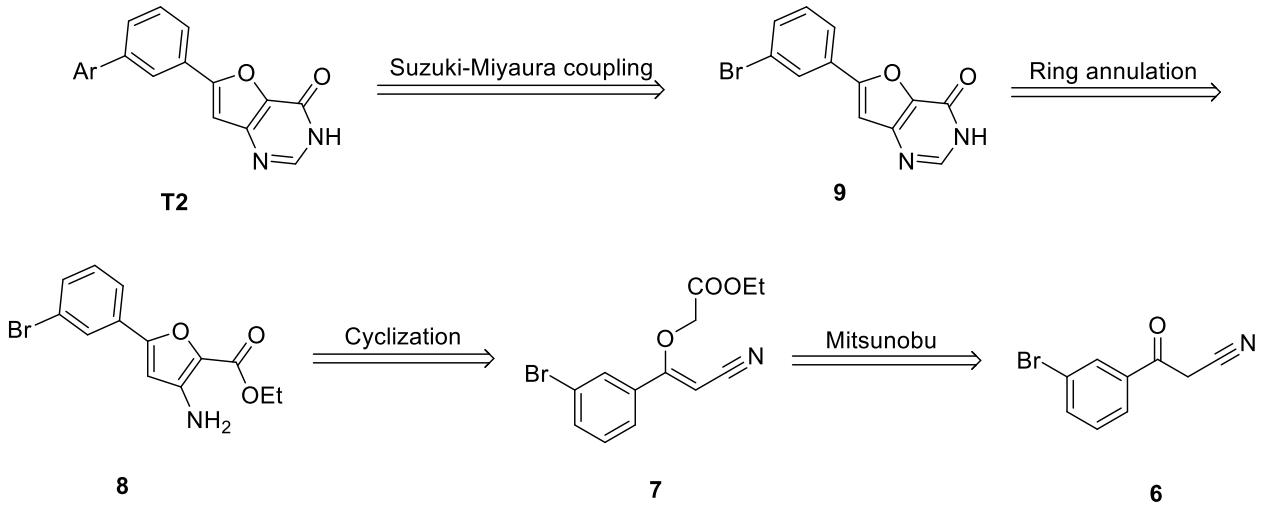
The synthetic approach to prepare **T1** consists of five steps, see Scheme 1. The first three steps are to prepare intermediate **4**. Previous attempts to prepare analogues of **T1** first had to perform a Suzuki-Miyaura coupling on **4** and subsequently perform the ring annulation, as final step Suzuki-Miyaura couplings were unsuccessful in obtaining conversion towards the coupled product [32]. However, this requires an additional step for each desired analogue, making it a time and yield inefficient procedure. The aim of this work is also to investigate the Suzuki reaction on the annulated

intermediate **5** and to identify a synthetic procedure to easily prepare a variety of heteroaromatic analogues of **T1**.



*Scheme 1. Retrosynthetic approach to prepare **T1***

This work also aimed at preparing analogues of **T2**, where the final step would be a Suzuki-Miyaura coupling (Scheme 2). The synthesis starts with an *O*-alkylation under Mitsunobu conditions, while the final three steps are similar to the synthetic strategy for **T1**.

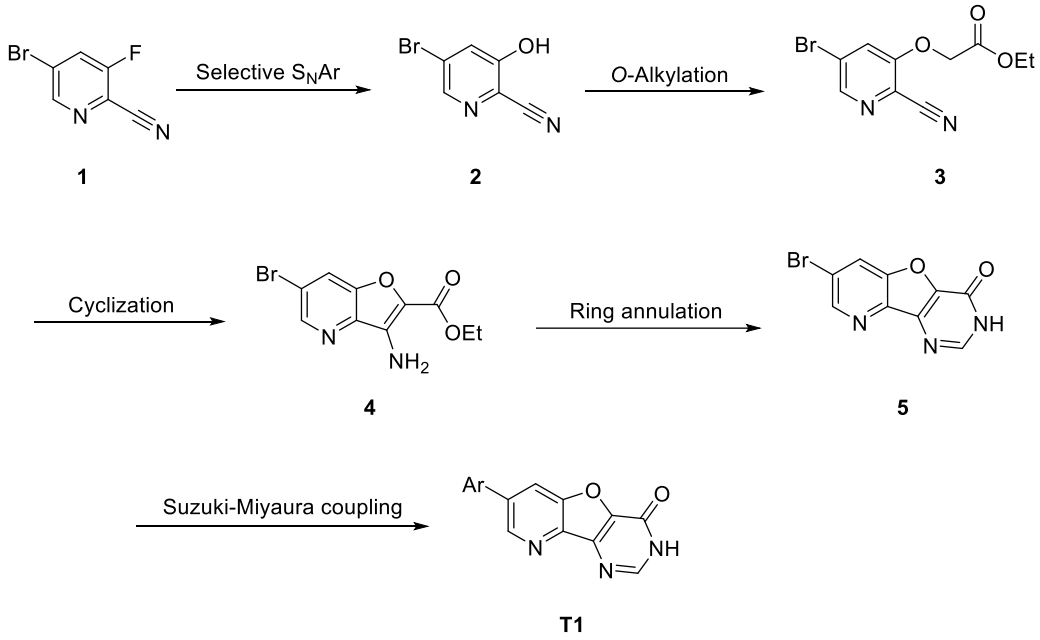


*Scheme 2. Retrosynthetic approach to prepare **T2***

2. Results and discussion

2.1. Synthesis towards T1

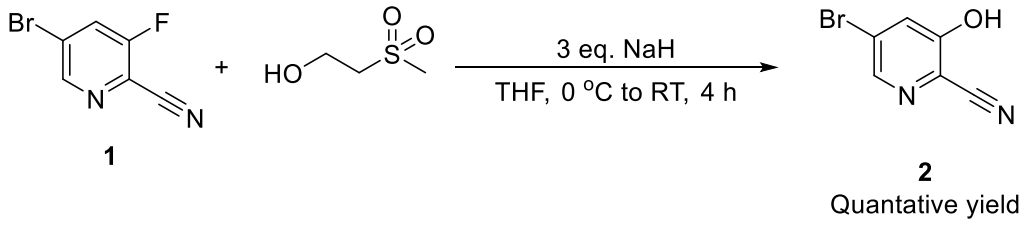
The suggested pathway to prepare **T1** was initiated by a selective nucleophilic aromatic substitution of the commercially available 5-bromo-3-fluoropicolinonitrile (**1**) to form the hydroxy picolinonitrile (**2**). Next, an *O*-alkylation of the hydroxy group with ethyl 2-bromoacetate was performed to generate ethyl 2-((5-bromo-2-cyanopyridin-3-yl)oxy)acetate (**3**). Deprotonation of the acidic α-hydrogen of the ester under elevated temperatures facilitated cyclization, forming the aromatic amine **4**. Subsequently, the third scaffold-ring was generated in an annulation reaction with ethyl formimidate. The bromo-substituted scaffold **5** was finally converted into target compounds **T1a-c** through a Suzuki-Miyaura coupling with various heteroaromatic boronic acids.



*Scheme 3. Synthetic pathway and experimental conditions to prepare **T1***

2.1.1. Selective nucleophilic aromatic substitution of 5-bromo-3-fluoropicolinonitrile

For conversion of the fluoro pyridine **1** to hydroxy pyridine **2**, a method reported by Rogers et al. was employed [33]. In this procedure methylsulfonyl ethanol is deprotonated by a base to generate an alkoxide which selectively displaces the fluor of electron deficient aryl fluorides. Next, an additional equivalent of base subtracts an alpha proton to the sulfonyl group and causes formation of a vinyl sulfoxide and elimination of the phenol product.

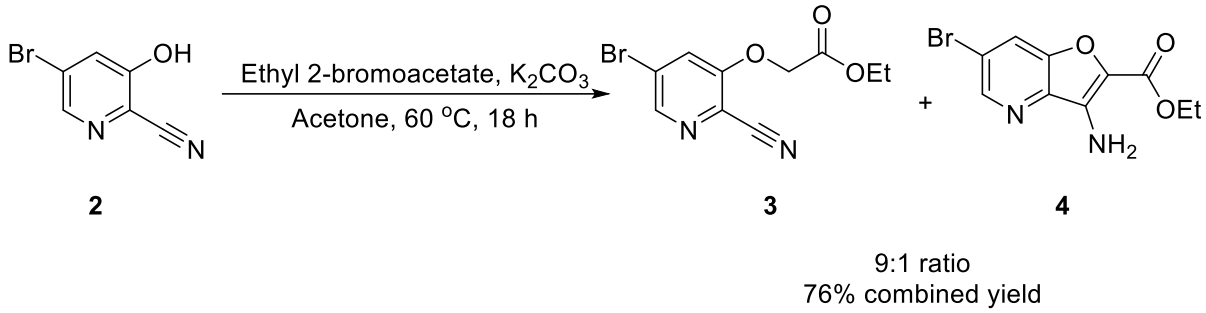


Scheme 4. Selective nucleophilic aromatic substitution of 5-bromo-3-fluoropicolinonitrile using 2-(methylsulfonyl)ethanol

After 4 hours reaction time, full conversion of the starting material was obtained. A facile acidic work-up with HCl resulted in the precipitation of the product, which was filtered off and used in the next step without further purification.

2.1.2. Alkylation of 5-bromo-3-hydroxypicolinonitrile

Subsequently, *O*-alkylation of hydroxy picolinonitrile (**2**) was done following a general S_N2 like reaction with ethyl 2-bromoacetate to prepare the ethyl oxyacetate **3**.

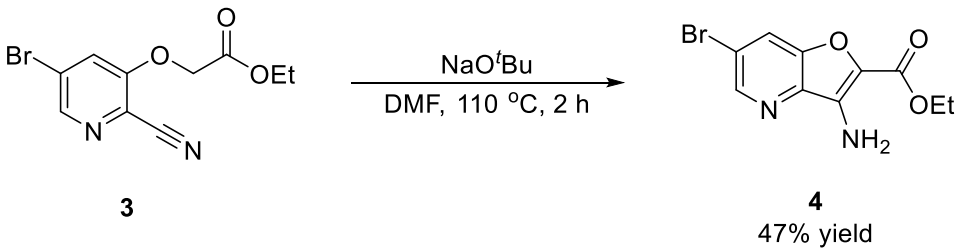


Scheme 5. Alkylation of 5-bromo-3-hydroxypicolinonitrile with ethyl 2-bromoacetate

Purification was done by quenching the reaction mixture with water and performing extractions with EtOAc, which yielded the product **3** in sufficient purity for the next step. However, it was observed by ^{1}H-NMR spectroscopy that another aromatic compound was present in a 1:9 ratio to compound **3**. This compound was identified to be the product of the next step, the cyclized product **4**. Evidently, under these mild conditions, the ester α-hydrogens can be deprotonated allowing cyclization to occur. This has inspired us to make further efforts to shorten the overall synthesis procedure and combine the alkylation and cyclization in a single step (see below).

