Design, synthesis and evaluation of thienopyridines as ligands of adenosine receptors

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Dissertation accepted in partial fulfilment of the requirements for the degree Master of Science in Pharmaceutical Chemistry at the North-West University

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PREFACE

This dissertation is submitted in article format in accordance with the General Academic Rules (4.10) of the North-West University (NWU). It comprises of an article (**Chapter 3**) that will be submitted to *Medicinal Chemistry Research* and compiled according to the authors' guidelines, accessible in **Annexure B** and available online in the journal's author information pack:

https://www.springer.com/journal/44/submission-guidelines#linksAndDownloads

All scientific research for the purpose of this dissertation was conducted by Ms Gaofenngwe Nkomba at the NWU's Potchefstroom campus after obtaining ethical approval for the study (**Annexure C**).

The co-authors have agreed to the inclusion of the article in this dissertation. A table of contributions and contributors for the original article to be submitted has also been included.

LETTER OF AGREEMENT FROM CO-AUTHORS

LETTER OF AGREEMENT

March 2022

To whom it may concern

Co-authorship on original article:

The undersigned are co-authors of the original article to be submitted to *Medicinal Chemistry Research*. The co-authors hereby give Mrs Gaofenngwe Nkomba permission to submit this article as part of her dissertation in fulfilment of the requirements for the degree Master of Science in Pharmaceutical Chemistry at the North-West University (NWU).

- Design, synthesis and evaluation of amino-3,5-dicyanopyryridines and thieno[2,3-b]pyridines as ligands of adenosine receptors

Sincerely,

Prof. Gisella Terre'Blanche

Dr. Helena D. Janse van Rensburg

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I wish to show my appreciation to all the staff members of Pharmaceutical Chemistry and fellow postgraduate students for the support, motivation and well wishes.

I am extremely grateful to my husband and our two beautiful boys. It has been a challenging time for us as a family, but I couldn't be where I am if it was not for their love, prayers and encouragement throughout my studies. My deepest gratitude to my dear mother who has always instilled good values and spirit of hard work to me.

Finally, I thank everyone who has supported me to complete the project directly or indirectly.

ABSTRACT

Despite the availability of various classes of antiepileptic drugs (AEDs), about one third of epileptic patients do not experience satisfactory seizure control with present treatments. This has been an important drive in the search for alternative epilepsy treatment. The endogenous nucleoside adenosine is a known anticonvulsant through activation of adenosine A_1 receptors. The development of adenosine derivatives such as N6-cyclohexyladenosine (CHA) as anticonvulsants had limitations which include pronounced peripheral side effects, mainly cardiovascular effects. Over the years, non-nucleoside agonists have been investigated as an alternative set of compounds which are highly potent and selective to specific adenosine receptor (AR) subtypes.

The aim of this study was to investigate the use of amino-3,5-dicyanopyridine and thieno[2,3-*b*]pyridine derivatives as potential A_1 AR agonists. A total of 23 test compounds were synthesised (**6a**–**s** and **7a**–**d**) and 7 of these were novel (**6d** and **6k**–**p**), while 4 compounds (**7a**–**d**) have been synthesised before but have never been tested for AR affinity.

The effect of intramolecular cyclisation that occurs during synthesis of thieno[2,3-*b*]pyridines from the intermediate compounds, amino-3,5-dicyanopyridines, in relation to AR binding was evaluated. Overall, amino-3,5-dicyanopyridine displayed superior activity towards rA_1 ARs compared to thieno[2,3]pyridines. The general poor activity of thieno[2,3-b]pyridines suggest that the intramolecular cyclisation results in molecular stiffening or rigidity which negatively affects binding to the receptors, perhaps, due to steric hindrance. For instance, compound **6f** (open ring) had a six-fold better inhibition constant (K_i) value of 48 nM for the A_1 subtype compared to its closed ring counterpart compound **7d** ($rA_1 K_i$ = 305 nM). Generally, most amino-3,5-dicyanopyridines exhibited greater affinity toward the rA_1 AR (K_i <10 nM) than the rA_{2A} AR. Compound **6c** had the overall best rA_1 affinity ($rA_1 K_i$ = 0.076 nM). 7 novel compounds synthesised (**6d**, **6k**, **6l**, **6m**, **6n**, **6o**, **6p**) proved to be highly selective with low nanomolar rA_1 affinity (K_i values between 0.179 to 21.0 nM). Compounds **6n**, **6q** and **7c** acted as potent, highly selective agonists at A_1 ARs; however, compounds **6c**, **6d** and **6o** (notably all containing a 3-OCH_3 group at position R^2) behaved as rA_1 antagonists. Due to their high selectivity for A_1 receptors, amino-3,5-dicyanopyridines could be investigated further for use as A_1 ligands in pharmaco-resistant epilepsy with the right structural optimisations and formulations.

Key terms: Epilepsy, Antiepileptic drugs, Adenosine A_1/A_{2A} receptor agonists, Amino-3,5-dicyanopyridine derivatives, Thieno[2,3-b]pyridine derivatives, Intramolecular cyclisation

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LIST OF ABBREVIATIONS

$[^3H]$DPCPX	$[^3H]$-8-cyclopentyl-1,3-dipropylxanthine
$[^3H]$NECA	5'-N-$[^3H]$-ethylcarboxamidoadenosine
$[^3H]$ZM241385	[2-(3)H]-4-(2-[7-Amino-2-(2-furyl)-[1,2,4]-triazolo-[2,3-a]-[1,3,5]-triazin-5-ylamino]ethyl)phenol
^{13}C	carbon-13
^1H	hydrogen-1 / protium
2-CLA	2-chloroadenosine
A_1 AR	adenosine A_1 receptor substype
A_{2A} AR	adenosine A_{2A} receptor subtype
A_{2B} AR	adenosine A_{2B} receptor subtype
A_3 AR	adenosine A_3 receptor subtype
AC	adenylyl cyclase
AD	Alzheimer's disease
AEDs	antiepileptic drugs
ADME	absorption, distribution, metabolism, and excretion
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APCI	atmospheric pressure chemical ionisation
AR	adenosine receptor
ATP	adenosine triphosphate
BAY60-6583	3,6-diamino-5-cyano-4-[4-(cyclopropylmethoxy)phenyl]thieno [2,3-b]pyridine-2-carboxamide
BBB	blood-brain barrier
BG	basal ganglia
BOILED-Egg	Brain Or IntestinaL EstimateD permeation
Br	bromine

C=O	carbonyl
Ca^{2+}	calcium ion
CADO	2-chloroadenosine
cAMP	cyclic adenosine-3,5 monophosphate
CCPA	2-chloro-N^6-cyclopentyladenosine
CHA	N^6-cyclohexyladenosine
CHO cells	Chinese hamster ovary cells
Ci	curie
Cl	chlorine
Cl^-	chloride ion
CNS	central nervous system
CO_2	carbon dioxide
CPA	N^6-cyclopentyladenosine
CCPA	2-chloro-N6-cyclopentyladenosine
CPA	N6-cyclopentyladenosine
Csp^3	Fraction of carbon atoms in the sp3 hybridization
CV	cardiovascular
CYP	cytochrome P450 enzyme system
(d)	doublet
D_2 receptors	dopamine D_2 receptors
DCM	dichloromethane
(dd)	doublet of doublets
DDC	dopa decarboxylase
(ddd)	double double doublet
DMF	dimethylformamide

DMSO	dimethyl sulfoxide
DMSO-d_6	deuterated dimethyl sulfoxide
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
DSC	differential scanning calorimetry
DTG	derivative thermogravimetry
EC_{50}	50% maximal effective concentration
EEG	electronencephalography
eFK2-K	elongation factor 2 kinase
EtOAc	ethyl acetate
EtOH	ethanol
Et_3N	triethylamine
F	fluorine
FeS	iron sulphide
GABA	gamma-aminobutyric acid
GI	gastrointestinal
GPCR	G protein-coupled receptor
GTP	guanosine 5’-triphosphate
h	human
H	hydrogen
HCl	hydrochloric acid
HIA	human gastrointestinal absorption
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
HSM	hot stage microscopy
Hz	hertz

IC_{50}	half maximal inhibitory concentration
(J)	coupling constant
K_i	inhibition constant
KD	ketogenic diet
KOH	potassium hydroxide
KATP	ATP sensitive potassium channel
KW-6002	istradefylline
LogP	partition coefficient
$logP_{o/w}$	partition coefficient between n-octanol and water
log*S*	solubility in water
(m)	multiplet
MeOH	methanol
$MgCl_2$	magnesium chloride
MHz	megahertz
MMPD	2-amino-4-(3-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile
MN	malononitrile
mp	melting point
MRS5474	4-{2-chloro-6[(dicyclopropylmethyl)amino]-9H-purin-9-yl}bicyclo[3.1.0]hexane-2,3-diol
MS	mass spectrometry
MW	molecular weight
m/z	mass-to-charge ratio
N	nitrogen
Na^+	sodium ion
NECA	5’-N-ethylcarboxamidoadenosine

NH_2	amino
nM	nano molar
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NT	nucleoside transporter
NWU	North-West University
NWU-AnimCareREC	North-West University Animal Care, Health and Safety Research Ethics Committee
OCH_3	methoxy
O	oxygen
OH	hydroxy
PAINS	pan assay interference compounds
PD	Parkinson's disease
PDA	photodiode-array detection
PE	petroleum ether
P-gp	permeability glycoprotein
PK	pharmacokinetics
PKA	protein kinase
PLC	phospholipase C
ppm (δ)	parts per million
PRE	pharmaco-resistant epilepsy
(q)	quartet
QOL	quality of life
r	rat
Rf	retention factor
R-PIA	R-phenylisopropyladenosine

(s)	singlet
S	sulphur
SANS	South African National Standard
SAR	structure-activity relationships
SDZWAG-994	N-cyclohexyl-2'-O-methyladenosine
SEM	standard error of the mean
SI	selectivity index
(t)	triplet
(td)	triple of doublets
TGA	thermogravimetric analysis
TLC	thin layer chromatography
TMS	tetramethylsilane
TPSA	topological polar surface area
WLOGP	Wildman & Crippen partition coefficient
XLOGP3	LogP value of a compound using a logP of a reference compound
ZM 241385	4-[2-[[7-amino-2-(2-furanyl)[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-yl]amino]ethyl]-phenol

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CHAPTER 1

1. INTRODUCTION

1.1 Background and rationale

Adenosine receptors (ARs) are a family of purinergic G protein-coupled receptors (GPCRs) with adenosine as the endogenous agonist (Sheth *et al.*, 2014). GPCRs are the largest family of membrane proteins and they are responsible for most cellular responses to hormones, neurotransmitters and environmental stimulants (Rosenbaum *et al.*, 2009). All GPCRs are characterized by the presence of seven membrane-spanning α-helical segments separated by alternating intracellular and extracellular loop regions (Rosenbaum *et al.*, 2009). They are considered to have great potential as therapeutic targets for a broad spectrum of diseases (Du & Xie, 2012).

There are four known types of ARs in humans which are A_1, A_{2A}, A_{2B} and A_3 (Jacobson & Gao, 2006). These receptors are widely expressed throughout the human body and they regulate several physiological and pathological functions of the body (Chen *et al.*, 2013). Among their widespread functions include the regulation of heart rate, blood pressure and body temperature, lipolysis, renal blood flow, immune function, sleep regulation, and platelet function (Betti, 2016; Boison, 2005). A_1 and A_{2A} ARs are high affinity receptors activated by the physiological concentration of adenosine (Catarzi *et al.*, 2019; Sheth *et al.*, 2014). Adenosine has the lowest affinity for A_{2B} ARs and intermediate affinity for the A_3 subtype (Catarzi *et al.*, 2019). ARs are able to form homodimers with each other (e.g., A_1 /A_{2A} ARs) and heterodimers with other GPCRs, (e.g., A_{2A}/D_2 and A_1/β-adrenergic receptors) (Sheth *et al.*, 2014). The involvement of ARs and adenosine levels in a variety of human diseases (asthma, neurodegenerative disorders, psychosis and anxiety, immune and inflammatory disorders, cardiac ischaemic diseases, sleep disorders, cancer) and many other pathophysiological states has opened a promising field in the discovery of novel potential drugs that bind to ARs (Klotz, 2000).

Adenosine is a long-known endogenous anticonvulsant substance responsible for seizure arrest (Rombo *et al.*, 2018). This hypothesis is supported by various experimental studies showing that adenosine levels rise dramatically during seizures (Swiader *et al.*, 2014). The seizure-related adenosine elevations are believed to be a feedback mechanism that limits seizure intensity or duration (Masino *et al.*, 2014). The strongest inhibitory action of adenosine occurs in the excitatory glutamatergic system, where it is capable of completely blocking synaptic transmission, and therefore, in general the activation of ARs leads to reduced excitability of neurons (Swiader *et al.*, 2014). It is believed that adenosine regulates seizure activity by activating A_1 receptors leading to the inhibition of glutamate release (Fabera *et al.*, 2019). Studies have revealed that systemic

injection of selective A_1 receptor agonists limits seizures and convulsions in numerous types of experimental seizures and epilepsies (Vianna *et al.*, 2005). A_1 receptors are believed to be strongly associated with antiseizure effects due to their clear effects on neuronal activity, high affinity, and widespread distribution (Masino *et al.*, 2014). A_{2A} receptors have been shown to increase epileptiform activity in the brain (Stockwell *et al*, 2017), and therefore, A_{2A} receptor antagonists would be expected to have antiseizure effects (Masino *et al.*, 2014). ARs are thus a powerful therapeutic target for treatment of conditions involving neural over activity such as epilepsy. It is worth noting that existing anticonvulsant drugs do not have substantial affinities for ARs but AR-based therapy especially with A_1 receptor activity may provide a great therapeutic potential in the treatment of medically refractory epilepsy as well as epilepsy in general.

In a recent study, 3,5-dicyanopyridine derivatives were found to exhibit affinity for ARs (Dal Ben *et al.*, 2019). 3,5-Dicyanopyridine compounds (**Figure 1-1**) are generally 2-aminopyridines presenting two cyano groups at the 3- and 5-positions, a phenyl group in the 4-position, and in the 6-position a further substituent starting with a methylthio spacer followed by groups of various volumes and chemical-physical properties. There are a number of 3,5-dicyanopyrine derivatives that have been developed, such as Capadenoson (BAY 68-4986), Neladenoson, MMPD, BAY 606583, piclodenoson, etc. Nelodenoson has displayed partial A_1 receptor agonist activity (Dal Ben *et al.*, 2019; Jacobson *et al.*, 2019a) and it is in clinical trials for treatment of chronic heart failure (Jacobson *et al.*, 2019a). Capadenoson is a selective agonist of the adenosine A_1 receptor (Dal Ben *et al.*, 2019; Jacobson *et al.*, 2019a). BAY-606583 is a non-nucleoside compound and the most potent and selective A_{2B} AR agonist (Betti *et al.*, 2018; Catarzi *et al.*, 2019). This compound has reached the preclinical stage for treating angina pectoris (Betti *et al.*, 2018). MMPD is a partial A_1 AR agonist (Jacobson *et al.*, 2019a). Piclodenoson has been found to have moderate selectivity for A_3 ARs as an agonist and is currently in clinical trials for treatment of autoimmune anti-inflammatory diseases and psoriasis (Jacobson *et al.*, 2019a). Another A_1 AR partial agonist that has been developed is Neladenoson bialanate which is a prodrug of neladenoson and is currently being evaluated in clinical trials for treatment of heart failure (Meibom *et al.*, 2017). The LUF series; namelyLUF5844 and LUF5845, are also 3,5-dicyanopyridine derivatives which have been found to be potent as A_1 and A_{2B} AR agonists, respectively (Dal Ben *et al.*, 2019).

3,5-Dicyanopyridine derivatives

Thieno[2,3-b]pyridine derivatives

Figure 1-1: General structure of 3,5-dicyanopyridines and thieno[2,3-b]pyridines

3,5-Dicyanopyridines also serves as intermediates in the synthesis of thieno[2,3-b]pyridine derivatives. Thienopyridines (**Figure 1-1**) have attracted considerable interest due to their very broad spectrum of biological activities (Alinaghizadeh *et al.*, 2016); which include antitumor, antibacterial, antiviral, antihypertensive, antidiabetic, anti-inflammatory, antidermatophytic, antimalarial activities (Masch *et al.*, 2019), in addition the treatment of central nervous system (CNS) disorders (Hassan *et al.*, 2013). Some are selective progesterone receptor agonists (Wang *et al*, 2009), and factor 2 kinase (eEF2-K) inhibitors (Lockman *et al.*, 2010) as well as intermediates in the synthesis of non-nucleoside inhibitors of human cytomegalovirus and related herpes polymerases (Schnute *et al.*, 2005). Despite their aforementioned promising biological activities, the thienopyridine core only received scanty attention as scaffolds for the design of AR ligands, with a recent study reporting lack of AR affinity for the thienopyridines, based on the evaluation of only one compound in this class (Betti *et al.*, 2018). Therefore, there is a need for more structure activity relationship (SAR) studies on this bicyclic scaffold to get greater insight.

The focus of the current study is to design and synthesise thieno[2,3-b]pyridine derivatives with AR affinity from lead amino-3,5-dicyanopyridine derivatives and to determine SARs of the synthesised compounds in relation to their AR affinity. **Figure 1-2** indicates the modifications at R^2 and aryl positions on the thieno[2,3-b]pyridines scaffold that will be applied to investigate the influence of these substitutions on AR A_1 affinity.

Our proposed approach includes (**Figure 1-2**): (1) Thiophene ring closure with an amine group (polar functional group) at position 3: A pyridine ring (6- membered ring) will be fused with a 5-membered heterocyclic thiophene moiety to increase the rigidity and stability of the molecule, (2) substituting different functional groups at the meta- and para- positions of the 4-phenyl ring (R^2) and (3) substituting different aryl groups at position 2.

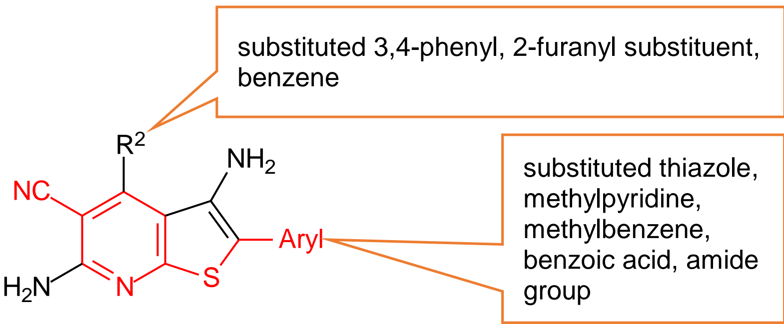

Figure 1-2: Proposed modification on the thieno[2,3-b]pyridine scaffold

1.2 Problem statement and hypothesis

Epilepsy is one of the most common disorders of the brain (Masino *et al.*, 2014) with various underlying causes, and the fact that most often the cause(s) are unknown, makes it difficult to treat all types of epilepsies successfully (Klaft *et al.*, 2016). About one third of epileptic patients are not adequately treated with currently available antiepileptic drugs (Swiader *et al.*, 2014). Therefore, there is a need for alternative treatment strategies that would allow achieving improved efficacy in epilepsy – especially the drug resistant forms. The approach that seems to be particularly attractive and promising for pharmaco-resistant epilepsy (PRE) involves interfering with the adenosinergic system (Swiader *et al.*, 2014). As mentioned earlier, one of the pathophysiological roles of adenosine is its anticonvulsant action and researchers have already demonstrated that adenosine agonists have potential clinical applications as antiepileptic agents (Vianna *et al.*, 2005). According to (Masino *et al.*, 2014), adenosine-based therapy has the capacity to treat all types of seizures; be it metabolic, genetic, or of unknown origin. Epilepsy is frequently associated with a psychiatric disorder and/or cognitive dysfunction (Manchanda, 2002) and one of the issues of present anti-epileptic drug treatments (AEDs) is that they often control seizures but may exacerbate psychiatric comorbidities (Devinsky, 2003). These adenosine-based strategies might shed light on desired therapies that reduce seizures and improve psychiatric comorbidity simultaneously (Masino *et al.*, 2014). Previously developed nucleoside-based A_1 AR agonists with antiseizure activity such as 2-chloroadenosine (2-CLA) have several disadvantages such as peripheral side effects particularly cardiovascular effects, poor brain permeability as well as lack of selectivity (Boison, 2005; Jacobson *et al.*, 2019b; Ribeiro *et al.*, 2002). These inadequacies suggest more research on drug designs targeting specific ARs to offer an advance in antiseizure therapies.

As previously stated, the thienopyridine core only received scanty attention as a scaffold for the design of AR ligands. This is especially important because bicyclic scaffolds such as benzofurans, tetralones and indanones were previously associated with affinity for ARs (**Figure 1-3**) and because of the structural similarities of these compounds with thienopyridines it is important to explore SARs of the thienopyridines in relation to AR activity in the management of neurological

conditions such as epilepsy. Novel benzofuran derivatives with selective A_{2A} receptor antagonist properties have shown good potency in catalepsy (Saku *et al.*, 2010). Another study by (Janse van Rensburg *et al.*, 2019) revealed that indanone derivatives are potent and selective A_1 and A_{2A} receptor antagonists for the potential treatment of neurological conditions. The structurally related tetralone derivatives also displayed affinity for A_1 and A_{2A} ARs as antagonists; hence, these compounds are potential drug candidates in the management of neurological conditions such as Parkinson's Disease (PD) (van Rensburg *et al.*, 2017).

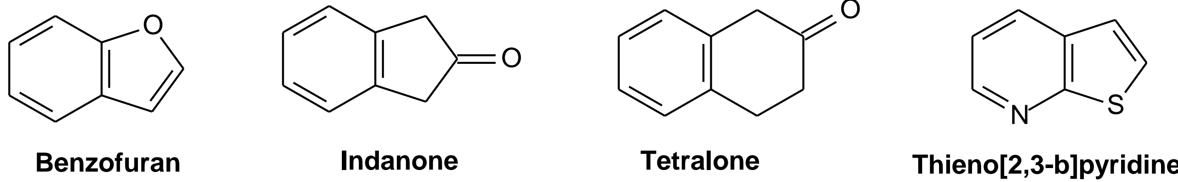

Figure 1-3: Structures of bicyclic scaffolds

3,5-Dicyanopyridine derivatives possess AR affinity and are intermediates in the synthesis of thieno[2,3-b]pyridine derivatives. Due to the chemical similarity of the 3,5-dicyanopyridines and thieno[2,3-b]pyridines, we hypothesize that a suitably substituted thieno[2,3-b]pyridine core can lead to derivatives with potent AR affinity.

1.3 Aims and objectives

Due to the widespread expression of ARs, drug therapy requires compounds endowed not only with high potency and efficacy at the various ARs but also with selectivity for specific AR subtypes. Non-nucleoside agonists provide an alternative set of compounds highly potent and selective to specific AR subtypes and that is what this research project hopes to achieve.

Therefore, the main aim of this research study is the design, synthesis, characterisation and evaluation of novel thieno[2,3-b]pyridine derivatives as potent and selective A_1 AR agonists for the potential treatment of neurological conditions, such as epilepsy. This study will give insight onto the SARs that govern A_1 affinity for future design of novel compounds.

The objectives of the study are as follows:

- Structure-based design of novel thieno[2,3-b]pyridine derivatives with potential agonist activity at A_1 ARs based on the most promising structures of intermediates compounds, 3,5-dicyanopyridines
- The synthesis of intermediates, as well as novel thieno[2,3-b]pyridine derivatives via multicomponent reactions
- The characterisation/verification of the synthesised compounds with proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectrometry, mass spectrometry (MS),

melting point (DSC analysis) and purity determination by high performance liquid chromatography (HPLC)

- The *in vitro* evaluation of the synthesised compounds by means of a standardised radioligand binding assay and functional guanosine triphosphate (GTP) shift assay using rat brain tissue expressing the implicated ARs
- Structure-activity relationship evaluation of the synthesised compounds

***

The present study investigates A_1 and/or A_{2A} AR agonists for the potential pharmacological treatment of neurological conditions, such as epilepsy. While this chapter provides the background, rationale, hypotheses and aims and objectives of the research, the second chapter dives deeper into the current literature on adenosine and epilepsy. Chapter 3 constitutes of a research article, which focuses on the synthesis and *in vitro* evaluation of novel and known thieno[2,3-b]pyridine analogues as adenosine A_1 and/or A_{2A} receptor agonists. Chapter 4 provides a brief summary of the present study as well as suggestions for future research.

1.4 References

Alinaghizadeh, F., Zahedifar, M., Seifi, M. & Sheibani, H. 2016. Cascade synthesis of thieno [2, 3-b] pyridines by using intramolecular cyclization reactions of 3-cyano-2-(organylmethylthio) pyridines. *Journal of the Brazilian Chemical Society*, 27(4):663-669.

Betti, M. 2016. *Design,Synthesis and Pharmacological evaluation of new adenosine receptor ligands*, Florence:University of Florence (Thesis -PhD).

Betti, M., Catarzi, D., Varano, F., Falsini, M., Varani, K., Vincenzi, F., Dal Ben, D., Lambertucci, C. & Colotta, V. 2018. The aminopyridine-3,5-dicarbonitrile core for the design of new non-nucleoside-like agonists of the human adenosine A2B receptor. *European Journal Medicinal Chemistry*, 150:127-139.

Boison, D. 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. *Neuroscientist*, 11(1):25-36.

Catarzi, D., Varano, F., Varani, K., Vincenzi, F., Pasquini, S., Dal Ben, D., Volpini, R. & Colotta, V. 2019. Amino-3,5-Dicyanopyridines Targeting the Adenosine Receptors Ranging from Pan Ligands to Combined A1/A2B Partial Agonists. *Pharmaceuticals (Basel)*, 12(159):1-22.

Chen, J.F., Eltzschig, H.K. & Fredholm, B.B. 2013. Adenosine receptors as drug targets--what are the challenges? *Nature Review Drug Discovery*, 12(4):265-286.

Dal Ben, D., Lambertucci, C., Buccioni, M., Marti Navia, A., Marucci, G., Spinaci, A. & Volpini, R. 2019. Non-Nucleoside Agonists of the Adenosine Receptors: An Overview. *Pharmaceuticals (Basel)*, 12(4):1-22.

Devinsky, O. 2003. Psychiatric comorbidity in patients with epilepsy: implications for diagnosis and treatment. *Epilepsy & Behavior*, 4:2-10.

Du, C. & Xie, X. 2012. G protein-coupled receptors as therapeutic targets for multiple sclerosis. *Cell research*, 22(7):1108-1128.

Fabera, P., Parizkova, M., Uttl, L., Vondrakova, K., Kubova, H., Tsenov, G. & Mares, P. 2019. Adenosine A1 Receptor Agonist 2-chloro-N6-cyclopentyladenosine and Hippocampal Excitability During Brain Development in Rats. *Frontiers in Pharmacology*, 10(656):1-11.

Hassan, A.Y., Sarg, M.T., Said, M.M. & El-Sebaey, S.A. 2013. Utility of thieno[2,3-b] pyridine derivatives in the synthesis of some condensed heterocyclic compounds with expected biological activity. *Universal Organic Chemistry*, 1(1):1-15.

Jacobson, K.A. & Gao, Z.G. 2006. Adenosine receptors as therapeutic targets. *Nature Review Drug Discovery*, 5(3):247-264.

Jacobson, K.A., Tosh, D.K., Jain, S. & Gao, Z.-G. 2019. Historical and current adenosine receptor agonists in preclinical and clinical development. *Frontiers in cellular neuroscience*, 13 (124):1-17.

Janse van Rensburg, H., D., Legoabe, L., Terre'Blanche, G. & Van der Walt, M. 2019. 2–Benzylidene–1–Indanone Analogues as Dual Adenosine A1/A2a Receptor Antagonists for the Potential Treatment of Neurological Conditions. *Drug Research*, 69(07):382-391.

Klaft, Z.J., Hollnagel, J.O., Salar, S., Caliskan, G., Schulz, S.B., Schneider, U.C., Horn, P., Koch, A., Holtkamp, M., Gabriel, S., Gerevich, Z. & Heinemann, U. 2016. Adenosine A1 receptor-mediated suppression of carbamazepine-resistant seizure-like events in human neocortical slices. *Epilepsia*, 57(5):746-756.

Klotz, K.N. 2000. Adenosine receptors and their ligands. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 362:382-391.

Lockman, J.W., Reeder, M.D., Suzuki, K., Ostanin, K., Hoff, R., Bhoite, L., Austin, H., Baichwal, V. & Willardsen, J.A. 2010. Inhibition of eEF2-K by thieno [2, 3-b] pyridine analogues. *Bioorganic & Medicinal Chemistry Letters*, 20(7):2283-2286.

Manchanda, R. 2002. Psychiatric disorders in epilepsy: clinical aspects. *Epilepsy & Behavior*, 3(1):39-45.

Masch, A., Nasereddin, A., Alder, A., Bird, M.J., Schweda, S.I., Preu, L., Doerig, C., Dzikowski, R., Gilberger, T.W. & Kunick, C. 2019. Structure-activity relationships in a series of antiplasmodial thieno[2,3-b]pyridines. *Malaria Journal*, 18(89):1-10.

Masino, S.A., Kawamura, M.J. & Ruskin, D.N. 2014. Adenosine receptors and epilepsy: current evidence and future potential. *International Review of Neurobiolology*, 119:233-255.

Meibom, D., Albrecht-Kupper, B., Diedrichs, N., Hubsch, W., Kast, R., Kramer, T., Krenz, U., Lerchen, H. G., Mittendorf, J., Nell, P. G., Sussmeier, F., Vakalopoulos, A. & Zimmermann, K. 2017. Neladenoson Bialanate Hydrochloride: A Prodrug of a Partial Adenosine A1 Receptor Agonist for the Chronic Treatment of Heart Diseases. *ChemMedChem*, 12:728-737.

Ribeiro, J., Sebastiao, A. & De Mendonça, A. 2002. Adenosine receptors in the nervous system: pathophysiological implications. *Progress in Neurobiology*, 68(6):377-392.

Rombo, D.M., Ribeiro, J.A. & Sebastião, A.M. 2018. Role of adenosine receptors in epileptic seizures. In: Borea, P., Varani, K., Gessi, S., Merighi, S. & Vincenzi, F., eds. *The Adenosine Receptors*. Cham: Humana Press. pp. 309-350.

Rosenbaum, D.M., Rasmussen, S.G. & Kobilka, B.K. 2009. The structure and function of G-protein-coupled receptors. *Nature*, 459(7245):356-363.

Saku, O., Saki, M., Kurokawa, M., Ikeda, K., Takizawa, T. & Uesaka, N. 2010. Synthetic studies on selective adenosine A2A receptor antagonists: Synthesis and structure–activity relationships of novel benzofuran derivatives. *Bioorganic & Medicinal Chemistry Letters*, 20(3):1090-1093.

Schnute, M.E., Cudahy, M.M., Brideau, R.J., Homa, F.L., Hopkins, T.A., Knechtel, M.L., Oien, N.L., Pitts, T.W., Poorman, R.A. & Wathen, M.W. 2005. 4-Oxo-4, 7-dihydrothieno [2, 3-b] pyridines as non-nucleoside inhibitors of human cytomegalovirus and related herpesvirus polymerases. *Journal of Medicinal Chemistry*, 48(18):5794-5804.

Sheth, S., Brito, R., Mukherjea, D., Rybak, L.P. & Ramkumar, V. 2014. Adenosine receptors: expression, function and regulation. *International Journal of Molecular Sciences*, 15(2):2024-2052.

Swiader, M.J., Kotowski, J. & Luszczki, J.J. 2014. Modulation of adenosinergic system and its application for the treatment of epilepsy. *Pharmacology Reports*, 66(3):335-342.

van Rensburg, M., Leung, E., Haverkate, N.A., Eurtivong, C., Pilkington, L.I., Reynisson, J. & Barker, D. 2017. Synthesis and antiproliferative activity of 2-chlorophenyl carboxamide thienopyridines. *Bioorganic & Medicinal Chemistry Letters*, 27(2):135-138.

Vianna, E.P.M., Ferreira, A.T., Dona, F., Cavalheiro, E.A. & Fernades, J. 2005. Modulation of Seizures and Synaptic Plasticity by Adenosinergic receptors in an experimental model of temporal lobe epilepsy induced by pilocarpine in rats. *Epilepsia*, 46(5):166-173.

CHAPTER 2

2. ADENOSINE AND EPILEPSY

2.1. Role of adenosine in the central nervous system

Adenosine receptors (ARs) are widely distributed in almost all human body systems such as the nervous, cardiovascular, respiratory, gastrointestinal, urogenital, and immune systems as well as other organs including bones, eyes, skin, joints, and blood cells (Ballesteros-Yanez *et al.*, 2017). This implies that these receptors are potentially able to control many physiological functions in the body such as neuronal, cardiac, metabolic, and renal activities as shown by **Figure 2-1** (Borea *et al.*, 2018).

The A_1 ARs are highly abundant in the brain (cortex, hippocampus), and in lower density in peripheral organs and tissues, such as the heart, lungs, kidney, and adipose tissues (Müller, 2001). The adenosine A_{2B} receptor has its major distribution in the large intestine and bladder, and A_3 ARs are present in the heart, lungs, liver, brain and testes (Sachdeva & Gupta, 2013; Sheth *et al.*, 2014). Each type of AR has different functions, although with some overlap (Catarzi *et al.*, 2019). For instance, both A_1 and A_{2A} ARs play roles in the heart, regulating myocardial oxygen consumption and coronary blood flow, while the A_{2A} AR also has broader anti-inflammatory effects throughout the body (Layland *et al.*, 2014). These two receptors also have important roles in the brain, regulating the release of neurotransmitters such as dopamine and glutamate (Swiader *et al.*, 2014). The A_{2B} and A_3 ARs are mainly located peripherally and are involved in processes such as inflammation and immune responses (Chen *et al.*, 2013).

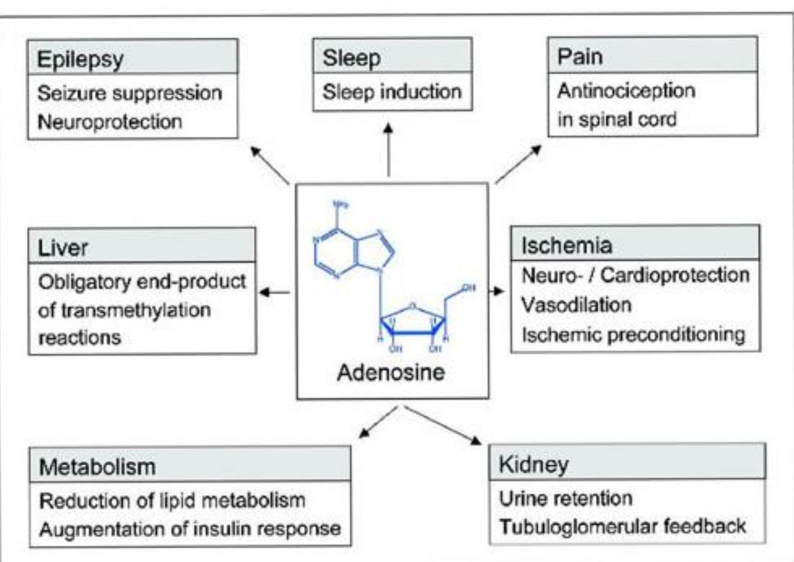

Figure 2-1: Adenosine receptor-mediated effects in various organ system. Reproduced with permission from (Boison, 2005).

ARs are a family of purinergic G protein-coupled receptors (GPCRs) with adenosine as the endogenous agonist (Sheth *et al.*, 2014). GPCRs are the largest family of membrane proteins and they are responsible for most cellular responses to hormones, neurotransmitters and environmental stimulants (Rosenbaum *et al.*, 2009). All GPCRs are characterized by the presence of seven membrane-spanning α-helical segments separated by alternating intracellular and extracellular loop regions as in **Figure 2-2** (Rosenbaum *et al.*, 2009). There are four known types of ARs in humans which are A_1, A_{2A}, A_2B and A_3 (Jacobson & Gao, 2006).