2.1.3. Cyclization of ethyl 2-((5-bromo-2-cyanopyridin-3-yl)oxy)acetate

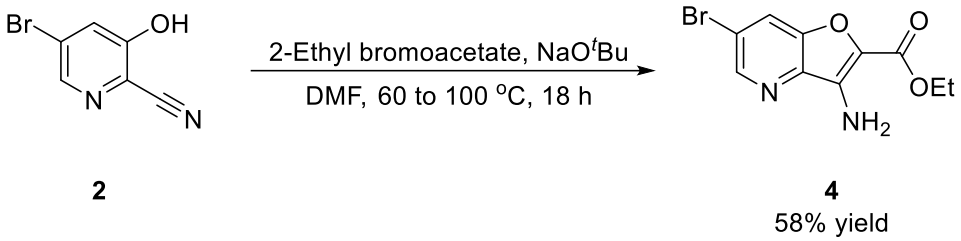
The ring closure in the two-step synthesis of compound **2** to form product **4** was performed using a catalytic amount of NaO^{t}Bu as base to form product **4** in 47% yield.



*Scheme 6. Ring cyclization to prepare the furan ring of compound **4***

To combine the *O*-alkylation and cyclization in a one-pot reaction, the reaction conditions of the separate steps were conjoined. First, using 1 equivalent of NaO^{t}Bu, the alkylation was performed by stirring the reaction mixture at 60 °C overnight. After full conversion was observed by TLC, an additional equivalent of NaO^{t}Bu was added and the temperature was increased to 110 °C for 4 hours. However, under these harsh conditions the product started to decompose. When the one-pot reaction

was executed again, but with 100 °C for 2 hours for the cyclization step, the aromatic amine **4** was obtained in 58% yield. Thus, by combining the two separate reactions as a one-pot procedure the yield can be increased from 36% to 58%, while shortening the overall synthetic pathway by one step.

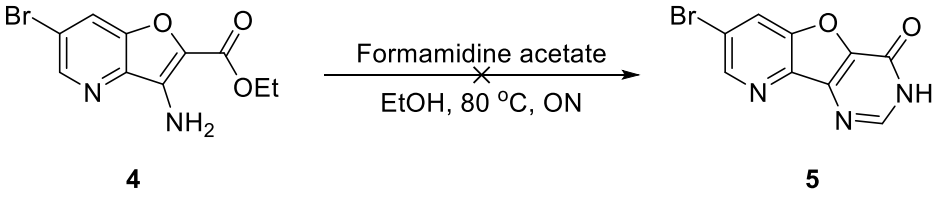


2 **4**
58% yield

Scheme 7. One-pot reaction of O-alkylation and cyclization

2.1.4. Ring annulation of ethyl 3-amino-6-bromofuro[3,2-b]pyridine-2-carboxylate

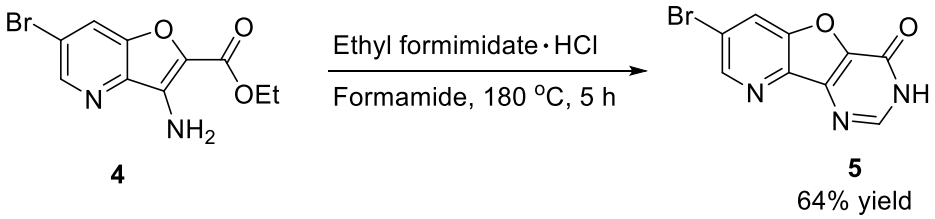
Next, the synthetic procedure will deviate from the original pathway. Whereas previously the Suzuki coupling is performed on compound **4**, using a variety of different heterocyclic boronic acids, here the ring annulation will be performed first. Previous work has developed a procedure for the ring annulation under mild conditions, using formamidine acetate in ethanol at 80 °C [34]. However, when the reaction was executed under these conditions no conversion or product formation was observed after 16 hours reaction time. Even elevating the temperature to 110 °C and 150 °C had no effect on the conversion.



4 **5**

*Scheme 8. Conditions for ring annulation using formamidine acetate to prepare the tricyclic structure **5***

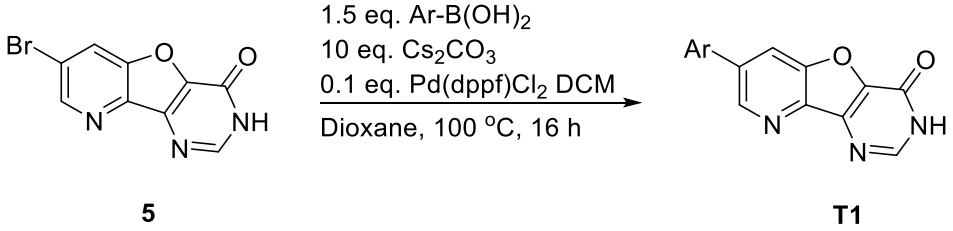
Instead, to prepare the tricyclic structure **5** a different procedure was applied that makes use of somewhat harsher conditions and ethyl formimidate as reactant. This does not require to first form the ethyl ester leaving group *in situ*. Performing the reaction at 180 °C overnight shows full conversion of the starting material by TLC. However, only minor product has formed. It is assumed that under these high temperatures and for this extended duration most of the product and starting material have decomposed. Shorter reaction time minimized product decomposition, yielding **5** with 64% yield, which crashed out of solution upon cooling to room temperature and could be isolated by filtration of the reaction mixture. Both ^{1}H- and ^{13}C-NMR of the product showed the presence of a small amount of aliphatic impurities and the product is assumed to contain decomposed, polyaromatic impurities which are insoluble in d_6-DMSO and thus not visible by NMR. Unfortunately, due to the insolubility of the tricyclic structure **5** in most solvents it could not be purified by flash chromatography. Recrystallization was attempted in methanol, ethanol, ethyl acetate and toluene without success. Ultimately, the product was used in the coupling reaction without further purification.



*Scheme 9. Conditions for ring annulation using ethyl formimidate to prepare the tricyclic structure **5***

2.1.5. Suzuki-Miyaura cross-coupling of 7-bromopyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one

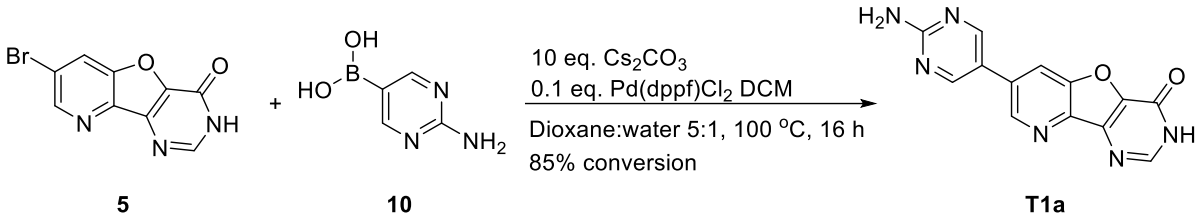
The final step in the preparation of **T1** is the Suzuki coupling. In this final step modification, a series of different heterocyclic boronic acids can be used to quickly create a series of **T1** analogues. Previous attempts using 2 equivalents of Cs_2CO_3 were unsuccessful at performing the coupling reaction and no product formation was observed.



*Scheme 10. General conditions for the Suzuki coupling to prepare **T1***

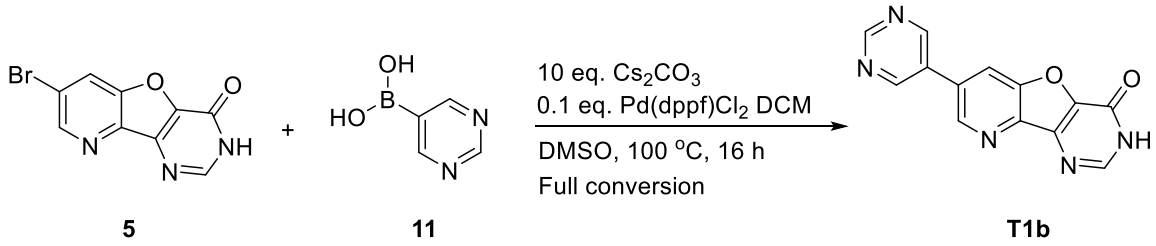
The first attempt to prepare **T1a** using (2-aminopyrimidin-5-yl)boronic acid (**10**) with 10 equivalents of Cs_2CO_3 showed that indeed some product had formed, albeit no full conversion was observed by TLC. Purification of the reaction mixture was initially troublesome as both the product and base are water soluble. Therefore, purification was done by reversed-phase chromatography of the reaction mixture with ACN:water + 0.1% TFA as eluent. Under these conditions the product eluted already at 1% ACN together with the rest of the impurities. The culprit was the large excess of Cs_2CO_3 being present in the reaction mixture. TFA is added to the eluent ensuring the protonation of the pyrimidone ring. However, the large excess Cs_2CO_3 counteracts the acidic eluent deprotonating the product, which strongly increases its polarity. Therefore, the product elutes in the first fractions together with the base Cs_2CO_3.

Since the conversion was poor, the reaction was attempted again with a different solvent. Dioxane is a rather nonpolar solvent, especially for such a polar compound. Solubility issues of both the starting material and the base could have led to poor conversion. A solvent mixture of dioxane:water 5:1 was used to enhance solubility, which led to a conversion of 85% of the starting material after 16 hours. The reaction mixture was then worked up by using 20 equivalents of HCl to neutralize the base Cs_2CO_3. This then forms CsCl salt and H_2CO_3, which in presence of water dissociates into CO_2 and water. Taking the product up in a small amount of DMSO, in which the CsCl salt is insoluble, and then performing RP chromatography with ACN:water + 0.1% TFA as eluent yielded the product. In order to submit the product for biological screening a purity of >95% needs to be obtained. The material was given to supervisor Jan-Åke Husmann for further purification by preparative HPLC.



*Scheme 11. Suzuki coupling of scaffold **5** to form **T1a***

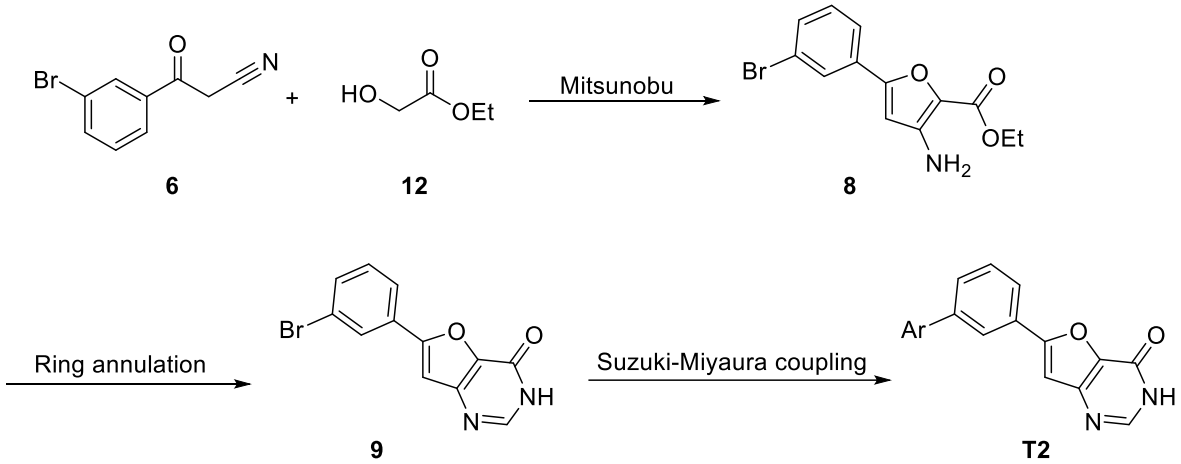
The next target compound, **T1b**, was obtained by performing a Suzuki coupling with pyrimidin-5-ylboronic acid (**11**). For this reaction DMSO was used, since it is more polar than dioxane:water and it could potentially circumvent solubility issues, as DMSO is the only known solvent the starting material is soluble in. Previous research has revealed DMSO to be an effective solvent for Suzuki coupling reactions [35]. The ^{1}H-NMR spectrum of the crude product shows full conversion of the starting material. The reaction mixture was worked-up with the same procedure as previously described and purified twice manually with RP-HPLC. However, the product was not obtained with high enough purity for biological screening, thus it has been submitted for preparative HPLC.



*Scheme 12. Suzuki coupling of scaffold **5** to form **T1b***

2.2. Synthesis towards T2

For the preparation of **T2** we planned to perform a one-pot reaction of first a Mitsunobu reaction on the α-cyanoketone **6** with ethyl glycolate followed by base induced cyclization to afford the aminofuran **8**. The subsequential part is similar as in the preparation of **T1**, namely a ring annulation followed by the Suzuki coupling.



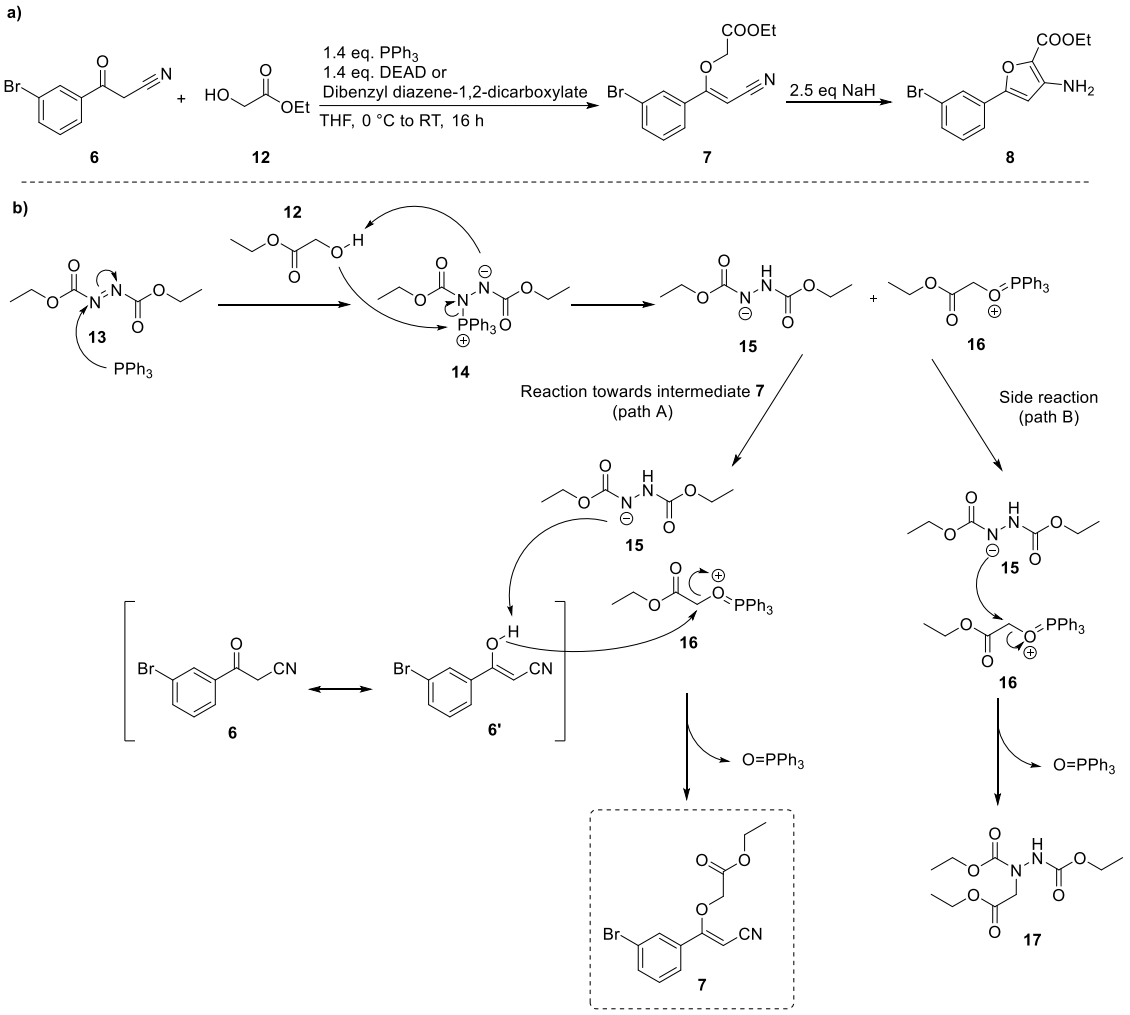
*Scheme 13. Synthesis pathway and experimental conditions to prepare **T2***

2.2.1. One-pot Mitsunobu reaction of 3-(3-bromophenyl)-3-oxopropanenitrile

A procedure is described by Redman et al. [36] which produces amino furans starting from α-cyanoketones. This procedure is a one-pot synthesis, where first an *O*-alkylation is performed under Mitsunobu conditions to form a vinyl ether, followed by a base-catalysed cyclization, see Scheme 14a. They found that comparable yields were obtained regardless of whether the synthesis was performed as a two-step reaction or as a one-pot reaction.

When the reaction was executed with dibenzyl diazene-1,2-dicarboxylate as azo coupling reagent, progression of the reaction was troublesome to keep track of by TLC. The starting material, intermediate, phosphine and coupling reagent all share a similar retention factor (R_f) around 0.5 using EtOAc:hexanes 3:7 as eluent. After 16 hours of reaction time, ^{1}H-NMR analysis revealed that only a minor amount of product was formed, approximately 10% conversion, see Figure 9. Peak A at 4.05 ppm belongs to C10 of the starting material and should shift over the course of the reaction to form the olefinic hydrogen at 5.05 ppm (peak B). In addition, C13 of the intermediate should become noticeable at 5.02 ppm (peak C). Heating the reaction for an additional 24 hours at 40 °C unfortunately had no effect on the reaction progress. When the reaction was performed with diethyl azodicarboxylate (DEAD) as coupling reagent only 2% conversion was obtained.

To make sure that the poor conversion was not attributed to the integrity of the reagents all materials were analysed by ^{1}H-NMR, however, no significant amount of impurities have been found. In the proposed mechanism of the Mitsunobu reaction, the phosphine first attacks the azo coupling reagent to form a betaine **14**, see Scheme 14. Betaine **14** reacts with the alcohol **12** to form anion **15** and phosphonium ion **16**. Phosphines are prone to oxidation to form a phosphine oxide. Phosphine oxides are not nucleophilic and cannot form the betaine. However, when the starting material was analysed by ^{31}P-NMR spectroscopy, less than 1% of the triphenylphosphine was found to have oxidized.



*Scheme 14. a) One-pot procedure of Mitsunobu type reaction followed by cyclization to form **8** b) proposed mechanism for the formation of the intermediate **7** and the undesired side reaction that can occur [37]*

Next, we considered that water in the THF could be the culprit, thus the reaction was performed again with dibenzyl diazene-1,2-dicarboxylate as azo coupling reagent and in dry THF that was obtained from an anhydrous solvent delivery system (SPS-800). This seemed to have a minor positive effect on the conversion, though the majority of the starting material was still present. The second step of the one-pot synthesis was performed, the addition of sodium hydride to form the amino furan. The resulting product was purified by flash column chromatography. However, in spite of several attempts to purify the crude product by column chromatography, the amino furan product **8** could not be separated from the phosphine and the azo coupling reagent as they share similar polarity.

Low conversion of the starting material could be attributed to the formation of undesired side products. Mitsunobu reactions generally have low yield when the acid that is used as starting material has a high pK_a. When the acid has a pK_a of over 11, protonation of the anion **15** is slow [38] and an undesired side reaction takes place, see Scheme 14b (path B). The anion **15** reacts in a nucleophilic substitution reaction with phosphonium ion **16** instead, and a C-N bond is formed. This procedure does not make use of a conventional Brønsted-Lowry acid, but instead relies on keto-enol tautomerization, where the enol needs to be deprotonated. When tautomerization is slow, the rate of path A is decreased and the reagents are lost to the formation of the side product **17** and the desired product is not formed. The degree of competition of the side reaction can be determined by

conducting a ^{31}P NMR experiment. Over the course of the reaction the concentration of the benzoyl acetonitrile **6** should not change while the phosphine is transformed into phosphine oxide.