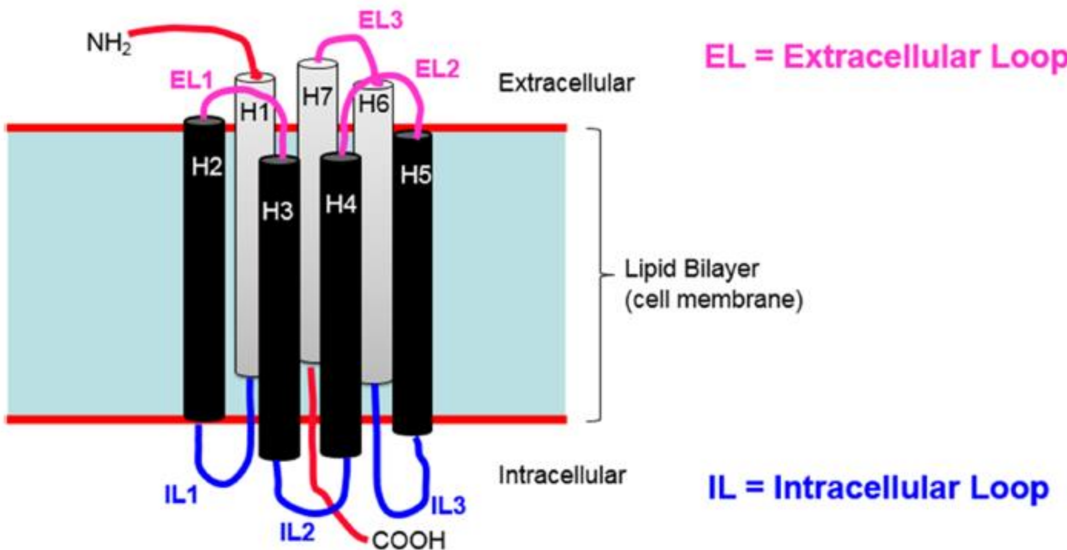

Figure 2-2: General Structure of GPCRs. Reproduced with permission from (Clark, 2017).

The ARs are far more abundant in the brain than in any other organ or cell type in mammals (Cunha, 2016). Of all ARs, the A_1 ARs are the most abundant and widely distributed in the brain, and they are highly expressed in the cortex, hippocampus, cerebellum, nerve terminals, spinal cord, and glia (Cunha, 2016). They are localized both pre- and postsynaptically as well as in non-neuronal cells such as astrocytes (Tomé *et al.*, 2010). This wide range of locations reflects the multiple physiological effects orchestrated by it, including inhibition of synaptic transmission and inhibition and neuronal excitability, sedation, anticonvulsant and anxiolytic effects, analgesia and regulation of sleep. (Ballesteros-Yanez *et al.*, 2017). A_{2A} ARs are more abundant in the striatum (Sebastiao & Ribeiro, 2009) but also detectable in the olfactory tubercle, cerebral cortex, neurons and glial cells (Sheth *et al.*, 2014). Activation of A_{2A} ARs, results in the increased release of neurotransmitters such as glutamate, acetylcholine, gamma amino butyric acid (GABA) and noradrenaline (Sheth *et al.*, 2014). A low level of expression of A_{2B} and A_3 ARs have been detected in the brain (Stockwell *et al.*, 2017).

Intracellularly, adenosine is produced through two metabolic pathways. The first involves dephosphorylation of adenosine monophosphate (AMP) by cytosolic 5′-nucleotidase and the second involves the hydrolysis of S-adenosylhomocysteine by the enzyme S-adenosyl-L-

homocysteinase hydrolase (Effendi *et al.*, 2020; Stiles, 1992). Extracellularly, adenosine is produced through ecto-nucleotidases-mediated hydrolysis of released adenosine triphosphate (ATP) (Ernst *et al.*, 2010). Adenosine can be eliminated through several pathways. It can be transported back into the cell by a specific nucleoside transporter (NT) (Pastor-Anglada & Pérez-Torras, 2018) or by simple diffusion cross the cell membrane (Stiles, 1992). Adenosine can also be deaminated by adenosine deaminase to inosine, which is further broken down to hypoxanthine, xanthine and uric acid, which is excreted by the kidneys (Klabunde, 2011). Finally, adenosine can be metabolised by adenosine kinase and rephosphorylated to AMP which helps maintain the adenine nucleotide pool in cells (Klabunde, 2011) (**Figure 2-3**).

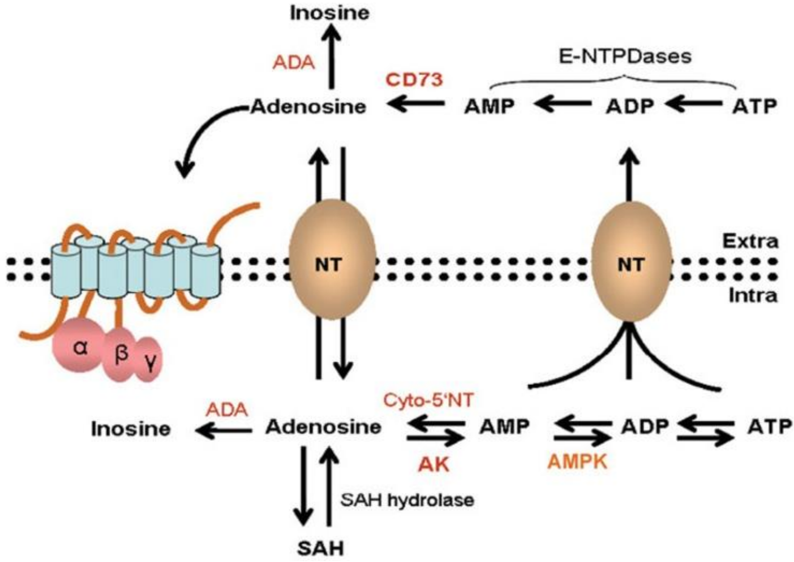

Figure 2-3: Adenosine synthesis and metabolic pathways. Reproduced with permission from (Ham & Evans, 2012).

The released adenosine mediates its physiological effects through the activation of the aforementioned four ARs, A_1, A_{2A}, A_{2B} and A_3 (Borea *et al.*, 2018). Activation of these receptors alters the activity of secondary signalling transductors. Adenosine exerts different functions depending on the type of AR and subsequent cellular signalling involved (Ballesteros-Yanez *et al.*, 2017). A_1 and A_3 ARs interact with G_i and G_0 members of the protein family and inhibit adenylyl cyclase, reduce cyclic AMP levels causing a decrease in protein kinase A (PKA) activity. A_{2A} and A_{2B} receptors are coupled to G_s proteins, through which they stimulate adenylyl cyclase and increase cAMP levels which results in activation of protein kinase A (PKA) (Borea *et al.*, 2018; Sheth *et al.*, 2014) (**Figure 2-4**).

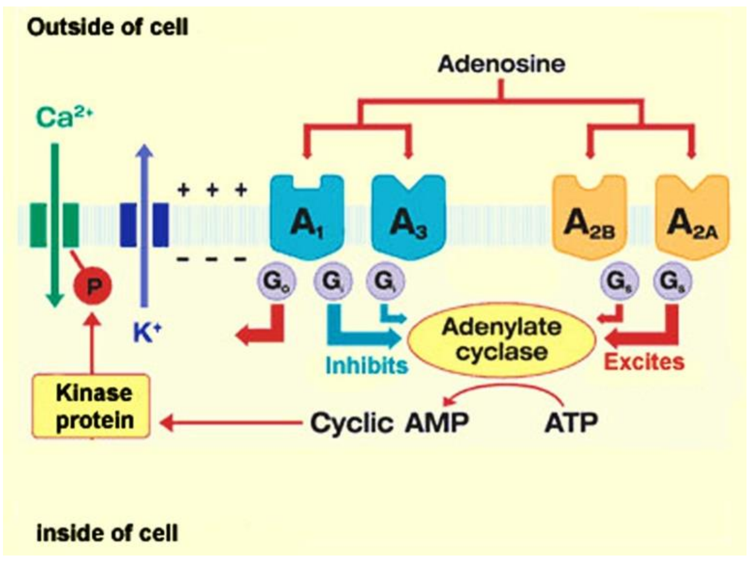

Figure 2-4: Adenosine receptor signalling. Reproduced with permission from (Ham & Evans, 2012).

During conditions of cellular stress such as in hypoxic, ischemic, or inflamed environments, adenosine is produced to reduce tissue injury and promote repair (Betti, 2016). Under these stressful conditions, intracellular concentrations of adenosine increase, and adenosine is released from the cell by the active transport of into the extracellular space and subsequent activation of ARs (Betti, 2016; Sachdeva & Gupta, 2013). There is evidence that adenosine serves as an endogenous neuromodulator and neuroprotective agent in the central nervous system (CNS) (Wardas, 2002). It modulates the cellular properties of pre- and/or postsynaptic neurons (or glial cells) and regulates synaptic neurotransmission (Domingos, 2016). Its neuromodulation role is triggered by neuronal activity and depends on its release to the extracellular space. Adenosine levels in the brain extracellular space increase dramatically during excitotoxic events, such as ischemia/hypoxia, seizures, or trauma to prevent neuronal injury, hence its neuroprotective role (Benarroch, 2008). It is believed that neuroprotective effects of adenosine may be produced by the stimulation of A_1 receptors and blockade of A_{2A} receptors (Wardas, 2002). Agents which potentiate the action of adenosine by either inhibiting its degradation (adenosine deaminase and kinase inhibitors), preventing its transport through nucleoside transporter, or directly activating the adenosine receptors, offer protection against ischemic or excitotoxic neuronal damage (Wardas, 2002). Within the CNS, adenosine has a role in mechanisms of seizure susceptibility, sleep induction, basal ganglia function, pain perception, cerebral blood flow, and respiration (Benarroch, 2008).

It is thus evident that adenosine receptors modulate neuronal and synaptic function of the brain in a range of ways that may make them implicated in the pathophysiology of neurological diseases (Stone *et al.*, 2009). Therefore, ARs are potential therapeutic targets for treatment of cerebral ischemia, idiopathic pain, drug addiction and neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, Huntington disease, and epilepsy (Ribeiro *et al.*, 2002). There have

been attempts to generate ligands that will target ARs as therapeutic agents to treat some of these disorders (Stone *et al*., 2009). This concept gives an opportunity to identify therapies that will alter the outcomes of these diseases, therefore providing a hopeful future for the patients who suffer from these diseases (Sebastiao & Ribeiro, 2009).

2.2. Adenosine receptors as therapeutic targets

As stated, ARs are widely expressed throughout the body and adenosine exerts a broad spectrum of physiological and pathophysiological functions by activating these receptors. The involvement of ARs and adenosine levels in a variety of human diseases (asthma, neurodegenerative disorders, psychosis and anxiety, immune and inflammatory disorders, cardiac ischaemic diseases, sleep disorders, cancer) and many other pathophysiological states has opened a promising field in the discovery of novel potential drugs that bind to ARs (Klotz, 2000). For each of the four AR subtypes, selective agonists and antagonists have been developed as new therapeutic drug concepts for various human diseases as shown by **Figure 2-5** (Jacobson, 2009).

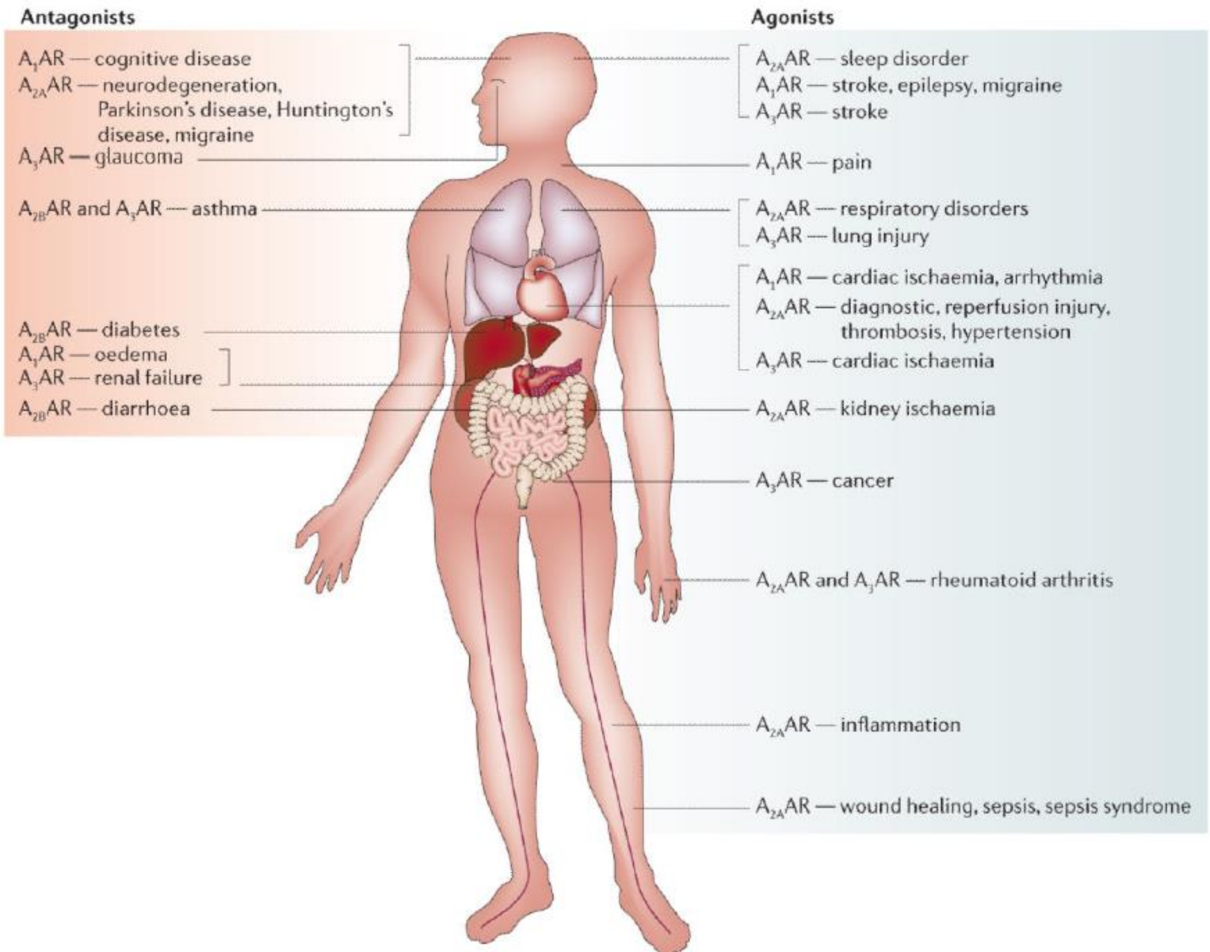

Figure 2-5: Potential therapeutic applications of ARs. Reproduced with permission from (Jacobson & Gao, 2006).

To date, a limited number of adenosine ligands have been approved for therapy (**Figure 2-6**). Short acting parenteral adenosine is used clinically for the treatment of supraventricular tachycardia (Jacobson *et al.*, 2019b). Others include, the A_{2A} AR agonist Regadenoson (approved as a coronary vasodilator) and the A_{2A} AR antagonist Istradefylline (KW-6002) as an anti-Parkinson drug (Dal Ben *et al.*, 2019). Methylxanthines such as caffeine and theophylline acts as antagonists at ARs. Caffeine is clinically used for the treatment of premature apnoea (Chen *et al.*, 2013) and also used as an analgesic co-adjuvant combined with other common analgesics such as paracetamol, ibuprofen or acetylsalicylic acid (Monteiro *et al.*, 2016). It is also widely consumed on a regular basis (coffee, energy drinks) for its stimulant activity on the CNS (Chen *et al.*, 2013). Theophylline is used in the treatment of asthma. Other clinically used drugs such as dipyridamole and methotrexate exert their effects by altering extracellular adenosine concentrations and signalling (Chen *et al.*, 2013).

Regadenoson **Dipyridaole**

Istradefylline **Caffeine**

Figure 2-6: Adenosine ligands approved for clinical use

2.3. Epilepsy

Epilepsy is defined as a chronic neurological disorder characterized by recurrent, unprovoked seizures due to excessive discharge of cerebral neurons (Masino *et al.*, 2014), which alter perception, consciousness, and motor activity. The excessive or hypersynchronous neuronal activity occur as a consequence of an imbalance between excitation and inhibition in the brain

(Spanoghe *et al.*, 2021). Common symptoms of a seizure often include sudden unusual feelings, uncontrollable twitching and even unconsciousness (Clossen & Reddy, 2017). It affects about 50 million people worldwide; hence, it may be said to be one of the most common neurological diseases globally (Organization, 2019). In South Africa, epilepsy affects 1 in every 100 people (Eastman, 2005). The underlying causes of epilepsy are categorized as genetic, structural/metabolic such as brain injury, infections, etc. and unknown causes (Masino *et al.*, 2014).

The types of epilepsy are generally divided into two groups. The first one is called generalised seizures which occur when excessive electrical activity involves the entire brain during which the person may lose consciousness. There are several types of generalised seizures such as absent, clonic, tonic, myoclonic, etc. The second group is called partial (focal) seizures where excessive electrical activity is limited to one side of the brain causing either simple partial seizures or complex partial seizure. (Dawda & Ezewuzie, 2010). Temporal lobe epilepsy is the most common form of partial seizures.

2.3.1. Cellular mechanisms of seizure generation

The major excitatory neurotransmitter in the brain is the amino acid glutamate while the major inhibitory neurotransmitter is gamma amino butyric acid (GABA). Mechanisms involved in pathogenesis of seizure activity result from increased excitatory synaptic neurotransmission of glutamate, decreased GABA mediated inhibitory neurotransmission and altered voltage -gated channel (K+, Na+, T-type Ca2+, Cl^-) activity. All these result in excessive excitability of the neurons which can trigger a seizure (Yardimoglu *et al.*, 2012).

2.3.2. Treatment of epilepsy

The current treatment of epilepsy consists of anti-epileptic drugs (AEDs) (also known as anticonvulsants) such as sodium channel blockers (carbamazepine), calcium channel blockers (gabapentin), glutamate receptor blockers (lamotrigine), GABA reuptake inhibitors (tiagibine), GABA transaminase inhibitors (vigabatrin) and GABA receptor agonists (barbiturates) (**Figure 2-7**). Most AEDs are thought to exert their effects through three possible mechanisms: (1) enhancement of GABA mediated inhibitory neurotransmission, (2) decrease in glutamate mediated excitatory neurotransmission and (3) modulation of voltage-gated ion channels (White *et al.*, 2007). The therapeutic strategy with AEDs is to bring balance between neuronal excitation and inhibition (Sills, 2017).

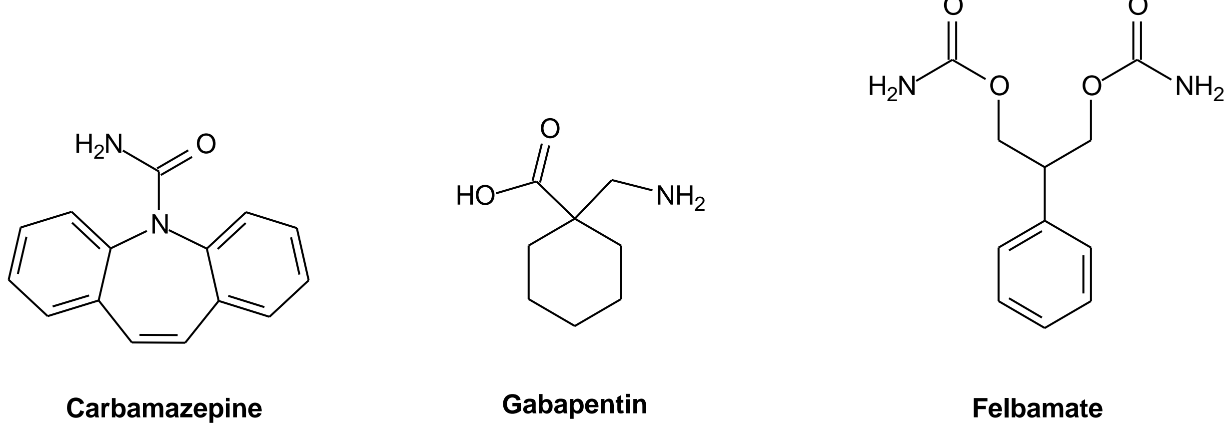

Figure 2-7: Examples of existing AEDs

Currently there is no available cure for epilepsy as AEDs do not adequately prevent seizure development, or permanently halt the occurrence of seizures (Clossen & Reddy, 2017). These therapies are employed to control symptoms of the disease (i.e. suppression of seizures) (Sills, 2017). Therefore, the goal of therapy in epilepsy is the management of seizures to maximize quality of life. Because epilepsy has various underlying causes, and most often the cause(s) are unknown, it is difficult to treat all epilepsies similarly or even successfully (Klaft *et al.*, 2016; Masino *et al.*, 2014). About 70% of epileptic patients benefit from AEDs, while 30% of patients remain medically refractory to AEDs, due to inefficacy in controlling seizures (Klaft *et al.*, 2016). In addition, pronounced side effects may limit the optimum utilisation of the available AEDs (Gouder *et al.*, 2003).

2.3.3. Pharmaco-resistant epilepsy

As mentioned earlier, despite the availability of different classes of AEDs, about one third of epileptic patients is pharmaco-resistant (PRE). Pharmacoresistance can be defined as failure to control seizures after introduction of two or three anticonvulsants that are suitable for the type of epilepsy, prescribed and taken at maximum daily therapeutic doses (Pati & Alexopoulos, 2010). PRE mostly occur in focal (partial) epilepsy patients with Temporal Lobe epilepsy (TLE) being the most common one (Domingos, 2016).

Factors that have been associated with PRE include early onset of seizures, having more than one type of seizure, history of poor seizure control, and history of status epilepticus (Janmohamed *et al.*, 2020). Epileptic patients with a history of brain infection or head trauma (Roy *et al.*, 2019), with certain abnormalities such as cortical dysplasia and electroencephalography (EEG) are also likely to develop drug resistance (Janmohamed *et al.*, 2020; Pati & Alexopoulos, 2010). Other factors are related to the disease itself such as alterations of drug targets and medication related issues such as development of tolerance (Pati & Alexopoulos, 2010). Notably, common causes of treatment failure, such as poor compliance or inappropriate selection of first-line antiepileptic drugs, should be ruled out first before declaring pharmaco-resistance.

There are about three patterns of drug resistance that exist in epilepsy. The *de novo* resistance is where the first drug introduced after diagnosis fails (Schmidt & Löscher, 2005) and most often patients will be resistant to all antiepileptic drugs (Pati & Alexopoulos, 2010). The progressive drug resistance occurs when epilepsy is initially controlled but then gradually becomes refractory and it is most common in childhood epilepsies (Schmidt & Löscher, 2005). It suggested that AED efficacy may be affected by progression of the disease. With the last drug resistance pattern, waxing and waning, resistance alternates between a remitting (pharmacoresponsive) and relapsing (pharmaco-resistant) course (Altwaijri *et al.*, 2020). It may occur due to changes in drug bioavailability and development of tolerance (Pati & Alexopoulos, 2010).

Currently available AEDs are not effective in PRE, and therefore, other pharmacological and non-pharmacological treatments are applied (Domingos, 2016). Pharmacological interventions include aggressive treatment with combination of available antiepileptic drugs and local drug delivery which entails direct delivery of drugs into the epileptogenic brain tissue (Pati & Alexopoulos, 2010). Non-pharmacological treatments include surgical procedures which is the most widely applied but because of a high risk of loss of brain functions not many patients can be treated surgically (Gouder *et al.*, 2003). Other non-pharmacological strategies include vagus nerve stimulation for adults and adolescents over the age of 12 years, focal cooling, and gamma knife therapy (Domingos, 2016). The ketogenic diet (KD) with high fat and low carbohydrate-protein content also provides a non-pharmacologic alternative, and it is usually reserved for young patients with refractory epilepsy (Goswami & Sharma, 2019). It is suggested that KD antiseizure effects include increased GABA production and inhibition of glutamate release through reducing glutamate uptake via vesicular glutamate transporters by direct action of ketone bodies (Juge *et al.*, 2010), direct opening of metabolic ATP-sensitive potassium (KATP) channels with increasing ketone levels (Masino, Susan A & Rho, 2019).

Effects of uncontrolled epilepsy include cognitive deterioration, psychosocial dysfunction, as well as increased morbidity and mortality (Hitiris *et al.*, 2007). PRE has major socioeconomic implications compared to other neurological disorders (Rubio-Donnadieu, 2013). PRE patients incur a cost eight times greater than those whose epilepsy is controlled (Pati & Alexopoulos, 2010). Other indirect expenses include loss of productivity and unemployment by patients or relatives and friends who care for the patient with PRE (Domingos, 2016).

A strategy aimed at preventing or controlling seizures in drug-resistant epilepsy would be an important therapeutic advance. The significant 30% of epileptic of patients who do not experience satisfactory seizure control with present treatments has been an important drive for the search for alternative epilepsy treatment (Klaft *et al.*, 2016). A lot of research for treatment of intractable epilepsy has focused on the role of adenosine in epilepsy which will be discussed in the next section.

2.3.4. Role of adenosine in epilepsy

Adenosine is a long-known endogenous anticonvulsant substance that effectively inhibits excitatory transmission in the brain (Rombo *et al.*, 2018). Boison (2012) suggested that in a normal healthy brain, the development and spread of seizures is prevented by a tonic anticonvulsant effect mediated by this endogenous nucleoside. This hypothesis is supported by various experimental studies showing that adenosine levels rise dramatically during seizures (Swiader *et al.*, 2014). Normal extracellular adenosine concentrations range from 25 to 250 nM, but during situations of metabolic stress, such as during epileptic seizures concentrations rise rapidly to micromolar levels (Boison, 2012). A study by During and Spencer (1992), where microdialysis probes were implanted in the hippocampi of patients with intractable partial epilepsy, revealed a 6- to 31-fold increase of extracellular adenosine during seizure activity. The seizure-related adenosine elevations are believed to be a feedback mechanism that limits seizure intensity or duration (Masino *et al.*, 2014).

The mechanism of adenosine action on regulating seizure activity is mediated through activation of A_1 ARs which is considered to be an inhibitory biological endogenous mechanism, which decreases excitability, limits the duration of seizures and mediates seizure arrest (Ribeiro *et al.*, 2002). This is validated by upregulation of A_1 AR expression immediately after induction of seizures which is a clear indication that in response to seizures, there is a potentiation of the anticonvulsive effects of the adenosinergic system by increasing the amount of A_1 ARs (Spanoghe *et al.*, 2021). In fact, some researchers suggest that an imbalance or altered brain levels of adenosine (low adenosine in the extracellular space) as well as low A_1 AR density may aggravate seizure activity (Boison, 2005). A study by Kochanek *et al.* (2006) on mice revealed that deletion of A_1 ARs leads to spontaneous seizures, increased spreading of induced seizures, aggravated seizure-induced brain damage and even the development of lethal status epilepticus. According to Tomé *et al.* (2010) there seems to be a decreased density of A_1 ARs in chronic epilepsy which further confirms the A_1 AR-mediated inhibitory system.

There are two principal inhibitory anticonvulsant mechanisms exerted by activation of A_1 ARs. Firstly, the released adenosine binds to presynaptic A_1 receptors, which blocks the influx of Ca^{2+} through voltage-dependent calcium channels leading to inhibition of glutamate release, and hence decreased excitation of postsynaptic glutamate receptors (Domingos, 2016; Wardas, 2002). Secondly, postsynaptic activation of A_1 ARs by adenosine opens K^+ channels leading to potassium efflux which results in resting membrane potential hyperpolarization rendering both ionotropic glutamate receptors (NMDA & AMPA) less responsive (Fabera *et al.*, 2019; Spanoghe *et al.*, 2021; Tomé *et al.*, 2010). Both decreased neurotransmitter release and membrane potential hyperpolarization lead to decreased excitatory synaptic transmission and lower probability of seizure generation onset and propagation (Domingos, 2016); hence, the seizure suppression role

of adenosine. Apart from the CNS effects, activation of peripheral adenosine A_1 ARs may lead to a reduction of heart rate, blood pressure, and body temperature (Koeppen *et al.*, 2009; Tupone *et al.*, 2013) which further contribute to seizure suppression (Gouder *et al.*, 2003).

According to Dingledine *et al.* (2014) there tends to be a vicious cycle of epileptogenesis, for seizures are known to cause cell death of critical neurons which in turn increase the likelihood for further seizure activity leading to recurrent chronic epileptic episodes. The advantage of adenosine in seizure control is the fact that it has both seizure-suppressive and neuroprotective functions and therefore able to interrupt this vicious circle (Boison, 2005) (**Figure 2-8**). Seizure suppression of adenosine is mostly mediated through presynaptic activity, while neuroprotection is mostly through postsynaptic activation (Weltha *et al.*, 2019).

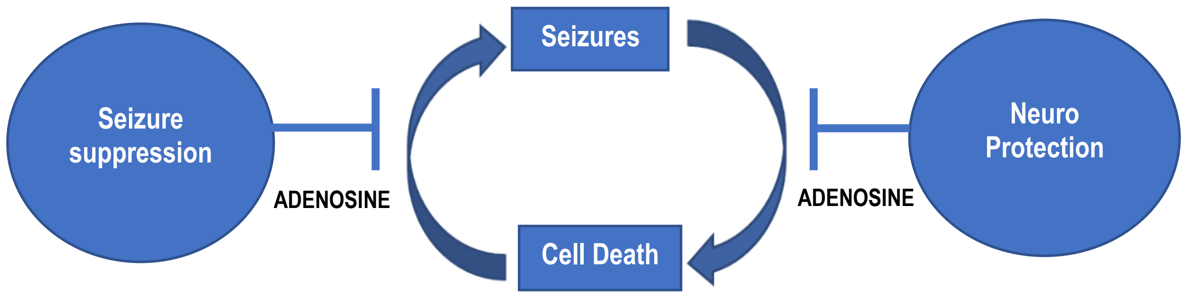

Figure 2-8: Seizure suppression and neuroprotection functions of adenosine. Adpated from (Boison, 2005).

Studies have revealed that systemic injection of selective A_1 AR agonists limits seizures and convulsions in numerous types of experimental seizures and epilepsies (Vianna *et al.*, 2005). A_1 receptors are believed to be strongly associated with antiseizure effects due to their clear effects on neuronal activity, high affinity, and widespread distribution (Masino *et al.*, 2014). On the other hand, adenosine acting through A_{2A} receptors have been shown to aggravate seizure activity in the brain (Spanoghe *et al.*, 2021; Stockwell *et al.*, 2017) and therefore, A_{2A} AR antagonists would be expected to have antiseizure effects (Masino *et al.*, 2014). Etherington and Frenguelli (2004) studied the effects of ZM 241385 (adenosine A_{2A} AR antagonist) on seizure activity on the rat hippocampus, and it turned out that the duration of epileptiform was shortened.

It is in this light that adenosine receptors are a powerful therapeutic target for treatment of conditions involving neural overactivity such as epilepsy. It is worth noting that existing anticonvulsant drugs do not have substantial affinities for ARs but is clear that treatments that facilitate adenosine-based neuromodulation may be effective in preventing seizures especially through A_1 AR activation. As mentioned earlier, about 30% of epileptic patients remain pharmaco-resistant to currently available AEDs. In a study by Klaft *et al.* (2016), effects of an A_1 AR agonist, SDZ WAG 994, were tested on human temporal cortex slices insensitive to a high dose of carbamazepine (50µM) and the A_1 AR agonist efficiently suppressed carbamazepine-resistant seizures. Another A_1 AR agonist, MRS5474, displayed efficacy in a pharmaco-resistant epilepsy

when tested on mice (Domingos, 2016). Gouder *et al.* (2003), also used a mouse model to test effectiveness of 2-chloro-N6-cyclopentyladenosine (CCPA), a selective A_1 AR agonist in drug-resistant temporal lobe epilepsy and the study demonstrated that activation of adenosine A_1 ARs leads to the suppression of seizure activity in drug-resistant epilepsy. Therefore, with all the evidence it can only be concluded that AR-based therapy may provide a great therapeutic potential for patients who do not gain satisfactory seizure control with currently available AEDs since it has shown to successfully suppress pharmaco-resistant seizures. One of the advantages of influencing AR function is that it can modulate neuronal activity directly and shift seizure threshold regardless of the seizure's aetiology, whether environmental, genetic, or originating from unknown causes (Masino *et al.*, 2014).

Epilepsy is frequently associated with a psychiatric disorder such as, depression, anxiety, and personality disorder (Manchanda, 2002). Existence of epilepsy and psychiatric disorders comorbidities occurs in 20 to 50% of patients (Bragatti *et al.*, 2011). According to (Devinsky, 2003), one of the issues with present AEDs is that they often control seizures but may exacerbate psychiatric comorbidities. One of the benefits of potential adenosine-based mechanisms is that they can improve not only epilepsy but also various types of neurological disorders as well (Masino, S. *et al.*, 2009). Shen *et al.* (2012) also confirmed that adenosine augmentation therapies ameliorate the cognitive symptoms and psychomotor-related symptoms in schizophrenia. Therefore, adenosine-based strategies might shed light on desired therapies that reduce seizures and improve psychiatric comorbidity simultaneously (Masino *et al.*, 2014).

In summary, it is clear that activation of adenosine receptors can be explored as new approaches to epilepsy treatment. This is due to the ability of adenosine agonists to inhibit neurotransmitter release, especially the release of the excitatory neurotransmitter glutamate (Knutsen *et al.*, 1995). There has been some evidence that status epilepticus is caused by loss of adenosine anticonvulsant mechanisms which supports that the purinergic agents hold considerable potential as anticonvulsants (Knutsen *et al.*, 1995). Due to this emerging implication of adenosine in the management of seizures, a new field of adenosine-based therapies has been investigated including adenosine itself, adenosine receptor agonists and antagonists and adenosine kinase inhibitors (Pagonopoulou *et al.*, 2006).

2.4. Development of adenosine receptor agonists

Adenosine, a naturally occurring nucleoside, is the endogenous agonist of the four types of ARs. Chemically it is known as 6-amino-9-beta-D-ribofuranosyl-9-H-purine. It is made up of a nucleobase adenine linked to a sugar ribose by a β-N9-glucosidic bond (Effendi *et al.*, 2020). There is growing evidence that AR agonists are attractive therapeutic targets for a number of conditions, including pain, cardiac arrhythmias, myocardial perfusion imaging, cardiac ischemia,

inflammation and certain types of cancer (Gao & Jacobson, 2011). An increasing number of AR agonists are currently in clinical trials for these various conditions (**Figure 2-9**).

Capadenoson
A_1 AR agonist

BAY-606583
A_{2B} AR agonist

Spongosine
A_{2A} AR agonist

Piclidenoson
A_3 AR agonist

Figure 2-9: AR agonists in clinical trials

Pharmacological manipulations of ARs have long been targeted in treating epilepsy, with A_1 AR activation being the most effective (Masino *et al.*, 2014). According to Swiader *et al.* (2014), numerous adenosine agonists acting through A_1 ARs have proven to be potent anticonvulsant compounds in a wide variety of animal models of epilepsy. Adenosine antagonists are considered to be proconvulsants. A study by (Swiader *et al.*, 2014), revealed that aminophylline which is an AR antagonist increases the duration and severity of seizures and in the same study proconvulsant effects of aminophylline were prevented by A_1 AR agonists, therefore convulsions were considered to be due to the blockade of A_1 ARs.

Initially the approach for discovering AR agonists has been restricted to modification of the physiological agonist adenosine (Müller, 2001) and these derivatives, nucleoside-based agonists, represent the great majority of molecules developed and reported to date (Dal Ben *et al.*, 2019). The development of these agonists has been limited by the essential requirement of the retention of the ribose moiety of adenosine for agonist activity (Gao & Jacobson, 2011; Jacobson *et al.*,

1992; Müller, 2001). Most of the useful analogue's modification are limited to substitutions at the N6- or C2-position (**Figure 2-10**) of the adenine moiety (Jacobson *et al.*, 1992) and at the 3′-, 4′- or 5′-position of the ribose moiety (Gao & Jacobson, 2011). Alterations of either structure or stereochemistry result in a loss of receptor binding potency and possibly intrinsic activity (Poulsen & Quinn, 1998).