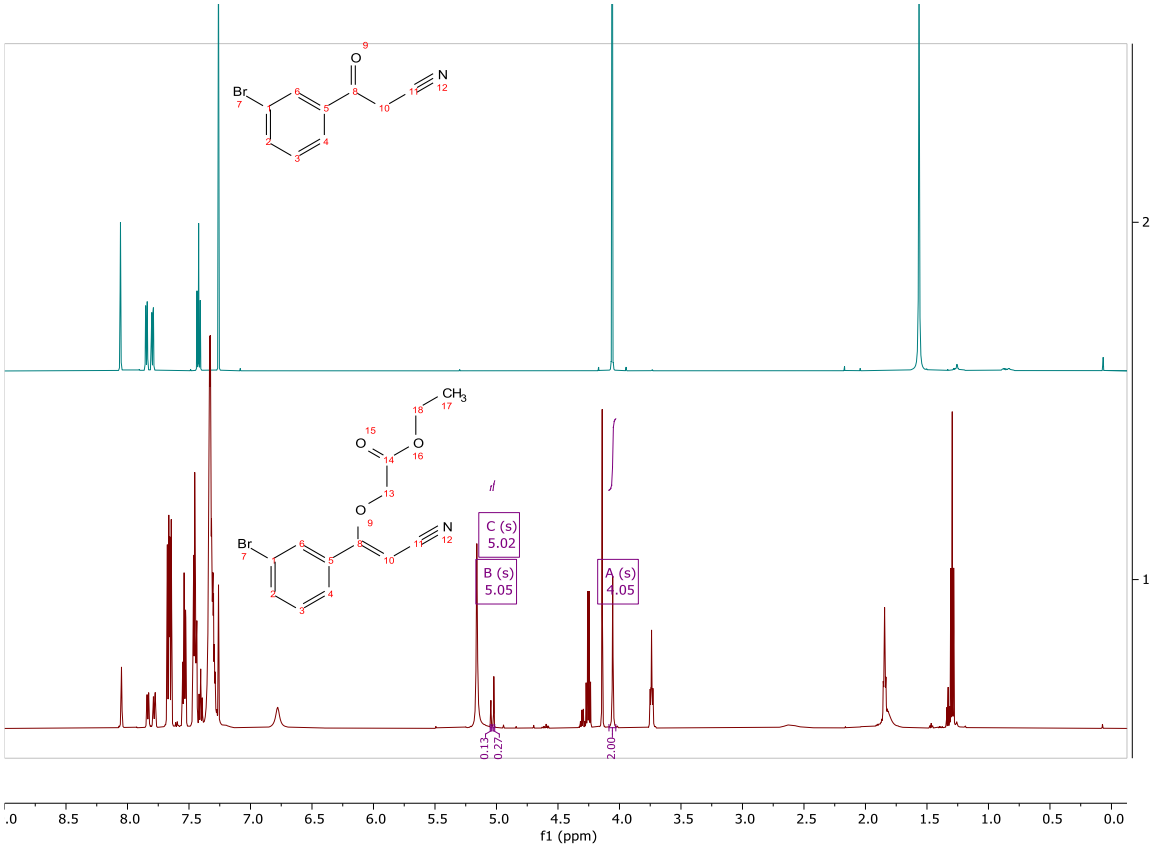


*Figure 9. ^{1}H-NMR spectrum of (top) starting material **6** and (bottom) the crude product mixture of **7***

3. Conclusion

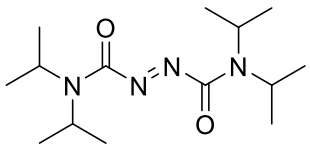
A method has been developed to prepare **T1** that shortens the overall synthesis path from five steps to four by combining the *O*-alkylation and cyclization as a one-pot reaction. Whereas previous attempts to synthesize scaffold **T1** have failed to perform a Suzuki coupling on the precursor **5** as a final step modification, experimental conditions were identified allowing a quicker preparation of a series of analogues containing varying heterocyclic aromatic substitutions. For the Suzuki coupling it is essential that a large excess of base (10 equivalents of Cs_2CO_3) is present. The Pd-catalyzed coupling requires 2 equivalents to run and additional base is required since the 1H of the pyrimidone ring is deprotonated during the reaction. In addition, a more polar solvent than dioxane is required to counteract the solubility issues of the polar reagents. Both a solvent mixture of dioxane:water 5:1 and DMSO show to be highly effective solvents for conversion of the starting material in the Suzuki-Miyaura coupling.

Unfortunately, these newfound conditions could not be applied toward preparation of **T2** as the first step of the synthesis, the *O*-alkylation under Mitsunobu conditions, could not be achieved with sufficient yields and purity of the product.

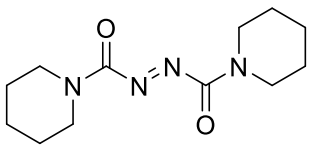
4. Future perspective

Target compounds **T1a** and **T1b** were prepared successfully, however the purity of the compounds is not sufficient for SPR assay to determine the binding affinity to the FMN riboswitch. As a purity of >95% is required. The compounds have been submitted for purification by preparative HPLC and can be analysed afterwards. Alternatively, to obtain compound **T1a** and **T1b** with high purity, scaffold **5** can first be SEM-protected on the pyrimidone ring before the Suzuki-Miyaura coupling is performed. Not only could this clean-up the coupling reaction, since a large excess of base will not be necessary, it also reduces the polarity of the intermediate product, making it more soluble in non-polar solvents and make it easier to purify with normal phase chromatography. Although this approach will add two additional steps to the synthetic procedure, a protection and a deprotection reaction, it could be worth the additional effort in order to obtain the final product with a high degree of purity. Generally, SEM-protection and deprotection reactions are quickly performed with good yields [39]. Furthermore, **T1c** could be prepared using the method that is described herein, but it was not prioritised due to poor solubility of the compound in DMSO, making it lose interesting as a drug.

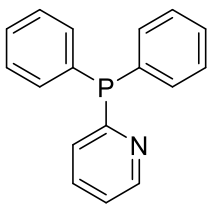
Unfortunately, the Mitsunobu reaction to form compound **7** was not successful. To prepare this intermediate for further synthesis of **T2**, different conditions can be tested. The issue might be a slow keto-enol tautomerization, leading to a competitive side reaction consuming the azo coupling reagent while forming undesired side product. Procedures are described to perform a Mitsunobu reaction on weak acids [40], which could be successful for this application. The key lies in the use of an azo coupling reagent that not only has an increased basicity but, more importantly, a lower nucleophilicity, such as TIPA [41] and ADDP [42]. Substitution of the ethoxy moiety of the coupling reagent with an amino group increases the basicity of the anion and bulky N-substitutions counteract the increased nucleophilicity as result from the enhanced basicity. However, the lower reactivity towards the phosphine should be taken into account. The order of addition in the reaction is important. In this case, the betaine should be pre-formed by stirring the azo coupling reagent with the phosphine until it precipitates out of solution. In turn, a more nucleophilic phosphine, such as tributylphosphine, can be used to accelerate betaine formation. Furthermore, the use of these aliphatic reagents also resolves the second problem of the Mitsunobu reaction, namely, the purification problems. Removal of the aliphatic hydrazine by-product and tributylphosphine is easily achieved by SiO_2 column chromatography. Another method to remove phosphine and phosphine oxide contaminants from solution is reported [43]. This procedure makes use of an aromatic phosphine with a basic moiety, such as diphenyl-2-pyridylphosphine, without a reduction in reaction rate or loss of yield. During work-up the phosphine (oxide) is easily removed by performing an acidic aqueous extraction.



N,N,N',N'-tetraisopropylazodicarboxamide (TIPA)



1,1'-(azodicarbonyl)dipiperidine (ADDP)



Diphenyl-2-pyridylphosphine

5. Experimental

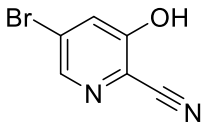
General remarks

Chemicals and solvents were purchased from Sigma-Aldrich and used as delivered unless otherwise stated. All moisture sensitive reactions were carried out under argon atmosphere in oven-dried (130 °C) equipment that has been cooled down under vacuum.

Anhydrous THF was obtained from an anhydrous solvent delivery system (SPS-800 system from M. Braun GmbH, Garching, Germany). Thin layer chromatography (TLC) analyses were performed on aluminium sheets coated with Merck TLC silica gel 60 F254 or Macherey-Nagel Alugram RP-18 and visualization was achieved by using ultraviolet light (254 nm). Flash column chromatography was performed either manually using silica gel from Merck (Silica gel 60, 0.040–0.063 mm) or was performed on a PuriFlash XS 420 system (Interchim, Montlucon Cedex, France) using pre-packed columns as specified for each compound. Preparative high-performance liquid chromatography (HPLC) was performed on a Thermo 321 multisolvent pump with an UltiMate 3000 variable wavelength detector using an XBridge Prep C18 5 µm OBD (19 x 250 mm, 5 µm) column with mixtures of acetonitrile and water (both containing 0.1% TFA) as eluent. Analytical HPLC was performed on a 1290 Infinity II Flexible pump with a 1260 Infinity II DAD WR detector using a ZORBAX RRHD Eclipse plus 300-SB C18 (50 x 2.1 mm, 300 Å, 1.8 µm) column with mixtures of acetonitrile and water (both containing 0.1% TFA) as eluent. Positive and negative ion electrospray ionization mass spectrometry was conducted on a AccuTOF™ JMS-T100LC from JEOL, USA, Inc. (Peabody, MA, USA) mass spectrometer that was operated with an orthogonal electrospray ionization source (ESI). The NMR experiments were recorded on Bruker BioSpin Ascend spectrometer operating at 600 MHz for ^{1}H equipped with an inverse-detected triple resonance (TCI) cryoprobe. ^{1}H and ^{13}C chemical shifts (δ) are reported in ppm with reference to the solvent residual peak ($CDCl_3$: δH 7.26 and δC 77.16; $(CD_3)_2SO$: δH 2.50 and δC 39.98). All coupling constants are given in Hz.

5.1. Synthesis towards T1

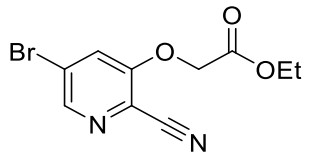
5.1.1. 5-Bromo-3-hydroxypicolinonitrile (2)



Hydroxypyridine **1** was prepared following a procedure reported in literature [33]. To a suspension of NaH (60% in mineral oil, 1.49 g, 37.3 mmol, 3 eq.) in dry THF (45 mL) was added 2-methylsulfonylethanol (1.85 g, 14.9 mmol, 1.2 eq.). The mixture was cooled to 0 °C in an ice/water bath, and 5-bromo-3-fluoropicolinonitrile (**1**) (2.50 g, 12.4 mmol, 1 eq.) was added slowly over 1.5 hours. The mixture was allowed to reach room temperature and stirred for an additional 2 hours before water (15 mL) and 4M HCl were added until pH 2 was reached and a precipitate formed. The resulting mixture was extracted with EtOAc (3x 50 mL) and the combined organic phases were washed with water (50 mL), brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give an off-white solid (3.328 g, quantative crude yield). The crude product was used in the next step without further purification. ^{1}H NMR (600 MHz, $(CD_3)_2SO$): δ = 12.43 (s, 1H), 8.31 (d, *J* =

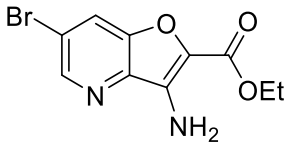
1.9, 1H), 7.78 (d, *J* = 1.9, 1H); ^{13}C NMR (151 MHz, $(CD_3)_2SO$): δ = 158.0, 142.7, 126.9, 124.7, 119.4, 115.5; HRMS (ESI): *m/z* $[M - H]^-$ calcd for $C_6H_3BrN_2O^-$: 196.9356, found 196.9360.

5.1.2. Ethyl 2-((5-bromo-2-cyanopyridin-3-yl)oxy)acetate (**3**)



Ethyl oxyacetate **3** was prepared following a procedure reported in literature [44]. To a solution of 5-bromo-3-hydroxypicolinonitrile (**2**) (3.33 g, 16.7 mmol, 1 eq.) in acetone (15 mL) was added potassium carbonate (9.25 g, 66.9 mmol, 4 eq.) and ethyl 2-bromoacetate (3.07 g, 18.4 mmol, 1.1 eq.). The reaction mixture was heated to 60 °C for 18 hours. After cooling, the solvent was evaporated under reduced pressure and the residual was taken up in EtOAc (20 mL), extracted with water (3x 15 mL), brine (15 mL), dried over $NaSO_4$, filtered and concentrated under reduced pressure to afford the title compound as a brown solid (3.336 g, 94% yield over 2 steps). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.39 (d, *J* = 1.7, 1H), 7.40 (d, *J* = 1.7, 1H), 4.80 (s, 2H), 4.30 (q, *J* = 7.2, 2H), 1.31 (t, *J* = 7.2, 3H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 166.9, 157.1, 145.1, 125.1, 123.8, 122.8, 114.4, 66.0, 62.4, 14.2; HRMS could not be obtained at this time with any of the used ionization techniques.

5.1.3. Ethyl 3-amino-6-bromofuro[3,2-b]pyridine-2-carboxylate (**4**)



Preparation from ethyl oxyacetate **3**

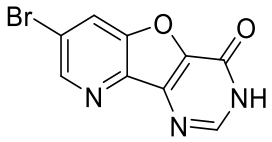
To a solution of ethyl 2-((5-bromo-2-cyanopyridin-3-yl)oxy)acetate (**3**) (3.35 g, 11.7 mmol, 1 eq.) in DMF (20 mL) was added NaO^{t}Bu (225 mg, 2.3 mmol, 0.2 eq.). The reaction mixture was heated to 110 °C for 2 hours. After cooling, water (20 mL) was added and the mixture was extracted with EtOAc (3x 30 mL). The combined organic layers were washed with brine (20 mL), dried over $NaSO_4$, filtered and the solvent was evaporated under reduced pressure to afford the crude product as a brown solid. Purification by flash column chromatography (EtOAc:hexanes 1:1 to 2:1) afforded the title compound as an off-white solid (1.583 g, 48% yield). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.59 (d, *J* = 1.8, 1H), 7.91 (d, *J* = 1.8, 1H), 5.18 (s, 2H), 4.46 (q, *J* = 7.2, 2H), 1.44 (t, *J* = 7.1, 3H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 161.1, 147.6, 146.9, 138.9, 128.0, 122.6, 119.4, 60.9, 14.6; HRMS could not be obtained at this time with any of the used ionization techniques.

One-pot synthesis from hydroxypyridine **2**

To a solution of 5-bromo-3-hydroxypicolinonitrile (**2**) (200 mg, 1.0 mmol, 1 eq.) and ethyl 2-bromoacetate (184 mg, 1.1 mmol, 1.1 eq.) in DMF (5 mL) was added NaO^{t}Bu (96 mg, 1.0 mmol, 1.0 eq.). The mixture was stirred and heated at 60 °C overnight. Next, an additional equivalent of NaO^{t}Bu (96 mg, 1.0 mmol, 1.0 eq.) was added and the heating was increased to 100 °C for 2 hours. After cooling, the mixture was concentrated under reduced pressure until approximately half volume was

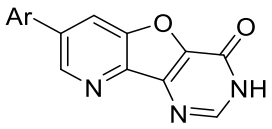
left. Water (5 mL) was added and the resulting mixture was extracted with EtOAc (3x 10 mL), washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash column chromatography (EtOAc:hexanes 1:1 to 2:1) afforded the title compound as an off-white solid (136 mg, 58% yield).

5.1.4. 7-Bromopyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (5)



In a sealed microwave tube, a solution of ethyl 3-amino-6-bromofuro[3,2-b]pyridine-2-carboxylate (**4**) (477 mg, 1.67 mmol, 1.0 eq.), and ethyl formimidate hydrochloride (458 mg, 4.18 mmol, 2.5 eq.) in formamide (10 mL) was purged with argon gas while stirring at room temperature for 20 minutes and thereafter heated at 180 ˚C for 5 hours. After the reaction mixture had cooled to room temperature, cold water (5 mL) was added and the mixture was filtered. The residual was air dried to afford the title compound as a fine black solid (284 mg, 64% yield). R_f = 0.7 (water:ACN + 0.1% TFA 2:1); ^{1}H NMR (600 MHz, $(CD_3)_2SO$): δ = 13.13 (s, 1H), 8.90 (d, *J* = 1.8, 1H), 8.78 (d, *J* = 1.8, 1H), 8.31 (s, 1H); ^{13}C NMR (151 MHz, $(CD_3)_2SO$): δ = 152.9, 150.1, 148.5, 147.6, 142.4, 141.9, 139.7, 124.0, 119.4; HRMS could not be obtained at this time with any of the used ionization techniques.

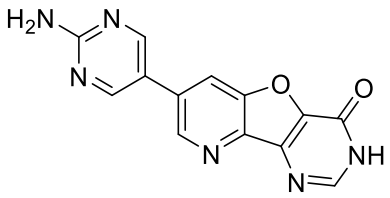
5.1.5. Suzuki-Miyaura cross coupling to form target compounds T1a-b



General procedure Suzuki-Miyaura cross coupling

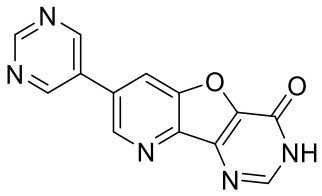
A microwave tube was loaded with 7-bromopyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (**5**) (26.6 mg, 0.1 mmol, 1 eq.), the boronic acid (0.15 mmol, 1.5 eq.), Cs_2CO_3 (325 mg, 1.0 mmol, 10 eq.) and $Pd(dppf)Cl_2 \cdot DCM$ (8.2 mg, 0.01 mmol, 0.1 eq.) and flushed with argon. Deoxygenated dioxane:water 5:1 or DMSO (3 mL) was added and the reaction mixture was heated to 100 °C for 16 hours. After the mixture was allowed to cool down to room temperature, HCl (2 eq.) was added and the solvent was evaporated under reduced pressure. The residual was purified using a Biotage Sfär C18 cartridge (30 µm, 12 g) eluting with acetonitrile in water + 0.1% TFA (5% to 60% over 30 minutes and then to 80% over 10 minutes at a flow rate of 10 mL/min).

5.1.5.1. 7-(2-aminopyrimidin-5-yl)pyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (*T1a*)



The title compound was prepared following the general procedure Suzuki-Miyaura coupling using dioxane:water 5:1 as solvent to give 85% conversion. The reaction mixture was purified by RP chromatography eluting at 80% ACN in water (+ 0.1% TFA) to give 30 mg of a brown oil, which needs further purification by prep-HPLC. ^{1}H NMR (600 MHz, $(CD_3)_2SO$): δ = 13.16 (s, 1H), 9.11 (d, *J* = 1.9, 1H), 8.81 (s, 2H), 8.61 (d, *J* = 1.9, 1H), 8.31 (s, 1H), 7.03 (s, 2H).

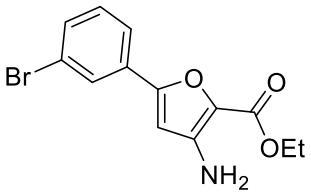
5.1.5.2. 7-(pyrimidin-5-yl)pyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (*T1b*)



The title compound was prepared following the general procedure Suzuki-Miyaura coupling using DMSO as solvent to give 88% conversion. The reaction mixture was purified by RP chromatography eluting at 20% ACN in water (+ 0.1% TFA) to give 7 mg of a white solid, which needs further purification by prep-HPLC. ^{1}H NMR (600 MHz, $(CD_3)_2SO$): δ = 13.18 (s, 1H), 9.37 (s, 2H), 9.30 (s, 1H), 9.26 (d, *J* = 1.9, 1H), 8.87 (d, *J* = 1.8, 1H), 8.34 (s, 1H).

5.2. Synthesis towards T2

5.2.1. *O*-alkylation under Mitsunobu conditions and cyclization to form ethyl 3-amino-5-(3-bromophenyl)furan-2-carboxylate (8)



The one-pot procedure to prepare compound **8** was executed as described by Redman et al. [36]. A solution of triphenyl phosphine (1.10 g, 4.2 mmol, 1.4 eq.) in dry THF (60 mL) was treated with dibenzyl diazene-1,2-dicarboxylate (1.25 g, 4.2 mmol, 1.4 eq.), ethyl glycolate (437 mg, 4.2 mmol, 1.4 eq.) and 3-bromobenzoylacetonitrile (672 mg, 3 mmol, 1.0 eq.) at 0 °C in an ice/water bath. The reaction mixture was allowed to warmed to room temperature and stirred overnight. Next, Sodium hydride

(60% in mineral oil, 300 mg, 7.50 mmol, 2.5 eq.) was added and the reaction mixture was stirred for 5 h. The reaction mixture was then quenched with water (1 mL) and concentrated to approximately half volume under reduced pressure. EtOAc (33 mL) was added and the resulting mixture was washed with water (33 mL). The aqueous layer was back-extracted with EtOAc (20 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$ and evaporated to give the crude product as an orange-brown semi-solid. The title compound could not be isolated from the crude product.