There are a number of nucleoside based A_1 AR agonists with anticonvulsant activity that have been developed over the years. Some of N6-substituted adenosine derivatives include N6-cyclohexyladenosine (CHA), N6-cyclopentyladenosine (CPA), and 2-Chloro-N6-cyclopentyladenosine (CCPA). The 2-substituted analogues include 2-chloroadenosine (2-CADO) and the 5′-substituted analogues include 5′-Nethylcarboxamidoadenosine (NECA). (Jacobson *et al.*, 1992). Non-selective AR agonists include adenosine itself, 2-CADO, NECA and R- Phenylisopropyladenosine (R-PIA). CCPA, CHA are some of the A_1 AR selective agonists (Knutsen *et al.*, 1995). CCPA is the most potent highly selective A_1 AR ligand with hA_1 AR K_i = 0.4 nM (Domingos, 2016; Fabera *et al.*, 2019). There are a number of studies where CCPA was used to suppress kainic acid (KA) induced pharmaco-resistant seizures in mice (Boison, 2005; Fabera *et al.*, 2019; Pagonopoulou *et al.*, 2006).

Adenosine **2-Chloroadenosine** **N6-Cyclopentyladenosine (CPA)**

Figure 2-10: Adenosine and adenosine derivatives with anticonvulsant action

The limitations of the use of adenosine derivatives AR agonists as anticonvulsant drugs are due to their very narrow therapeutic range (Swiader *et al.*, 2014) and they often produce pronounced peripheral side effects (mainly cardiovascular effects) as well as central side effects (like sedation) (Boison, 2005; Ribeiro *et al.*, 2002). In addition, typical ribose-containing A_1 AR agonists have low blood brain barrier permeability and hence limited use in CNS (Boison, 2005; Jacobson *et al.*, 2019a). Therefore, these drugs have not been pursued clinically (Masino *et al.*, 2014). One of the disadvantages of nucleoside agonists is that they have very short duration of action (Pagonopoulou *et al.*, 2006). Adenosine itself has a very short half-life of 1–2 seconds and is

rapidly metabolized by adenosine kinase and, to a lesser degree, adenosine deaminase to AMP and inosine, respectively (Jacobson & Gao, 2006).

These limitations of nucleoside-based A_1 AR agonists led to development of new strategies to produce potent and selective AR agonists with dominant CNS activity (Masino *et al*., 2014). Widespread AR expression requires compounds endowed not only with high potency and efficacy at the various ARs but also with selectivity for specific AR subtypes and CNS permeability (Dal Ben *et al*., 2019). Non-nucleoside agonists provide an alternative set of compounds highly potent and selective to specific AR subtypes (Gao & Jacobson, 2011). In this study thieno[2,3-b]pyridine derivatives were explored as alternative non-nucleoside A_1 AR agonists in management of seizure disorders.

2.5. 3,5-Dicyanopyridines

3,5-Dicyanopyridine derivatives serve as intermediates in the synthesis of thieno[2,3-b]pyridine derivatives, and exhibit interesting affinity for ARs. There are a number of 3,5-dicyanopyrine derivatives that have been developed, such as Capadenoson (BAY 68-4986), Neladenoson, MMPD, BAY 606583, piclodenoson, etc. Nelodenoson has displayed partial A_1 AR agonist activity (Dal Ben *et al*., 2019; Jacobson *et al*., 2019b) and it is in clinical trials for treatment of chronic heart failure (Jacobson *et al*., 2019b). Capadenoson is a selective agonist of adenosine A_1 AR (Dal Ben *et al*., 2019; Jacobson *et al*., 2019b). BAY-606583 is non-nucleoside and is the most potent and selective A_{2B} AR agonist (Betti *et al*., 2018; Catarzi *et al*., 2019). This compound has reached the preclinical stage for treating angina pectoris (Betti *et al*., 2018). MMPD is a partial A_1 AR agonist (Jacobson *et al*., 2019b). Another A_1 AR partial agonist is Neladenoson bialanate, a prodrug of neladenoson, and is currently evaluated in clinical trials for treatment of heart failure (Meibom *et al*., 2017). LUF series, LUF5844 and LUF5845, are also 3,5-dicyanopyridine derivatives which have been found to be potent as A_1 and A_{2B} AR agonists respectively (Dal Ben *et al*., 2019).

The strategy for developing compounds with good A_1 AR affinity is usually based on structural modifications at the R^1, R^2, Aryl and X positions of the parent 3,5-dicyanopyridine structure (**Figure 2-11**).

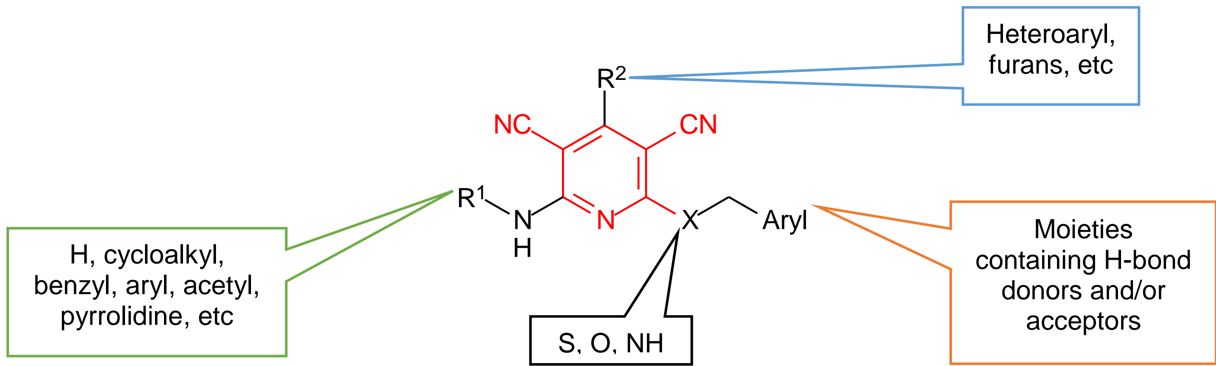

Figure 2-11: General 3,5-dicyanopyridine scaffold (Betti *et al.*, 2019)

A study done by Betti *et al.* (2019), revealed that compounds with S- at position X have higher affinity as compared to compounds with O- or NH- at the position (**Figure 2-12**). The activity of the amino-3,5-dicyanopyridine compounds is influenced by the substituent at position R^2 especially for A_1 AR agonism (Betti *et al.*, 2018)

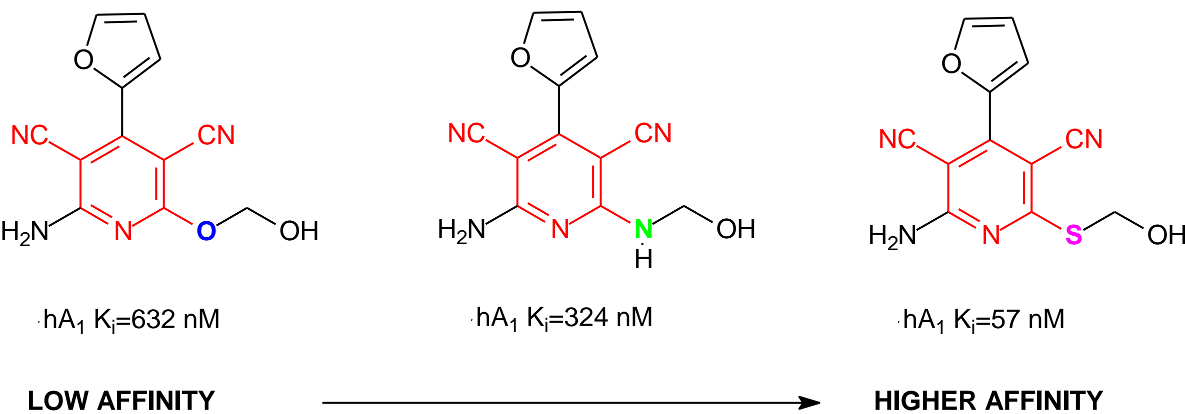

Figure 2-12: Characteristics of compound with O, NH or S at position X (Betti *et al.*, 2019)

The modifications that maintained the selectivity and potency of the capadenoson derivatives include substitution on the 4-phenyl ring of Capadenoson (K_i A_1 AR =1.4 nM) by replacing the para-hydroxyethyloxy substituent with a hydroxy group or methoxy group with K_i A_1 AR = 1.5 nM and EC_{50} A_1 AR = 2.9 nM, respectively (Dal Ben *et al.*, 2019). Substitution of capadenoson's 2-amino group with a pyrroline ring led to development of the A_1 AR agonist with increased potency and selectivity, nelodenoson - A_1 AR EC_{50} = 0.1 nM (Dal Ben *et al.*, 2019; Meibom *et al.*, 2017). Modification of the compound with a para- methoxy group on the 4-phenyl ring by replacing –Cl with -I at the aryl position resulted in a compound with K_i A_1 AR = 3.9 nM (Dal Ben *et al.*, 2019). Further modification which resulted in a potent A_1 AR agonist, LUF5844 (K_i A_1 AR = 2.0 nM), was achieved by inserting a methoxy group in the meta- position of the 4-phenyl ring and replacing a thiazole substituted group of capadesonon with a imidazole ring (Dal Ben *et al.*, 2019; Guo *et al.*, 2018). Another partial A_1 AR agonist, MMPD (K_i A_1 AR = 0.49 nM and EC_{50} = 1.0 nM), was developed by replacing the imidazole ring of LUF5844 with a methylpyridine (Dal Ben *et al.*, 2019;

Guo *et al.*, 2018). Still on capadenoson derivatives, maintaining the para-hydroxyethyloxy substituent at R^2 and replacing -Cl of the thiazole substituted group with -COOH, resulted in a compound with K_i A_1 AR = 0.02 nM (Meibom *et al.*, 2017). The same study indicated that replacing the para-hydroxyethyloxy substituent of capadenoson with dihydroxy derivative results in a compound with EC_{50} A_1 AR = 0.02 nM.

Other 3,5-dicyanopyridine compounds found to display good affinity for A_1 ARs were achieved by maintaining an imidazole group at aryl positions and inserting different functional groups at R^2 (Catarzi *et al.*, 2019). Examples include 4-fluorophenyl at R^2 with K_i A_1 AR = 0.78 nM, 4-phenyl substituent at R^2 (K_i A_1 AR = 2.4 nM) and 4-methylsulfane phenyl at R^2 (K_i A_1 AR = 8.32 nM) (Catarzi *et al.*, 2019). Inserting a 2-furanyl moiety at R^2 and substituting the imidazole group with a benzoic acid moiety at the aryl position produced a compound with a K_i A_1 AR value of 4.12 nM (Betti *et al.*, 2019). LUF6037, with 3,4-methylenedioxy-phenyl group at R^2 and $–CH_2CH(CH_3)OH$ at the aryl position displayed K_i A_1 AR = 0.021 nM (Klaasse, 2008) (**Figure 2-13**).

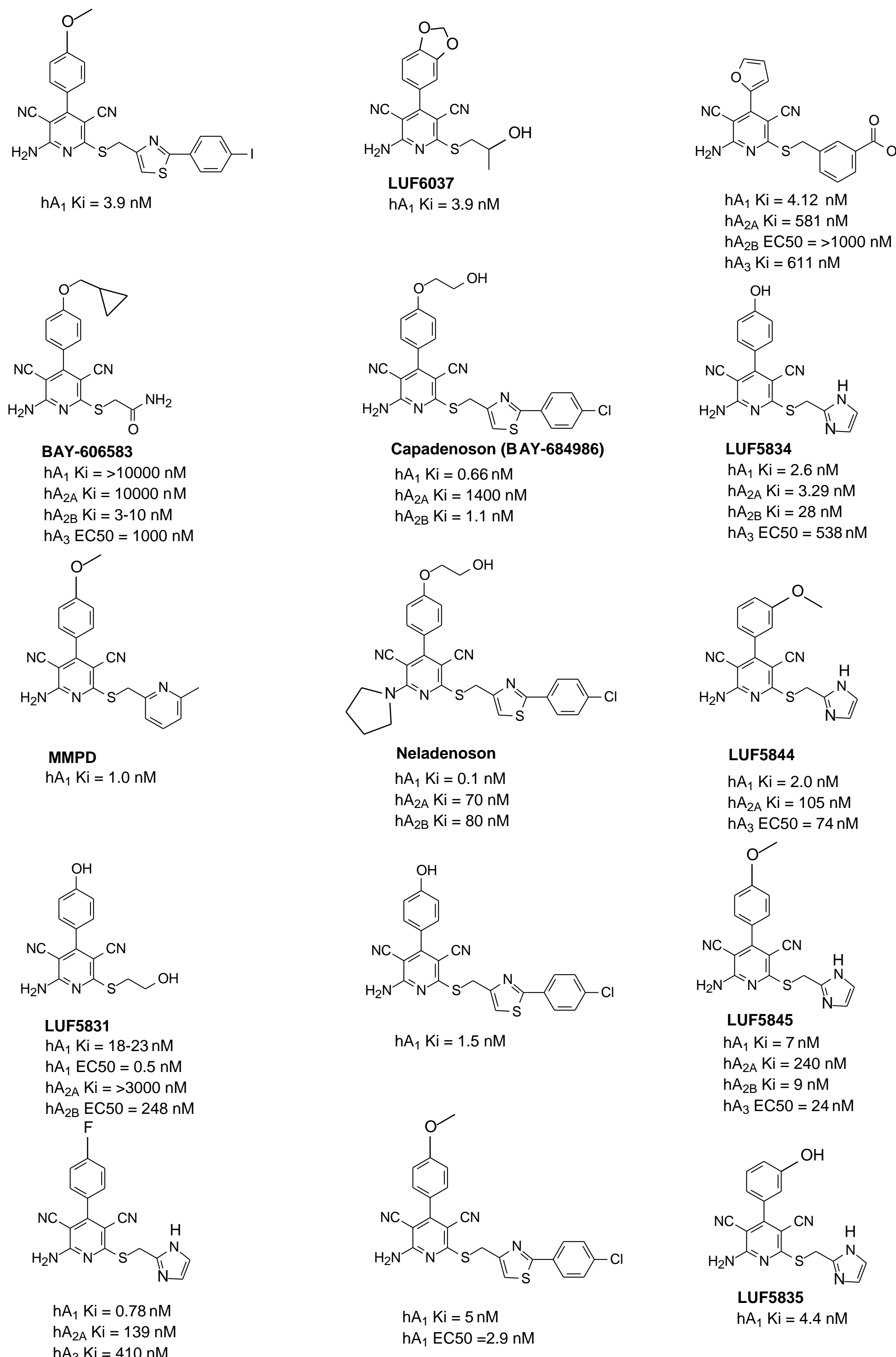

Figure 2-13: Examples of 3,5-dicyanopyridine derivatives with A_1 AR affinities in literature (Betti, 2016; Betti *et al.*, 2019; Catarzi *et al.*, 2019; Dal Ben *et al.*, 2019; Guo *et al.*, 2018; Klaasse, 2008)

2.6. Thienopyridines

Pyridines are heterocyclic six-membered aromatic compounds containing a single nitrogen atom. Derivatives of pyridine attract significant interest because of their great practical usefulness, primarily, due to their various biological activities (Litvinov *et al.*, 2005). Pyridine derivatives of different heterocyclic nucleuses have shown potent pharmacological properties like antifungal, antitubercular, antibacterial, antimicrobial, insecticidal (Alinaghizadeh *et al.*, 2016) and antimalarial (Masch *et al.*, 2019) (**Figure 2-14**). Among pyridine derivatives, the fused analogues are often of much greater interest biologically than the corresponding monocyclic compounds. This is due the presence of varying pharmacophoric groups in different positions and their ability to react with a wider range of receptors. Among heterocyclic systems there are thienopyridines which are derivatives of pyridine fused to thiophene systems (Litvinov *et al.*, 2005).

Figure 2-14: Structure of Pyridine & Thienopyridine.

Thienopyridines as a class of heterocyclic compounds, attract considerable interest due to their very broad spectrum of biological activities (Alinaghizadeh *et al.*, 2016). There are six isomeric thienopyridine structures characterized by different annulation modes: thieno[2,3-b]pyridine , thieno[3,2-b]pyridine, thieno[2,3- c]pyridine, thieno[3,2-c]pyridine, thieno[3,4-b]pyridine, and thieno[3,4-c] pyridine (Litvinov *et al.*, 2005) (**Figure 2-15**).

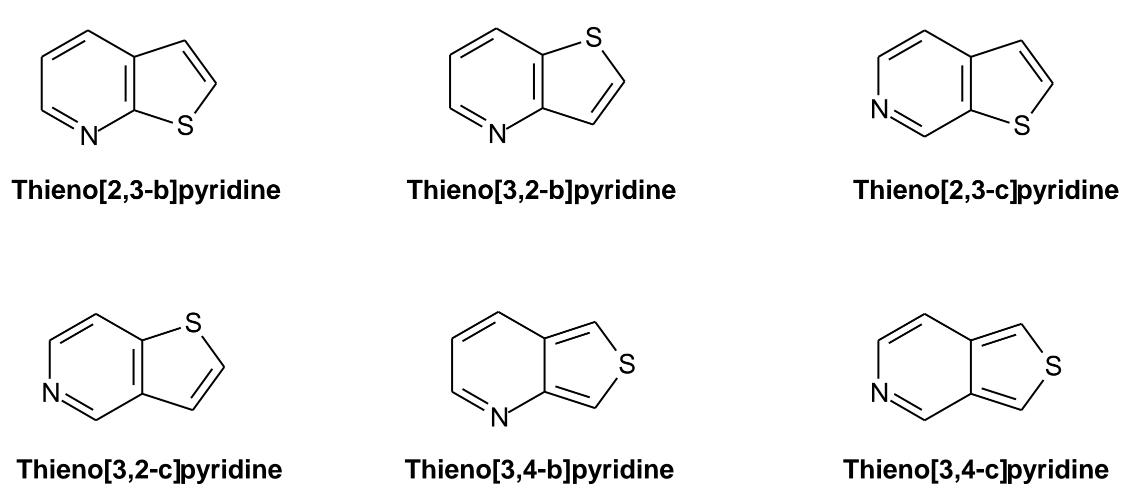

Figure 2-15: Annulation modes of thienopyridines.

The high pharmacological potential of thienopyridine derivatives made these compounds a privileged scaffold (Litvinov *et al.*, 2005). The clinically available thienopyridine derivatives are ticlopidine and clopidogrel (**Figure 2-16**) which act as non-competitive adenosine diphosphate receptor antagonists to inhibit platelet aggregation (Kam & Nethery, 2003).

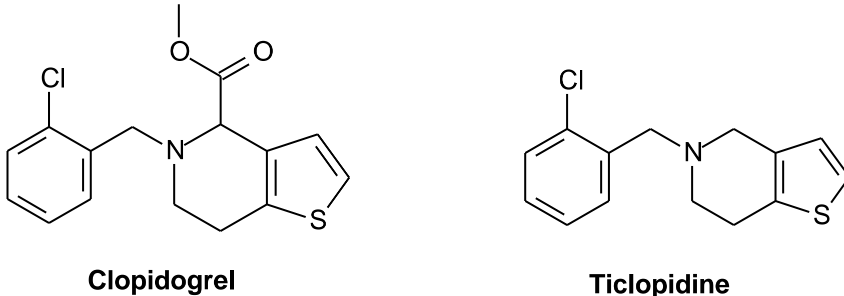

Figure 2-16: Structures of thienopyridine derivatives in clinical use.

Thieno[2,3-b]pyridine derivatives (**Figure 2-17**) have attracted attention due to their pharmacological activities including; antitumor, antibacterial (Al-Trawneh *et al.*, 2011), antiviral (Amorim *et al.*, 2017; Bernardino *et al.*, 2004), vasodilator and antihypertensive (Adachi *et al.*, 1988), antidiabetic (Bahekar *et al.*, 2007), anti-inflammatory (Liu *et al.*, 2013), antidermatophytic (Ouf & Gaber, 2005), antimalarial activities (Masch *et al.*, 2019) in addition to treatment of CNS disorders (Hassan *et al.*, 2013). Some are selective progesterone receptor agonists (Wang *et al.*, 2009), and factor 2 kinase (eEF2-K) inhibitors (Lockman *et al.*, 2010) and intermediates in the synthesis of non-nucleoside inhibitors of human cytomegalovirus and related herpes polymerases (Schnute *et al.*, 2005).

Figure 2-17: Thieno[2,3-b]pyridine scaffold

Little data has been reported in the literature on the adenosine receptor affinity of thieno[2,3-b]pyridines. Betti and co-workers (2018) synthesised several 3,5-dicyanopyridine derivatives based on the selective A_{2B} AR agonist, BAY60-6583, devoid of any A_1, A_{2A} and A_3 AR affinity. In addition they only cyclised BAY60-6583 to yield the corresponding bicyclic thieno[2,3-b]pyridine (3,6-Diamino-5-cyano-4-[4-(cyclopropylmethoxy)phenyl]thieno [2,3-b]pyridine-2-carboxamide) (**Figure 2-18**). Their results showed that the bicyclic thieno[2,3-b]pyridine lacked affinity for all 4 AR subtypes and suggested that the cyclisation made the structure more rigid and thus detrimental for interaction with ARs (Betti *et al.*, 2018).

Figure 2-18: Cyclisation of BAY60-6583 to 3,6-Diamino-5-cyano-4-[4-(cyclopropylmethoxy)phenyl]-thieno [2,3-b]pyridine-2-carboxamide. (a – KOH, EtOH)

Due to chemical similarity between the 3,5-dicyanopyridine and thieno[2,3-b]pyridine cores and only one thienopyridine compound screened for AR affinity, we envisaged that a suitably substituted thieno[2,3-b]pyridine core could lead to derivatives that could exhibit potent AR affinity. The focus of the current study is to design and synthesize thieno[2,3-b]pyridine derivatives with AR affinity from intermediate compounds which are amino-3,5-dicyanopyridine derivatives.

2.7. Synthetic approaches for target compounds

The synthesis of the target compounds, thieno[2,3-b]pyridine derivatives will be carried out through multicomponent condensation of malononitrile with hydrogen sulfide, corresponding aldehyde, and a suitable halide, in the presence of triethylamine. Thieno[2,3-b]pyridine derivatives have been previously prepared in a multistep procedure from either a thiophene or a pyridine ring and further ring closure leading to the heterocyclic fused system (Alinaghizadeh *et al.*, 2016). Unfortunately, many of these methods suffer from limitations such as long reaction times, low to moderate yields and co-occurrence of several side products (Alinaghizadeh *et al.*, 2016). The multicomponent reactions offer advantages of mild conditions, short reaction time and high yield product with desired purity when compared with a synthesis procedure that uses a sequence of two-component reactions towards the same product (Weber, 2002).

2.8. Summary

In summary, it is clear that activation of ARs can be explored as a new approach to epilepsy treatment. This is due to the ability of adenosine agonists to inhibit neurotransmitter release, especially the release of the excitatory neurotransmitter glutamate (Knutsen *et al.*, 1995). There has been some evidence that status epilepticus is caused by loss of adenosine anticonvulsant mechanisms which supports that the purinergic agents hold considerable potential as anticonvulsants (Knutsen *et al.*, 1995). Due to this emerging implication of adenosine in the management of seizures, a new field of adenosine-based therapies has been investigated

including adenosine itself, adenosine receptor agonists and antagonists and adenosine kinase inhibitors (Pagonopoulou *et al.*, 2006).

2.9. References

Adachi, I., YamamoriI, T., Hiramatsu, Y., SakaiI, K., Mihara, S., KawakamiI, M., Masui, M., Uno, O., Ueda, M. 1988.Studies on dihydropyridines. III. Synthesis of 4, 7-dihydrothieno [2, 3-b]-pyridines with vasodilator and antihypertensive activities. *Chemical and Pharmaceutical Bulletin*, 36(11):4389-4402.

Al-Trawneh, S.A., El-Abadelah, M.M., Zahra, J.A., Al-Taweel, S.A., Zani, F., Incerti, M., Cavazzoni, A. & Vicini, P. 2011. Synthesis and biological evaluation of tetracyclic thienopyridones as antibacterial and antitumor agents. *Bioorganic & Medicinal Chemistry*, 19(8):2541-2548.

Alinaghizadeh, F., Zahedifar, M., Seifi, M. & Sheibani, H. 2016. Cascade synthesis of thieno [2, 3-b] pyridines by using intramolecular cyclization reactions of 3-cyano-2-(organylmethylthio) pyridines. *Journal of the Brazilian Chemical Society*, 27(4):663-669.

Altwaijri, W.A., Yahya, B.J., Alasmari, L.B., Alsultan, R.N., Alsuhaibani, S.M., Alsemih, R.M. & Moukaddem, A.K. 2020. Quality of life in paediatrics with intractable epilepsy in a large paediatric university hospital in Riyadh, Saudi Arabia. *Journal of Family Medicine and Primary Care*, 9(11):5523-5536.

Amorim, R., de Meneses, M. D.F., Borges, J.C., da Silva Pinheiro, L.C., Caldas, L.A., Cirne-Santos, C.C., de Mello, M. V.P., de Souza, A.M.T., Castro, H.C. & de Palmer Paixao, I.C.N. 2017. Thieno [2, 3-b] pyridine derivatives: a new class of antiviral drugs against Mayaro virus. *Archives of Virology*, 162(6):1577-1587.

Bahekar, R.H., Jain, M.R., Jadav, P.A., Prajapati, V.M., Patel, D.N., Gupta, A.A., Sharma, A., Tom, R., Bandyopadhya, D. & Modi, H. 2007. Synthesis and antidiabetic activity of 2, 5-disubstituted-3-imidazol-2-yl-pyrrolo [2, 3-b] pyridines and thieno [2, 3-b] pyridines. *Bioorganic & Medicinal Chemistry*, 15(21):6782-6795.

Ballesteros-Yanez, I., Castillo, C.A., Merighi, S. & Gessi, S. 2017. The Role of Adenosine Receptors in Psychostimulant Addiction. *Frontiers in Pharmacology*, 8(985):1-18.

Benarroch, E.E. 2008. Adenosine and its receptors: multiple modulatory functions and potential therapeutic targets for neurologic disease. *Neurology*, 70(3):231-236.

Bernardino, A.M., Pinheiro, L.C., Ferreira, V.F., Azevedo, A.R., Carneiro, J., Souza, T.M. & Frugulhetti, I. 2004. Synthesis and antiviral activity of new 4-(phenylamino) thieno [2, 3-b] pyridine derivatives. *Heterocyclic Communications*, 10(6):407-410.

Betti, M. 2016. *Design,Synthesis and Pharmacological evaluation of new adenosine receptor ligands*, Florence:University of Florence (Thesis -PhD).

Betti, M., Catarzi, D., Varano, F., Falsini, M., Varani, K., Vincenzi, F., Dal Ben, D., Lambertucci, C. & Colotta, V. 2018.The aminopyridine-3,5-dicarbonitrile core for the design of new non-nucleoside-like agonists of the human adenosine A2B receptor. *European Journal Medicinal Chemistry*, 150:127-139.

Betti, M., Catarzi, D., Varano, F., Falsini, M., Varani, K., Vincenzi, F., Pasquini, S., di Cesare Mannelli, L., Ghelardini, C., Lucarini, E., Dal Ben, D., Spinaci, A., Bartolucci, G., Menicatti, M. & Colotta, V. 2019.Modifications on the Amino-3,5-dicyanopyridine Core To Obtain Multifaceted Adenosine Receptor Ligands with Antineuropathic Activity. *Journal of Medicinal Chemistry*, 62(15):6894-6912.

Boison, D. 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. *Neuroscientist*, 11(1):25-36.

Boison, D. 2012. Adenosine dysfunction in epilepsy. *Glia*, 60(8):1234-1243.

Borea, P.A., Gessi, S., Merighi, S., Vincenzi, F. & Varani, K. 2018. Pharmacology of adenosine receptors: the state of the art. *Physiological reviews*, 98(3):1591-1625.

Bragatti, J.A., Torres, C.M., Isolan, G.R. & Bianchin, M.M. 2011. Psychiatric Comorbidities of Epilepsy: A Review. *Journal of Neurology & Neurophysiology*,S2:1-10.

Catarzi, D., Varano, F., Varani, K., Vincenzi, F., Pasquini, S., Dal Ben, D., Volpini, R. & Colotta, V. 2019. Amino-3,5-Dicyanopyridines Targeting the Adenosine Receptors Ranging from Pan Ligands to Combined A1/A2B Partial Agonists. *Pharmaceuticals (Basel)*, 12(159):1-22.

Chen, J.F., Eltzschig, H.K. & Fredholm, B.B. 2013. Adenosine receptors as drug targets--what are the challenges? *Nature Review Drug Discovery*, 12(4):265-286.

Clark, T. 2017. G-Protein coupled receptors: answers from simulations. *Beilstein Journal of Organic Chemistry*, 13(1):1071-1078.

Clossen, B.L. & Reddy, D.S. 2017. Novel therapeutic approaches for disease-modification of epileptogenesis for curing epilepsy. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1863(6):1519-1538.

Cunha, R.A. 2016. How does adenosine control neuronal dysfunction and neurodegeneration? *Journal of Neurochemistry*, 139(6):1019-1055.

Dal Ben, D., Lambertucci, C., Buccioni, M., Marti Navia, A., Marucci, G., Spinaci, A. & Volpini, R. 2019. Non-Nucleoside Agonists of the Adenosine Receptors: An Overview. *Pharmaceuticals (Basel)*, 12(4):1-22.

Dawda, Y. & Ezewuzie, N. 2010. Epilepsy; Clinical features and Diagnosis. *Clinical Pharmacist*, 2:86-88.

Devinsky, O. 2003. Psychiatric comorbidity in patients with epilepsy: implications for diagnosis and treatment. *Epilepsy & Behavior*, 4:2-10.

Dingledine, R., Varvel, N.H. & Dudek, F.E. 2014. When and How Do Seizures Kill Neurons, and Is Cell Death Relevant to Epileptogenesis? *Advances in experimental medicine and biology,* 813:109-22.

Domingos, C.I.A. 2016. *Probing the synaptic target of a new putative antiepileptic drug: Modulation of the excitatory transmission in the hippocampus.* Lisboa: University of Lisboa (PhD Thesis).

During, M.J. & Spencer, D.D. 1992. Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Annals of Neurology*, 32(5):618-624.

Eastman, R. 2005. Epilepsy in South Africa. *Acta Neurologica Scandinavica*, 112(181):8-11.

Effendi, W.I., Nagano, T., Kobayashi, K. & Nishimura, Y. 2020. Focusing on adenosine receptors as a potential targeted therapy in human diseases. *Cells*, 9(3):1-36.

Ernst, P.B., Garrison, J.C. & Thompson, L.F. 2010. Much ado about adenosine: adenosine synthesis and function in regulatory T cell biology. *Journal of Immunology*, 185(4):1993-1998.

Etherington, L.A.V. & Frenguelli, B.G. 2004. Endogenous adenosine modulates epileptiform activity in rat hippocampus in a receptor subtype-dependent manner. *European Journal of Neuroscience*, 19(9):2539-2550.

Fabera, P., Parizkova, M., Uttl, L., Vondrakova, K., Kubova, H., Tsenov, G. & Mares, P. 2019. Adenosine A1 Receptor Agonist 2-chloro-N6-cyclopentyladenosine and Hippocampal Excitability During Brain Development in Rats. *Frontiers in pharmacology*, 10(656):1-11.

Gao, Z.G. & Jacobson, K.A. 2011. Emerging adenosine receptor agonists: an update. *Expert Opinion on Emerging Drugs*, 16(4):597-602.

Goswami, J.N. & Sharma, S. 2019. Current perspectives on the role of the Ketogenic diet in epilepsy management. *Neuropsychiatric disease and treatment*, 15:3273-3285.

Gouder, N., Fritschy, J.M. & Boison, D. 2003. Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. *Epilepsia*, 44(7):877-885.

Guo, M., Gao, Z.G., Tyler, R., Stodden, T., Li, Y., Ramsey, J., Zhao, W.J., Wang, G.J., Wiers, C.E., Fowler, J.S., Rice, K.C., Jacobson, K.A., Kim, S.W. & Volkow, N.D. 2018. Preclinical Evaluation of the First Adenosine A1 Receptor Partial Agonist Radioligand for Positron Emission Tomography Imaging. *Journal of Medicinal Chemistry*, 61(22):9966-9975.

Ham, J. & Evans, B.A. 2012. An emerging role for adenosine and its receptors in bone homeostasis. *Frontiers in Endocrinology (Lausanne)*, 3(113):1-8.

Hassan, A.Y., Sarg, M.T., Said, M.M. & El-Sebaey, S.A. 2013. Utility of thieno[2,3-b] pyridine derivatives in the synthesis of some condensed heterocyclic compounds with expected biological activity. *Universal Organic Chemistry*, 1(1):1-15.

Hitiris, N., Mohanraj, R., Norrie, J., Sills, G.J. & Brodie, M.J. 2007. Predictors of pharmacoresistant epilepsy. *Epilepsy research*, 75(2-3):192-196.

Jacobson, K.A. 2009. Introduction to adenosine receptors as therapeutic targets. *Handbook of Experimental Pharmacology*, (193):1-24.

Jacobson, K.A. & Gao, Z.G. 2006. Adenosine receptors as therapeutic targets. *Nature Review Drug Discovery*, 5(3):247-264.

Jacobson, K.A., Van Galen, P.J. & Williams, M. 1992. Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. *Journal of Medicinal Chemistry*, 35(3):407-422.

Jacobson, K.A., Tosh, D.K., Jain, S. & Gao, Z.-G. 2019a. Historical and current adenosine receptor agonists in preclinical and clinical development. *Frontiers in Cellular Neuroscience*, 13 (124):1-17.

Janmohamed, M., Brodie, M.J. & Kwan, P. 2020. Pharmacoresistance–Epidemiology, mechanisms, and impact on epilepsy treatment. *Neuropharmacology*, 168:1-9.

Juge, N., Gray, J.A., Omote, H., Miyaji, T., Inoue, T., Hara, C., Uneyama, H., Edwards, R. H., Nicoll, R.A. & Moriyama, Y. 2010. Metabolic control of vesicular glutamate transport and release. *Neuron*, 68(1):99-112.

Kam, P.C. & Nethery, C.M. 2003. The thienopyridine derivatives (platelet adenosine diphosphate receptor antagonists), pharmacology and clinical developments. *Anaesthesia*, 58(1):28-35.

Klaasse, E. 2008. *Non-ribose ligands for the human A1 receptors*, Potchefstroom:North West University (Thesis - PhD).

Klabunde, R. 2011. *Cardiovascular physiology concepts*. Philadelphia: Lippincott Williams & Wilkins.

Klaft, Z. J., Hollnagel, J. O., Salar, S., Caliskan, G., Schulz, S.B., Schneider, U.C., Horn, P., Koch, A., Holtkamp, M., Gabriel, S., Gerevich, Z. & Heinemann, U. 2016. Adenosine A1 receptor-mediated suppression of carbamazepine-resistant seizure-like events in human neocortical slices. *Epilepsia*, 57(5):746-756.

Klotz, K.N. 2000. Adenosine receptors and their ligands. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 362:382-391.

Knutsen, L.J.S., Lau, J., Sheardown, M.J., Eskesen, K., Thomsen, C., Weis, J.U., Judge, M.E., Klitgaard, H. 1995. Anticonvulsant Actions of Novel and Reference Adenosine Agonists. In: Belardinelli, L. & Pelleg, A., eds. *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology*. Norwell, MA USA: Kluwer Academic Publishers. pp. 479-487.

Kochanek, P.M., Vagni, V.A., Janesko, K.L., Washington, C.B., Crumrine, P.K., Garman, R.H., Jenkins, L.W., Clark, R.S.B., Homanics, G.E. & Dixon, C. E. 2006. Adenosine A1 receptor knockout mice develop lethal status epilepticus after experimental traumatic brain injury. *Journal of Cerebral Blood Flow & Metabolism*, 26(4):565-575.