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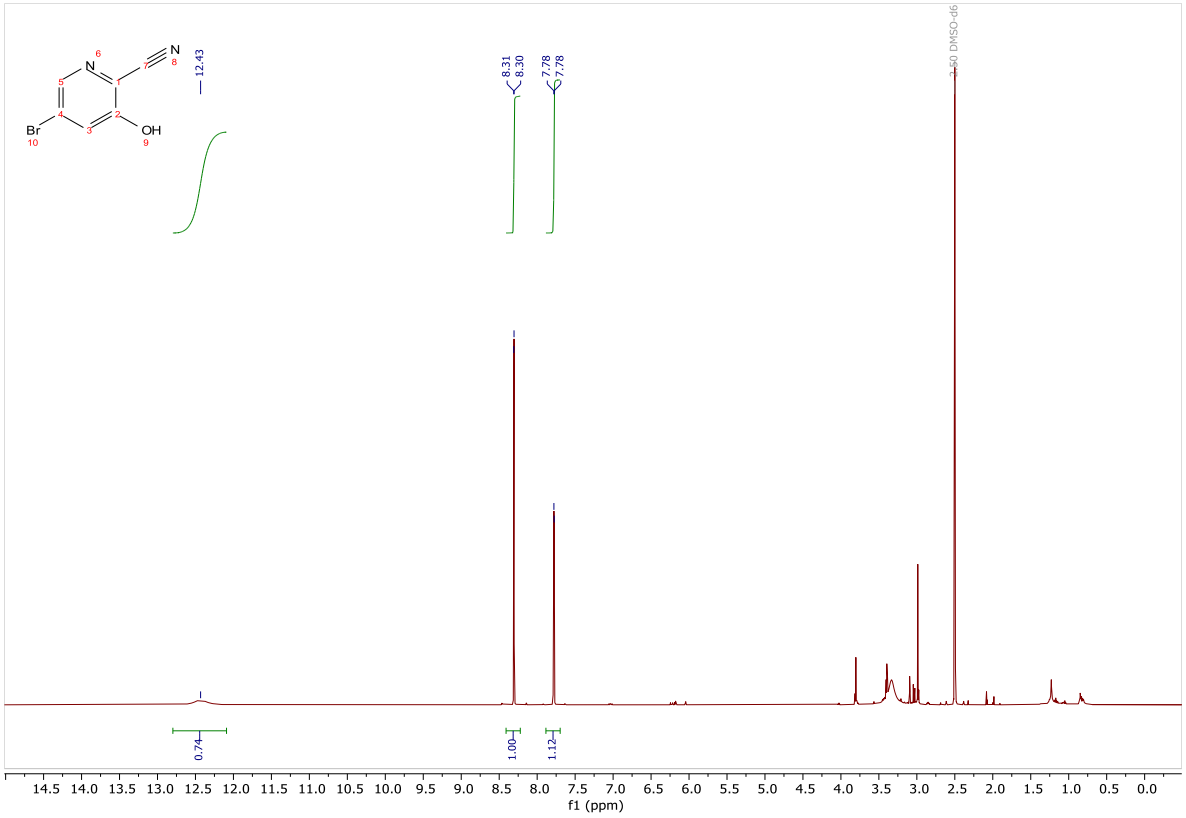
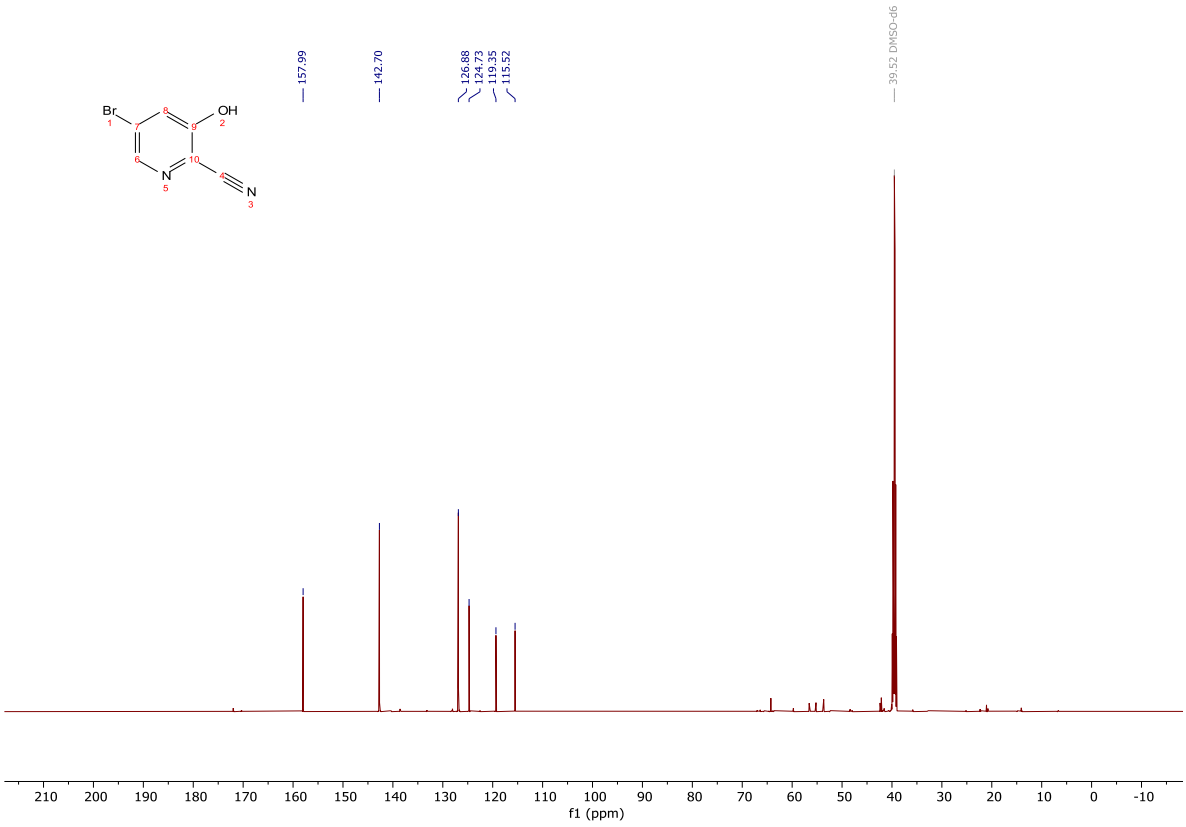
Appendix A – Spectral data

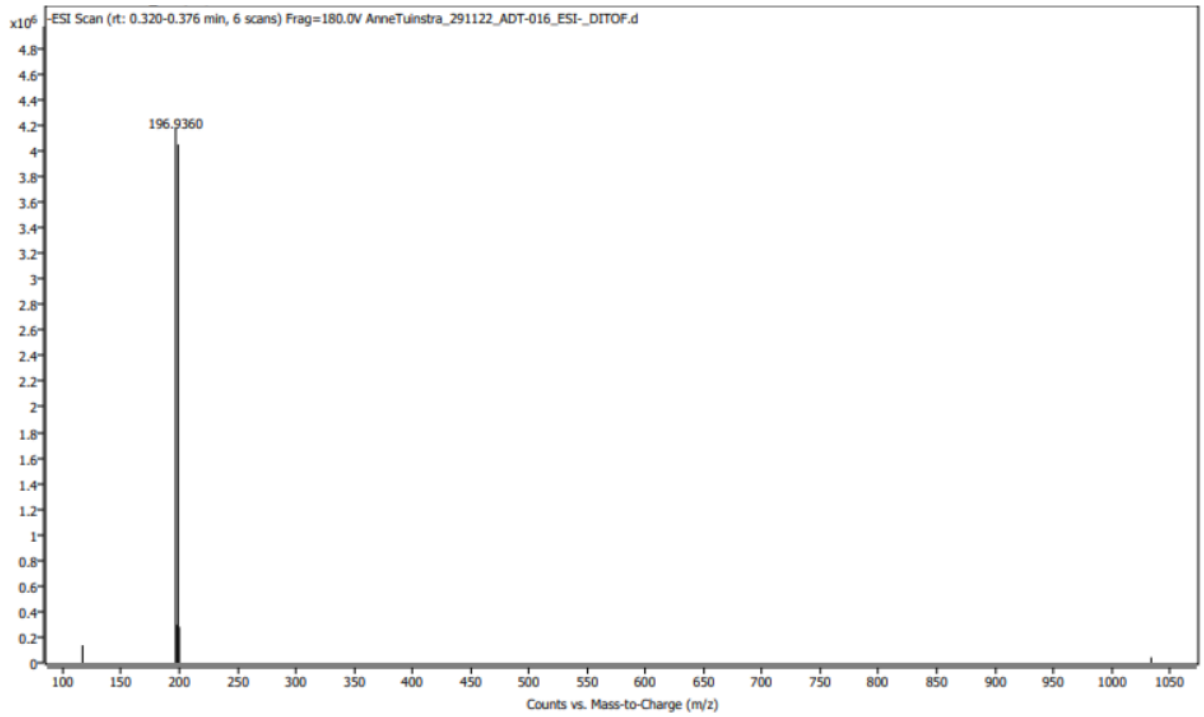
^{1}H NMR for 5-Bromo-3-hydroxypicolinonitrile (**2**)



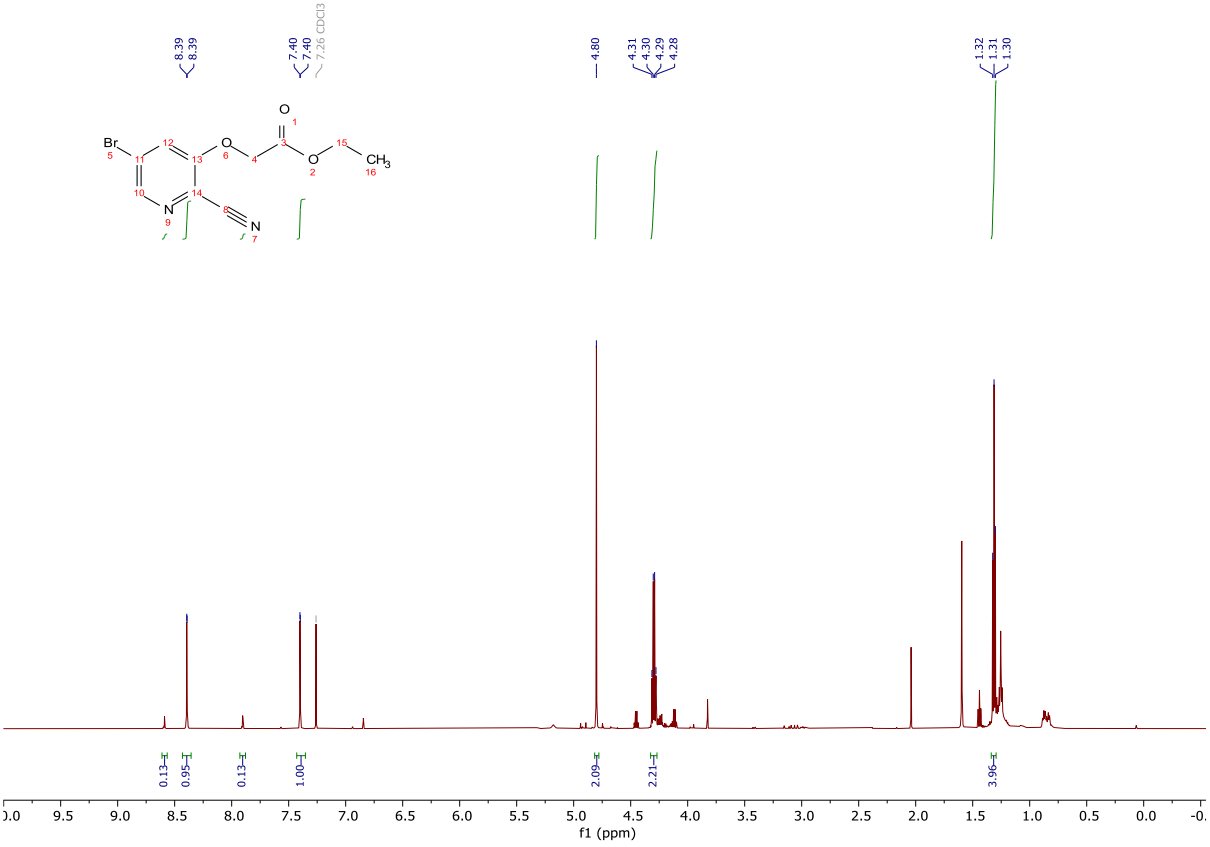
^{13}C NMR for 5-Bromo-3-hydroxypicolinonitrile (**2**)