Koeppen, M., Eckle, T. & Eltzschig, H.K. 2009. Selective deletion of the A1 adenosine receptor abolishes heart-rate slowing effects of intravascular adenosine in vivo. *PloS one*, 4(8):1-9.

Layland, J., Carrick, D., Lee, M., Oldroyd, K. & Berry, C. 2014. Adenosine: physiology, pharmacology, and clinical applications. *JACC: Cardiovascular Interventions*, 7(6):581-591.

Litvinov, V.P., Dotsenko, V.V. & Krivokolysko, S.G. 2005. Thienopyridines: synthesis, properties, and biological activity. *Russian Chemical Bulletin*, 54(4):864-904.

Liu, H., Li, Y., Wang, X., Wang, B., He, H., Liu, J., Xiang, M., He, J., Wu, X. Yang, L. 2013. Synthesis, preliminary structure–activity relationships, and in vitro biological evaluation of 6-aryl-3-amino-thieno [2, 3-b] pyridine derivatives as potential anti-inflammatory agents. *Bioorganic & Medicinal Chemistry Letters*, 23(8):2349-2352.

Lockman, J.W., Reeder, M.D., Suzuki, K., Ostanin, K., Hoff, R., Bhoite, L., Austin, H., Baichwal, V. & Willardsen, J. A. 2010. Inhibition of eEF2-K by thieno [2, 3-b] pyridine analogues. *Bioorganic & Medicinal Chemistry Letters*, 20(7):2283-2286.

Manchanda, R. 2002. Psychiatric disorders in epilepsy: clinical aspects. *Epilepsy & Behavior*, 3(1):39-45.

Masch, A., Nasereddin, A., Alder, A., Bird, M. J., Schweda, S. I., Preu, L., Doerig, C., Dzikowski, R., Gilberger, T.W. & Kunick, C. 2019. Structure-activity relationships in a series of antiplasmodial thieno[2,3-b]pyridines. *Malaria Journal*, 18(89):1-10.

Masino, S., Kawamura Jr, M., Wasser, C., Pomeroy, L. & Ruskin, D. 2009. Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. *Current Neuropharmacology*, 7(3):257-268.

Masino, S.A. & Rho, J.M. 2019. Metabolism and epilepsy: ketogenic diets as a homeostatic link. *Brain Research*, 1703:26-30.

Masino, S.A., Kawamura, M.J. & Ruskin, D.N. 2014. Adenosine receptors and epilepsy: current evidence and future potential. *International Review of Neurobiolology*, 119:233-255.

Meibom, D., Albrecht-Kupper, B., Diedrichs, N., Hubsch, W., Kast, R., Kramer, T., Krenz, U., Lerchen, H. G., Mittendorf, J., Nell, P. G., Sussmeier, F., Vakalopoulos, A. & Zimmermann, K. 2017. Neladenoson Bialanate Hydrochloride: A Prodrug of a Partial Adenosine A1 Receptor Agonist for the Chronic Treatment of Heart Diseases. *ChemMedChem*, 12:728-737.

Monteiro, J.P., Alves, M.G., Oliveira, P.F. & Silva, B.M. 2016. Structure-bioactivity relationships of methylxanthines: Trying to make sense of all the promises and the drawbacks. *Molecules*, 21(8):1-32.

Müller, C.E. 2001. A1 adenosine receptors and their ligands: overview and recent developments. *Il Farmaco*, 56(1-2):77-80.

Ouf, S.A. & Gaber, H.M. 2005. New fused pyridines: Synthesis of certain pyridothienopyrimidine derivatives as anti-dermatophytic agents. *Affinity: Journal of Theoritical and Applied Chemistry*, 62(518):337-345.

Pagonopoulou, O., Efthimiadou, A., Asimakopoulos, B. & Nikolettos, N. 2006. Modulatory role of adenosine and its receptors in epilepsy: possible therapeutic approaches. *NeuroscienceRresearch*, 56(1):14-20.

Pastor-Anglada, M. & Pérez-Torras, S. 2018. Who is who in adenosine transport. *Frontiers in Pharmacology*, 9(627):1-10.

Pati, S. & Alexopoulos, A.V. 2010. Pharmacoresistant epilepsy: from pathogenesis to current and emerging therapies. *Cleveland Clinic Journal Medicinal*, 77(7):457-567.

Poulsen, S.-A. & Quinn, R.J. 1998. Adenosine receptors: new opportunities for future drugs. *Bioorganic & Medicinal Chemistry*, 6(6):619-641.

Ribeiro, J., Sebastiao, A. & De Mendonça, A. 2002. Adenosine receptors in the nervous system: pathophysiological implications. *Progress in Neurobiology*, 68(6):377-392.

Rombo, D.M., Ribeiro, J.A. & Sebastião, A.M. 2018. Role of adenosine receptors in epileptic seizures. In: Borea, P., Varani, K., Gessi, S., Merighi, S. & Vincenzi, F., eds. *The Adenosine Receptors*. Cham: Humana Press. pp. 309-350.

Rosenbaum, D.M., Rasmussen, S.G. & Kobilka, B.K. 2009. The structure and function of G-protein-coupled receptors. *Nature*, 459(7245):356-363.

Roy, P.L., Ronquillo, L.H., Ladino, L.D. & Tellez-Zenteno, J.F. 2019. Risk factors associated with drug resistant focal epilepsy in adults: A case control study. *Seizure*, 73:46-50.

Rubio-Donnadieu, F. 2013. Pharmacoresistance and epilepsy. In. *Pharmacoresistance in Epilepsy*: Springer. pp. 1-9.

Sachdeva, S. & Gupta, M. 2013. Adenosine and its receptors as therapeutic targets: An overview. *Saudi Pharm J*, 21(3):245-253.

Schmidt, D. & Löscher, W. 2005. Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. *Epilepsia*, 46(6):858-877.

Schnute, M.E., Cudahy, M.M., Brideau, R.J., Homa, F.L., Hopkins, T.A., Knechtel, M.L., Oien, N.L., Pitts, T.W., Poorman, R.A. & Wathen, M.W. 2005. 4-Oxo-4, 7-dihydrothieno [2, 3-b] pyridines as non-nucleoside inhibitors of human cytomegalovirus and related herpesvirus polymerases. *Journal of Medicinal Chemistry*, 48(18):5794-5804.

Sebastiao, A.M. & Ribeiro, J.A. 2009. Adenosine receptors and the central nervous system. *Handbook of Experimental Pharmacology*, (193):471-534.

Shen, H. Y., Singer, P., Lytle, N., Wei, C. J., Lan, J. Q., Williams-Karnesky, R. L., Chen, J. F., Yee, B. K., Boison, D. 2012. Adenosine augmentation ameliorates psychotic and cognitive endophenotypes of schizophrenia. *Journal of Clinical Investigation*, 122(7):2567-2577.

Sheth, S., Brito, R., Mukherjea, D., Rybak, L.P. & Ramkumar, V. 2014. Adenosine receptors: expression, function and regulation. *International Journal of Molecular Sciences*, 15(2):2024-2052.

Sills, G.J. 2017. Mechanisms of action of antiepileptic drugs. In Rugg-Gunn, F.J. & Stapley H.B., eds. *Epilepsy 2011: From Bench to Bedside. A Practical Guide to Epilepsy*, London: International League Against Epilepsy. pp. 395-303.

Spanoghe, J., Larsen, L.E., Craey, E., Manzella, S., Van Dycke, A., Boon, P. & Raedt, R. 2021. The Signaling Pathways Involved in the Anticonvulsive Effects of the Adenosine A1 Receptor. *International Journal of Molecular Sciences*, 22(1):1-25.

Stiles, G. 1992. Adenosine receptors. *Journal of Biological Chemistry*, 267(10):6451-6454.

Stockwell, J., Jakova, E. & Cayabyab, F.S. 2017. Adenosine A1 and A2A Receptors in the Brain: Current Research and Their Role in Neurodegeneration. *Molecules*, 22(676):1-18.

Stone, T.W., Ceruti, S. & Abbracchio, M.P. 2009. Adenosine receptors and neurological disease: neuroprotection and neurodegeneration. *Handbook of Experimental Pharmacology*, 193:535-587.

Swiader, M.J., Kotowski, J. & Luszczki, J.J. 2014. Modulation of adenosinergic system and its application for the treatment of epilepsy. *Pharmacology Reports*, 66(3):335-342.

Tomé, Â.R., Silva, H. & Cunha, R.A. 2010. Role of the purinergic neuromodulation system in epilepsy. *The Open Neuroscience Journal*, 4(1):64-83.

Tupone, D., Madden, C.J. & Morrison, S.F. 2013. Central activation of the A1 adenosine receptor (A1AR) induces a hypothermic, torpor-like state in the rat. *Journal of Neuroscience*, 33(36):14512-14525.

Vianna, E.P.M., Ferreira, A.T., Dona, F., Cavalheiro, E.A. & Fernades, J. 2005. Modulation of Seizures and Synaptic Plasticity by Adenosinergic receptors in an experimental model of temporal lobe epilepsy induced by pilocarpine in rats. *Epilepsia*, 46(5):166-173.

Wang, Y., Duraiswami, C., Madauss, K.P., Tran, T.B., Williams, S.P., Deng, S., Graybill, T.L., Hammond, M., Jones, D.G. & Grygielko, E. 2009. 2-Amino-9-aryl-3-cyano-4-methyl-7-oxo-6, 7, 8, 9-tetrahydropyrido [2′, 3′: 4, 5] thieno [2, 3-b] pyridine derivatives as selective progesterone receptor agonists. *Bioorganic & Medicinal Chemistry Letters*, 19(17):4916-4919.

Wardas, J. 2002. Neuroprotective role of adenosine in the CNS. *Polish Journal of Pharmacology*, 54(4):313-326.

Weber, L. 2002. The application of multi-component reactions in drug discovery. *Current Medicinal Chemistry*, 9(13):1-9.

Weltha, L., Reemmer, J. & Boison, D. 2019. The role of adenosine in epilepsy. *Brain Research Bulletin*, 151:46-54.

White, H.S., Smith, M.D. & Wilcox, K.S. 2007. Mechanisms of action of antiepileptic drugs. *International Review of Neurobiology*, 81:85-110.

World Health Organization. 2019. Epilepsy: a public health imperative. Thailand: L'IV Com Sàrl.

Yardimoglu, M., Ilbay, G., Dalcik, C., Dalcik, H. & Kokturk, S. 2012. Yardimoglu, M. Ilbay, G., Dalcik, H. & Kokturk, S. 2012. Cellular and Molecular Mechanisms Underlying Epilepsy: An Overview with Our Findings, In Stevanovic, D. (ed.), *Epilepsy - Histological, Electroencephalographic and Psychological Aspects*, London. pp 1-30.

CHAPTER 3

3. ORIGINAL ARTICLE TO BE SUBMITTED

This chapter comprises of an original research article that will be submitted for publication to *Medicinal Chemistry Research*. The article was written in accordance with the said journal's guide for authors (**Annexure B**), which is available online in the journal's author information pack:

https://www.springer.com/journal/44/submission-guidelines#linksAndDownloads

All co-authors have given their permission to include the article in this dissertation. Supplementary material (**Annexure A**) that will be submitted with the manuscript have been included.

Please note that the article was written in British English and that the reference list has been compiled according to the Vancouver referencing format.

Design, synthesis and evaluation of amino-3,5-dicyanopyryridines and thieno[2,3-b]pyridines as ligands of adenosine receptors

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Abstract

Due to the implication of adenosine in seizure suppression, adenosine-based therapies such as adenosine receptor (AR) agonists have been investigated. This study aimed at investigating thieno[2,3-*b*]pyridine derivatives as non-nucleoside A_1 agonists that could be used in pharmaco-resistant epilepsy. Compound **7c** (thieno[2,3-*b*]pyridine derivative), displayed good binding affinity to the rA_1 AR (K_i = 61.9 nM). This could be a breakthrough for further investigation of this heterocyclic scaffold as potential ligand. *In silico* evaluation of this compound raised bioavailability concerns but performed well on drug-likeness tests. The effect of intramolecular cyclisation that occurs during synthesis of thieno[2,3-*b*]pyridines from the lead compounds, amino-3,5-dicyanopyridine derivatives (**6a-s**) in relation to AR binding was also evaluated. A significant loss of activity against rA_1/rA_{2A} ARs with cyclisation was revealed. Amino-3,5-dicyanopyridines exhibited greater affinity towards rA_1 ARs (K_i <10 nM) than rA_{2A}. Compound **6c** had the best rA_1 affinity (K_i = 0.076 nM). Novel compounds (**6d**, **6k**, **6l**, **6m**, **6n**, **6o**, **6p**) were highly selective towards rA_1 AR (K_i between 0.179 and 21.0nM). Based on their high selectivity for A_1 ARs, amino-3,5-dicyanopyridines may be investigated further as AR ligands in pharmaco-resistant epilepsy with the right structural optimisations and formulations.

Graphical abstract

amino, 3,5-dicyanopyridine scaffold

decrease in rA_1 AR affinity

thieno[2,3-b]pyridine scaffold

6a: R^2 = 4-methoxyphenyl (**Ki = 139 nM**)
6f: R^2 = 4-hydroxyphenyl (**Ki = 48 nM**)
6g: R^2 = benzo[d][1,3]dioxol-5-yl (**Ki = 26.6 nM**)

7a: R^2 = 4-methoxyphenyl (**Ki = 139 nM**)
7d: R^2 = 4-hydroxyphenyl (**Ki = 48 nM**)
7c: R^2 = benzo[d][1,3]dioxol-5-yl (**Ki = 26.6 nM**)

Keywords

Amino-3,5-dicyanopyryridines; Thieno[2,3-b]pyridines; Intramolecular cyclisation; Adenosine A_1/A_{2A} receptors; Epilepsy

Introduction

Adenosine receptors (ARs) are a family of G protein-coupled receptors (GPCRs) with the nucleoside adenosine as endogenous agonist [1]. There are four known types of ARs, namely A_1, A_{2A}, A_{2B} and A_3 [2] which have been linked to both inhibition (A_1 and A_3) and activation (A_{2A} and A_{2B}) of adenylyl cyclase activity [3]. These receptors are widely expressed throughout all human body tissues and organs; such as the brain, heart, lung, liver, kidney, eye, joints, and blood cells [4]. ARs also play a role in various pathological conditions such as inflammatory diseases, ischemia-reperfusion and neurodegenerative disorders [5], due to their broad spectrum of physiological and pathophysiological functions [6, 7]. All these physiological functions imply that ARs are potential drug targets for treatment of a variety of conditions such as asthma, neurodegenerative disorders, psychosis and anxiety, cardiac ischaemic diseases, sleep disorders, cancer and many other pathophysiological states that are believed to be associated with changes of adenosine levels [8].

The ARs are far more abundant in the brain than in any other cell type or organ in mammals [9], where it has a role in mechanisms of seizure susceptibility, sleep induction, pain perception, respiration and others [10]. Adenosine levels in the brain extracellular space increase dramatically during enhanced nerve activity conditions, such as ischemia, seizures, or trauma to prevent neuronal injury [10]. The neuroprotective effects of adenosine may be due to stimulation of A_1 receptors and blockade of A_{2A} receptors [11]. Therefore, ARs are potential therapeutic targets for treatment of neurological [12] as well as neurodegenerative diseases including epilepsy [13].

Epilepsy is defined as a chronic neurological disorder characterized by recurrent, unprovoked seizures due to excessive discharge of cerebral neurons [14], which alter perception, consciousness, and motor activity. It affects about 50 million people worldwide, hence it is one of the most common neurological diseases globally [15]. Currently there is no available cure for epilepsy. The current treatment of epilepsy consists of anti-epileptic drugs (AEDs) (also known as

anticonvulsants). These therapies are employed to control symptoms of the disease (i.e. suppression of seizures) [16].

Approximately one-third of epileptic patients on treatment remain poorly controlled [17]. Pharmaco-resistance epilepsy can be defined as failure to control seizures after introduction of two or three anticonvulsants that are suitable for the type of epilepsy, prescribed and taken at maximum daily therapeutic doses [18]. A strategy that prevents seizures in drug-resistant epilepsy would be an important therapeutic advance and altering purinergic signalling may be a viable option [19].

Adenosine is a long-known endogenous anticonvulsant substance that effectively inhibits excitatory transmission in the brain [20] through activation of A_1 ARs [3]. Firstly, the released adenosine binds to presynaptic A_1 receptors, which blocks the influx of Ca^{2+} through voltage-dependent calcium channels leading to inhibition of glutamate release, and hence, decreased excitation of postsynaptic glutamate receptors [11, 21]. Secondly, postsynaptic activation of A_1 receptors by adenosine opens potassium channels leading to K^+ efflux which results in resting membrane potential hyperpolarization rendering both ionotropic glutamate receptors (NMDA & AMPA) less responsive [22-24]. Both decreased neurotransmitter release and membrane potential hyperpolarization lead to decreased excitatory synaptic transmission and lower probability of seizure generation onset and propagation [21].

Therefore, adenosine receptor-based therapy – especially through A_1 AR activation – may provide therapeutic potential for patients who do not gain satisfactory seizure control with currently available AEDs [19, 21, 25].

Attempts have been made over the years to develop selective A_1 AR agonists that may be useful as antiepileptic agents. Initially the approach for discovering AR agonists as antiepileptics has been restricted to modification of the physiological agonist adenosine [26], and justly, these adenosine derivatives represent the great majority of molecules developed and reported to date [27]. The development of these agonists has been limited by the essential requirement of the retention of the ribose moiety of adenosine for agonist activity [26, 28, 29]. Examples of adenosine

derivatives include non-selective AR agonists such as 2-chloroadenosine (2-CADO) and A_1 AR selective agonists such as 2-chloro-N6-cyclopentyladenosine (CCPA) [30].

However, the development of adenosine-based AR agonists as novel therapeutic agents has been limited by their pronounced peripheral side effects (mainly cardiovascular effects such as bradycardia and hypotension) and central side effects (like sedation) [3, 6, 13] at doses that have relatively weak anticonvulsant and neuroprotective effects [3]. In addition, they exhibited low blood brain barrier permeability, and hence, limited use in the central nervous system (CNS) [6, 31]. Therefore, these drugs have not been pursued clinically [32].