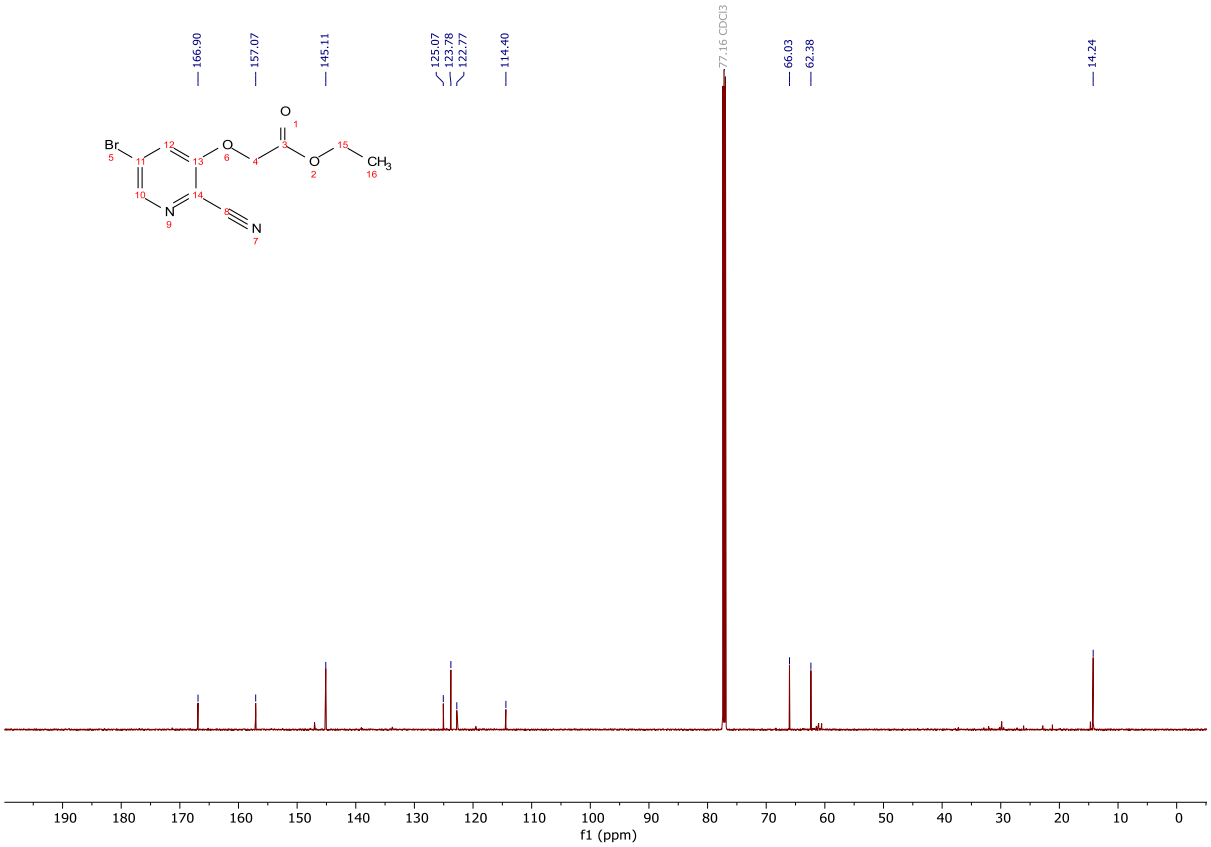
HRMS for 5-Bromo-3-hydroxypicolinonitrile (**2**)



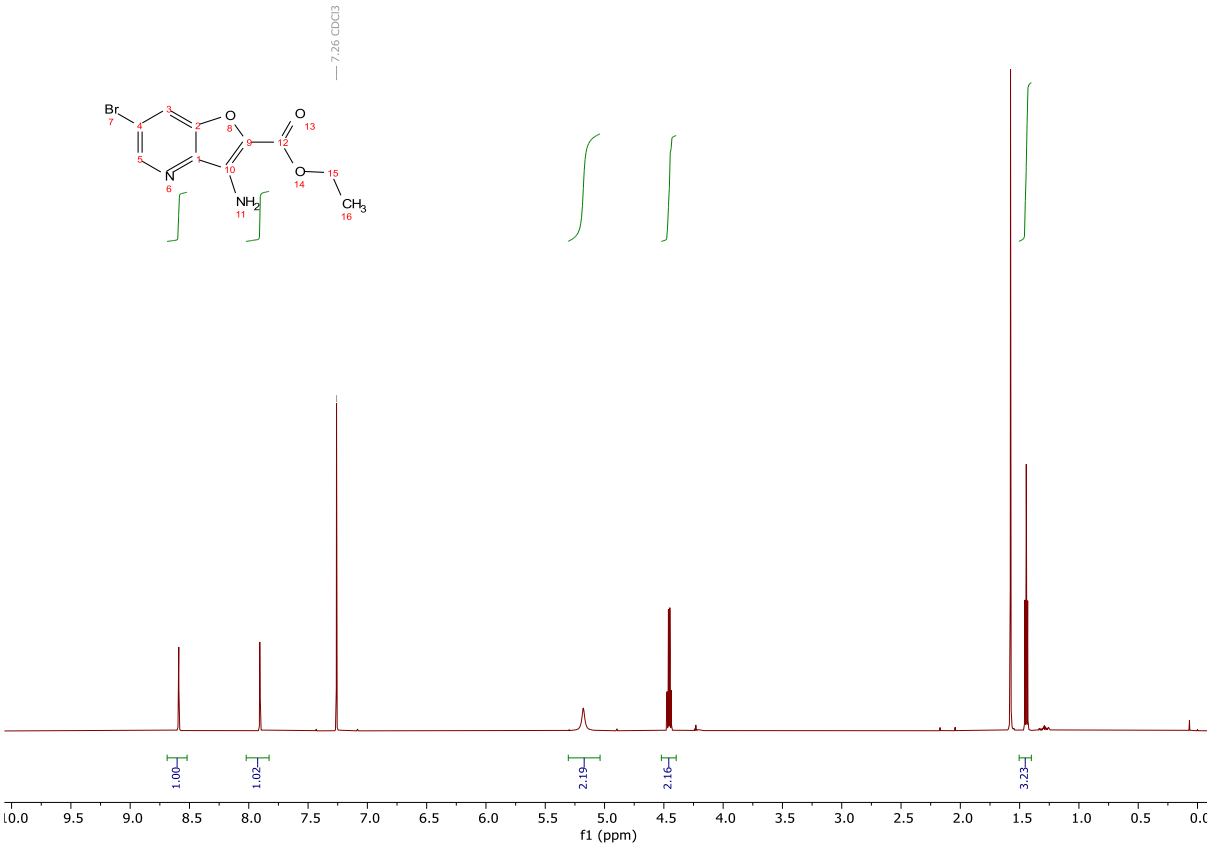
^{1}H NMR for Ethyl 2-((5-bromo-2-cyanopyridin-3-yl)oxy)acetate (**3**)



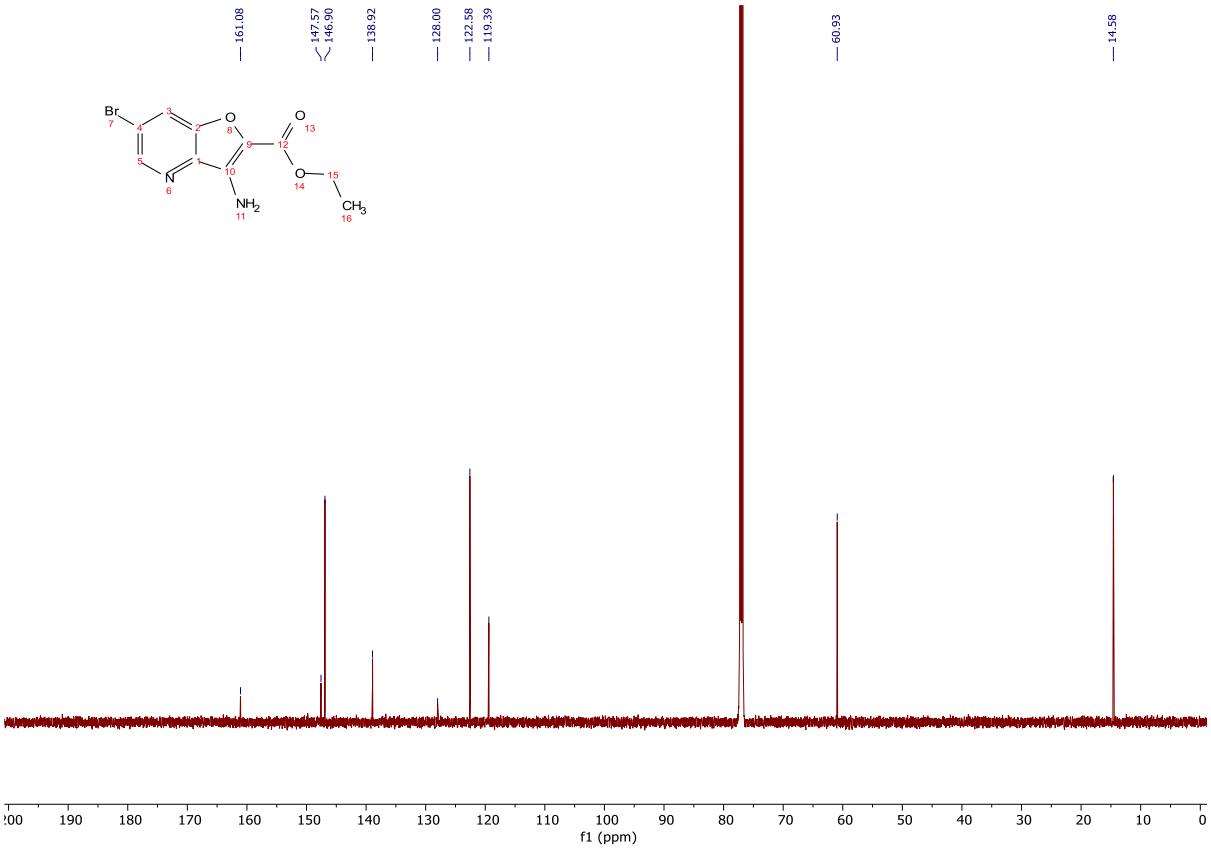
^{13}C NMR for Ethyl 2-((5-bromo-2-cyanopyridin-3-yl)oxy)acetate (**3**)



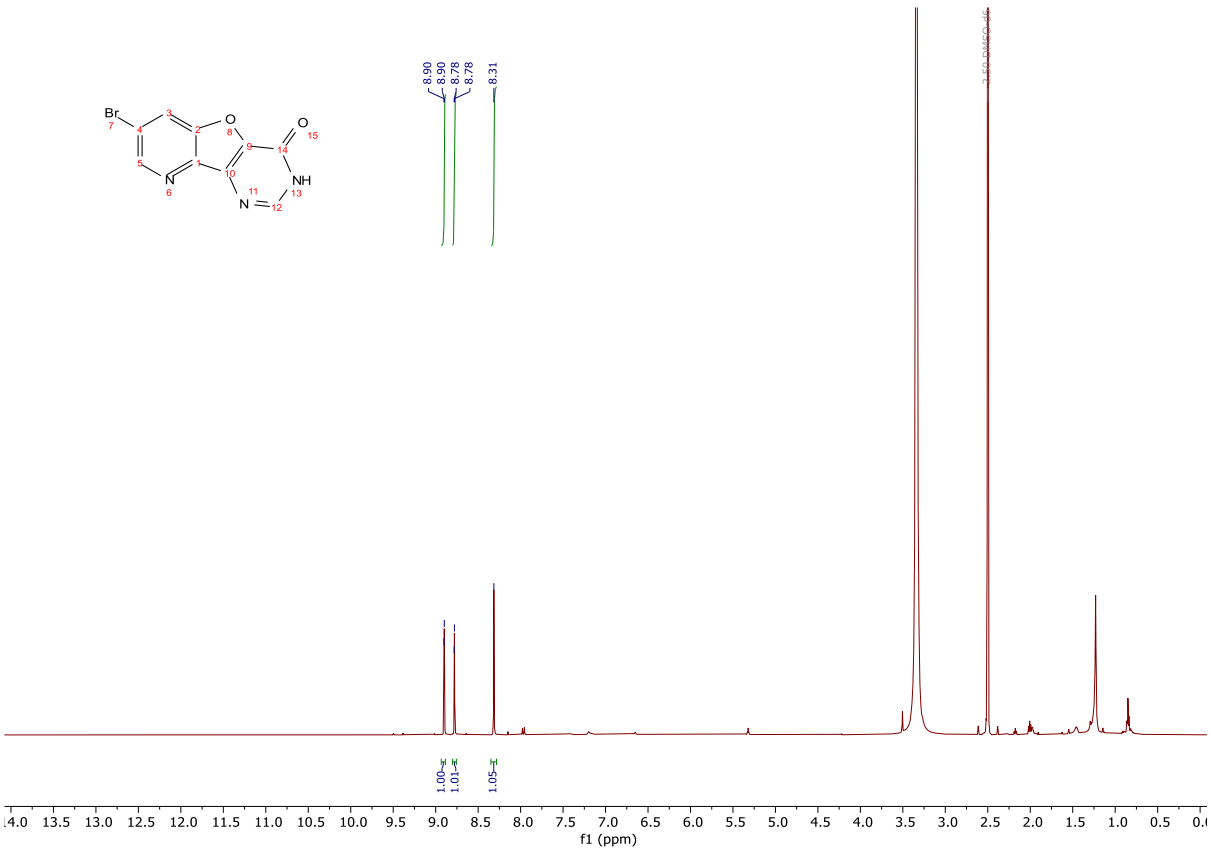
^{1}H NMR for Ethyl 3-amino-6-bromofuro[3,2-b]pyridine-2-carboxylate (**4**)



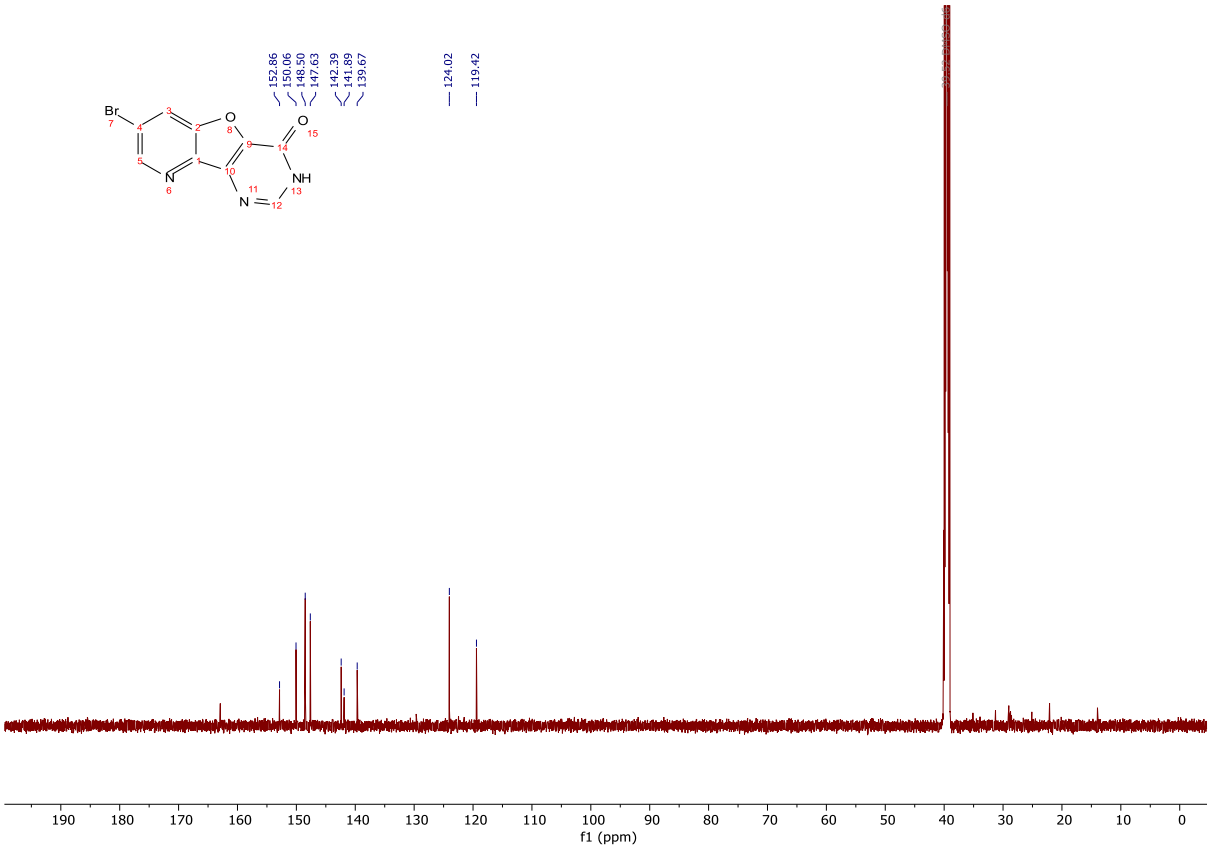
^{13}C NMR for Ethyl 3-amino-6-bromofuro[3,2-b]pyridine-2-carboxylate (**4**)



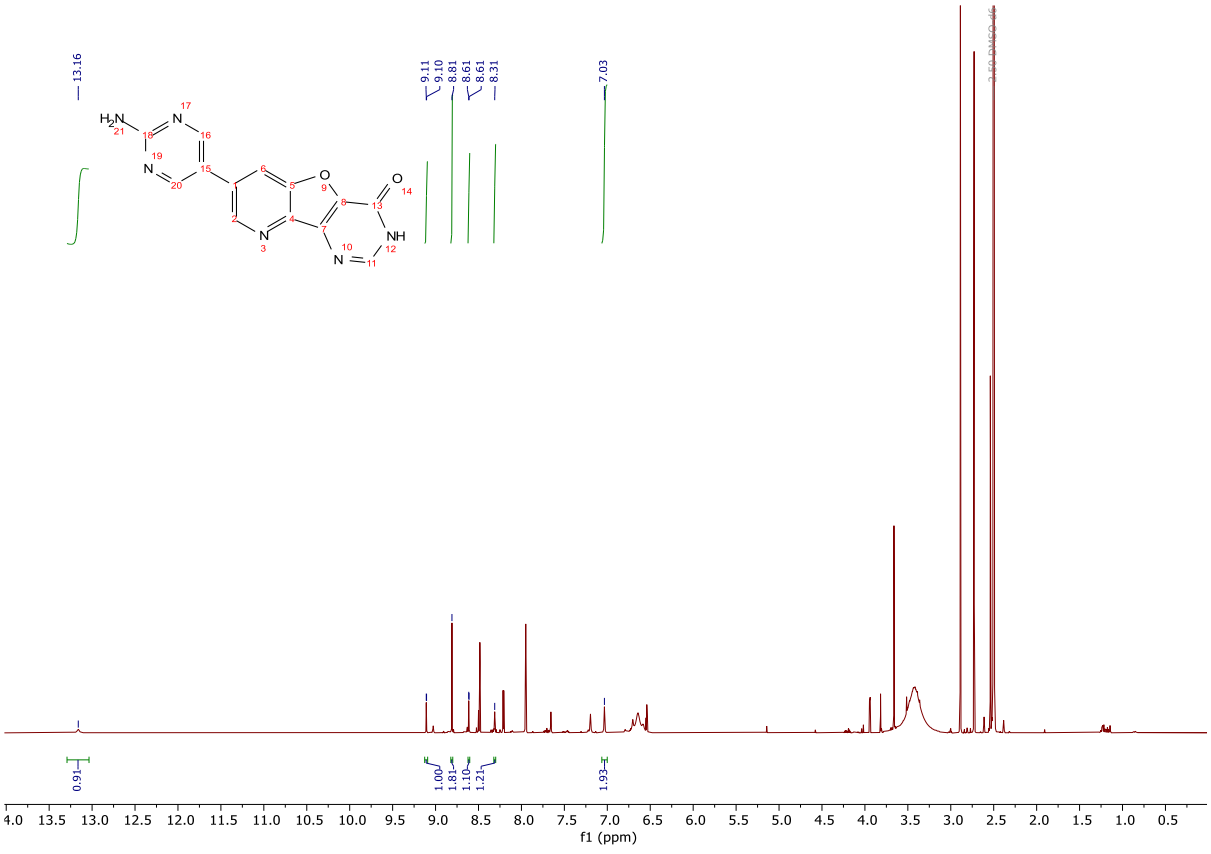
^{1}H NMR for 7-Bromopyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (**5**)



^{13}C NMR for 7-Bromopyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (**5**)



^{1}H NMR for 7-(2-Aminopyrimidin-5-yl)pyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (**T1a**)



^{1}H NMR for 7-(Pyrimidin-5-yl)pyrido[2',3':4,5]furo[3,2-d]pyrimidin-4(3H)-one (**T1b**)