The said limitations led to development of new strategies to produce potent and selective AR agonists with dominant CNS activity [14]. Non-nucleoside agonists provide an alternative set of compounds which are highly potent and selective for specific AR subtypes [28]. In this study thieno[2,3-*b*]pyridine derivatives were explored as alternative non-nucleoside A_1 AR agonists for the potential management of seizure disorders.

Thienopyridines as a class of heterocyclic compounds have attracted considerable interest due to their broad spectrum of biological activities [33]. The pharmacological potential of thienopyridine derivatives made these compounds a privileged scaffold in medicinal chemistry [34]. There are six isomeric thienopyridine structures, one of them being thieno[2,3-b]pyridine (Fig. 1) and its derivatives which have since attracted attention due to their antitumor, antibacterial [35], antiviral [36, 37], vasodilator and antihypertensive [38], antidiabetic [39], anti-inflammatory [40], antidermatophytic [41], antimalarial activities [42] in addition to treatment of CNS disorders [43].

N S

Thieno[2,3-b]pyridine

Fig. 1 Chemical structure of thieno[2,3-b]pyridine scaffold

Despite their aforementioned promising biological activities, the thienopyridine core has only received scanty attention as scaffold for the design of AR ligands. 3,5-Dicyanopyridine derivatives

which serve as intermediates in the synthesis of thieno[2,3-b]pyridine derivatives, were themselves found to exhibit interesting affinity for ARs. Due to chemical similarity between the 3,5-dicyanopyridin core and the thieno[2,3-b]pyridine core, we envisaged that a suitably substituted thieno[2,3-b]pyridine core could lead to derivatives which may exhibit AR affinity. Notably, bicyclic scaffolds such as benzofurans [44], tetralones and indanones were previously associated with affinity for ARs.

The main aim of this research study was to design, synthesise, characterise, and evaluate novel and known amino-3,5-dicyanopyryridines (intermediates) and thieno[2,3-*b*]pyridines (target compounds) as potent and selective A_1 AR agonists for the potential treatment of neurological conditions, such as epilepsy. Modifications at R_2 and the aryl position on the thieno[2,3-b]pyridines scaffold were influenced by the lead compounds amino-3,5-dicyanopyridine derivatives which displayed good affinity at A_1 AR (Fig. 2). The proposed modifications included thiophene ring closure (from lead compound) resulting in a fused 5-membered (thiophene) heterocyclic ring structure. Different functional groups were substituted at the meta and para positions of the 4-phenyl ring (R_2) and different aryl groups were substituted at position 2 (Fig. 2). The Structure activity relationship (SAR) of the synthesised compounds were evaluated in relation to A_1 and A_{2A} AR affinity.

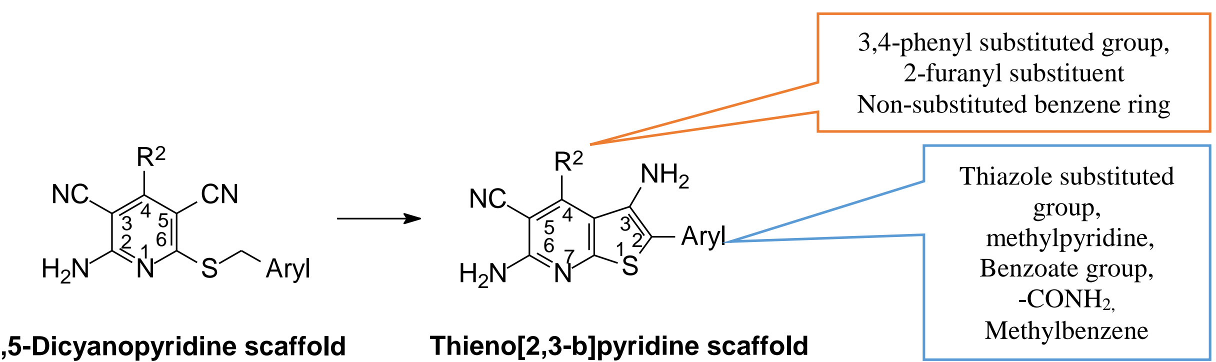

Fig. 2 Synthesis of thieno[2,3-b]pyridine derivatives from lead compounds and modification on the thieno[2,3]pyridine scaffold

Results and Discussion

Chemistry

The synthesis of the amino-3,5-dicyanopyridine derivatives (intermediates) **6a–6p** was done by multicomponent condensation of malononitrile with hydrogen sulphide, a corresponding aldehyde, and a suitable halide in the presence of triethylamine as catalyst [45]. As depicted in Scheme 1, initial addition of hydrogen sulphide to malononitrile gives cyanothioacetamide (**1**) which reacts with the aldehyde according to a Knoevenagel condensation reaction to yield **2**. Further addition of malononitrile results in **3** which undergoes chemoselective intramolecular cyclisation to 3,4-substituted phenyl-2,6-diamino-3,5-dicyano-4*H*-thiopyran (**4**). Recyclisation of the latter by the action of alkali (potassium hydroxide, dimethylformamide) leads to pyridine-2-thiolate (**5**). The subsequent regioselective alkylation of **5** at the sulphur atom with a suitable halide results in a sulphide (**6**). According to the method adopted from [45], the sulphide was supposed to undergo intramolecular cyclisation in the presence of an alkali (potassium hydroxide) to yield a thienopyridine (**7**) – a fused pyridine and thiophene ring heterocyclic compound – but all reactions except the one that yielded compound **7a**, did not go to completion. Instead, the method produced the intermediate compounds, namely amino-3,5-dicyanopyridine derivatives. Modifications such as increasing the potassium hydroxide concentration and contact time with the reaction mixture were made without success to try and bring the reactions to completion. Otherwise ring closure reactions were performed to convert the synthesised intermediate compounds to thieno[2,3-

b]pyridine derivatives using Scheme 2, where either 2–3 drops of potassium hydroxide were added to a solution of amino-3,5-dicyanopyridines in dimethylformamide and then the reaction was left to stand for several hours [46] or through heating a solution of amino-3,5-dicyanopyridines in ethanol containing potassium hydroxide under reflux for 3 hours [47]. Only 3 compounds, **7b–7d** were obtained through these attempts. Details of unsuccessful attempts have been summarised (see supplementary material).

From observation, only compounds with a carbonyl group at the aryl position managed to go to completion to thieno[2,3-b]pyridine derivatives (target compounds). This seems to be in line with the adopted method from [45, 46] since they used α-halo carbonyl compound as an alkylating agent to obtain thieno[2,3-b]pyridine derivatives in an one pot system. Most previously reported synthetic routes for thieno[2,3-b]pyridine derivatives involved the use of α-halo carbonyl compound as well [33, 48, 49]. It seems that the presence of a carbonyl compound at the aryl position has an influence on the intramolecular cyclisation of the intermediate compounds compared to aryl halides. This may be due to the fact that α-halo compounds are bifunctional since they can behave as both an electrophile and nucleophile in carbonyl condensation reactions. The target thieno[2,3-b] pyridines that were synthesised was based on intermediates with A_1 AR activity, hence the choice of halides used. Also, ring closure may have been accomplished with these compounds (**7a–7d**) due to presence of less bulky constituent (-$CONH_2$) at the aryl position as compared to other compounds with aromatic constituents at the same position. For Compounds **6q–6s** and **7a**, readily available cyanothioacetamide was used as starting material. One of the key starting materials for compounds **6q-6s**, 4-(chloromethyl)-2-(4-chlorophenyl)thiazole was synthesised by refluxing a mixture of 4-chlorobenzothioamide and 1,3-dichloroacetone in absolute ethanol for 2 hours (Scheme 3) [50].

The test compounds were obtained in relatively poor yields (**6a**, **6c–l**, **6n–s** and **7a–d**: 11.8–66.4%; with the exception of **6b** and **6m**: >80%), purified by recrystallization from a suitable solvent (either ethanol, methanol or hexane). The structure, molecular mass and purity of these compounds

were verified by ^{1}H and ^{13}C nuclear magnetic resonance spectra, mass spectroscopy and HPLC (see supplementary material). It should be noted that, protons on the NH_2-group (e.g., **6l**) and the OH-group (e.g., **6k**) are not always visible on a ^{1}H NMR spectrum as protons attached to a N-atom (or O-atom) are acidic, and thus, exchangeable [51]. Halogen-carbon bonds tend to cause splitting of ^{13}C NMR chemical shifts (e.g., **6p** and **7a**) due to deshielding by the F-atom on the directly bonded carbon nucleus [52] which results in multiple carbon peaks. This has the potential of causing difficulty in interpreting ^{13}C NMR spectra of fluorinated organic compounds.

Scheme 1 Reaction route for preparation of target thieno[2,3-b]pyridine derivatives [45]

Scheme 2: Synthesis of thieno[2,3-b]pyridines from intermediates

Scheme 3 Synthesis of 4-(chloromethyl)-2-(4-chlorophenyl)thiazole.

Biology

In vitro evaluation

Radioligand binding assays

A total of 23 test compounds were synthesised (**6a–s** and **7a–d**); 7 of these compounds were novel (**6d** and **6k–p**), while 4 compounds (**7a–d**) have been synthesised before but have never been tested for AR affinity. The affinities of the test compounds **6a–s** and **7a–d** at rat (r) A_1 and A_{2A} ARs were determined by radioligand binding assays and are expressed as inhibition constant (K_i, nM) values (Table 1). All test compounds displayed specific binding values <20% at a maximum tested concentration of 100 µM (rA_1 screening), and therefore, all underwent full biological assay for determination of inhibition constant values (Ki, nM). Compounds **6a–j** and **7a–d** displayed specific binding values <20% at a maximum tested concentration of 100 µM (rA_{2A} screening) and hence qualified for full rA_{2A} radioligand binding assay, unlike compounds **6k–s** with specific binding values >20%. The radioligand binding assays were validated with N_6-cyclopentyladenosine (CPA) (A_1 agonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (A_1 antagonist), istradefylline (A_{2A} antagonist) and caffeine (A_1/A_{2A} antagonist) as reference compounds and results were compared to literature values as shown by Table 1.

Table 1 K_i values of test compounds and reference compounds at rat A_1 and A_{2A} ARs

3,5-Dicyanopyridine derivatives (**6a-6s**) → Thieno[2,3-b]pyridine derivatives (**7a-7d**)

#	R^2	aryl	K_i ± SEM (nM)[a] (Specific binding (%))[b]			GTP shift[e]	SI[f]
			rA_1[c] vs 1 nM [^{3}H]DPCPX	rA_{2A}[d] vs 4 nM [^{3}H]NECA	rA_1[c] + 0.1 mM GTP vs 1 nM [^{3}H]DPCPX		
Amino-3,5-dicyanopyryridines							
6a			139 ± 18.8[a]	1473 ± 256[a]	-	-	11
6b			0.213 ± 0.019[a] (2.9)[g]	48.0 ± 11.1[a] (35)[g]	-	-	255
6c			0.076 ± 0.002[a] (0.49)[g] (0.21)[h]	48.3 ± 10.1[a] (71)[g] (52)[h]	0.069 ± 0.006[a]	1	636
6d			10.3 ± 0.643[a]	1205 ± 367[a]	11.3 ± 0.663[a]	1	117
6e			60.4 ± 3.83[a]	338 ± 79.1[a]	-	-	
6f			48.0 ± 4.36[a]	751 ± 12.0[a]	-	-	16
6g			26.6 ± 6.75[a]	429 ± 55.0[a]	-	-	16
6h			7.54 ± 0.768[a] (4.12)[i]	(581)[i]	-	-	
6i			4.57 ± 0.284[a]	634 ± 94.3[a]	-	-	139
6j			not determined (3.5)[g]	20.6 ± 6.56[a] (15)[g]	-	-	

6k			8.82 ± 0.760[a]	(22)[b]	-	-	-
6l			21.0 ± 5.56[a]	(27)[b]	-	-	-
6m			0.179 ± 0.013[a]	(80)[b]	-	-	-
6n			0.831 ± 0.076[a]	(35)[b]	1.94 ± 0.509[a]	2	-
6o			1.64 ± 0.228[a]	(25)[b]	2.25 ± 0.159[a]	1	-
6p			0.430 ± 0.012[a]	(30)[b]	-	-	-
6q			0.383 ± 0.069[a] (1.4)[j]	(44)[b]	1.82 ± 0.582[a]	5	-
6r			1.36 ± 0.040[a] (1.5)[j]	(44)[b]	-	-	-
6s			4.06 ± 0.759[a] (5.0)[j]	(27)[b]	-	-	-
Thieno[2,3-b]pyridines							
7a			1008 ± 58.3[a]	308 ± 93.6[a]			
7b			556 ± 28.1[a]	561 ± 12.1[a]	-	-	
7c			61.9 ± 2.11[a]	1062 ± 126[a]	145 ± 28.8[a]	2	
7d			305 ± 15.3[a]	162 ± 24.4[a]	-	-	
Reference compounds							
CPA (A_1 agonist)			6.5 ± 0.4[a] (15.3)[k] (7.9)[l]	-	36.5 ± 2.28[a]	6	-

DPCPX (A_1 antagonist)	0.5 ± 0.1[a] (0.6)[k] (0.3)[m]	-	0.4 ± 0.032[a]	1	-
Istradefylline (A_{2A} antagonist)	-	3 ± 0.9[a] (13; 2.2)[n] (11.1)[o]	-	-	-
Caffeine (A_1/A_{2A} antagonist)	52 800 ± 7 400[a] (44 000)[p] (41 000)[q] (26 000)[r]	27 800 ± 5 100[a] (43 000)[q] (22 000)[r] (33 000)[k]	-	-	0.5

[a] Inhibition constant (K_i, nM) represented as the mean ± standard error of the mean (SEM), n = 3 samples.

[b] Specific binding (%) of the radioligand at a maximum tested concentration of 100 μM is represented as the mean, n = 2 samples.

[c] rA_1: rat whole brain membranes expressing adenosine A_1 receptor.

[d] rA_{2A}: rat striatal membranes expressing adenosine A_{2A} receptor.

[e] GTP shift calculated by dividing the K_i (nM) in the presence of 0. 100 μM GTP by the K_i (nM) in the absence of 100 μM GTP.

[f] Selectivity index (SI) for the adenosine A_1 receptor subtype calculated by dividing the $rA_{2A}K_i$ (nM) by the rA_1K_i (nM).

[g] Literature value: human adenosine A_1 receptor and [^{3}H]DPCPX; human adenosine A_{2A} receptor and [^{3}H]ZM241385 [53]

[h] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX; rat adenosine A_{2A} receptor and [^{3}H]ZM241385 [53]

[i] Literature value: human adenosine A_1 receptor and [^{3}H]DPCPX; human adenosine A_{2A} receptor and [^{3}H]ZM241385 [54]

[j] Literature value: human adenosine A_1 receptor and [^{3}H]DPCPX [55]

[k] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [26]

[l] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [56]

[m] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [57]

[n] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [58]

[o] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [59]

[p] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [60]

[q] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [61]

[r] Literature value: rat adenosine A_1 receptor and [^{3}H]DPCPX [62]

Structure activity relationships (SAR)

Modifications were made at the R^2 and aryl positions of the test compounds to assess how different substituents can influence both rA_1 and rA_{2A} ARs binding affinity as well as selectivity. As shown in Table 1, all test compounds displayed greater affinity toward the rA_1 than rA_{2A} AR. Compound

6c had the best rA_1 AR affinity (K_i = 0.076 nM) of the present series. The latter compound together with **6b** displayed better rA_{2A} AR affinity than the other test compounds with K_i values of 48.3 nM (**6c**) and 48.0 nM (**6b**), respectively, but remain selective for the rA_1 AR. Comparing amino-3,5-dicyanopyryridines (**6a–s**) and thieno[2,3-b]pyridines (**7a–d**), it is evident that there was a significant decrease in both rA_1 and rA_{2A} AR affinity from the open ring structures to the closed ring structures. The only thieno[2,3]pyridine derivative that showed moderately good rA_1 AR affinity is compound **7c** ($rA_1 K_i$ = 61.9 nM). The general poor activity of thieno[2,3-b]pyridines relative to amino-3,5-dicyanopyryridins suggest that the ring closure affects binding to the receptors, perhaps, due to steric hindrance. (This may be confirmed by molecular docking studies in the future.)

SAR for amino-3,5-dicyanopyrines (intermediates)

For compounds **6a**, **6b**, **6**l and **6s**, the 4-methoxyphenyl group was maintained at position R^2 and different functional groups were substituted at the aryl position. Compound **6b** with a methylpyridine substituent at the aryl position exhibited low nanomolar activity toward the rA_1 AR (K_i = 0.213 nM) as well as selectivity for the rA_1 AR over the rA_{2A} AR (SI = 636). Affinity for the rA_1 and/or rA_{2A} ARs decreased when introducing a 4-chlorophenylthiazole group (**6s**: $rA_1 K_i$ = 4.06 nM), benzoic acid substituent (**6l**: $rA_1 K_i$ = 21.0 nM) and a carbonyl containing substituent (**6a**: $rA_1 K_i$ = 139 nM) which displayed the lowest affinity for rA1 AR. In terms of selectivity, **6b** also showed affinity toward the rA_{2A} AR (K_i = 48.0 nM) while compounds **6l** and **6s** were more selective towards rA_1 ARs, as seen from the calculated selectivity indexes.

Replacing 4-methoxyphenyl with 3-methoxyphenyl at position R^2 while maintaining the same aryl functional groups as **6a**, **6b** and **6l** above was also explored. Comparison of compound **6d** to **6a** (aryl = -$CONH_2$) showed a significant increase in binding affinity towards rA_1 ARs (**6d**: $rA_1 K_i$ = 10.3 nM vs **6a**: $rA_1 K_i$ = 139 nM) but had no effect on rA_{2A} AR affinity. In general, the presence of the 3-methoxyphenyl substituent resulted in increased affinity for rA_1 ARs but had no influence on rA_{2A} AR affinity as shown by **6c** vs **6b** and **6o** vs **6l**. Again, the substituents at the aryl position

had a similar effect on affinity as observed with the 4-methoxyphenyl containing compounds **6a**, **6l** and **6s** (in decreasing order of affinity: **6c** (methylpyridine) > **6o** (benzoic acid substituent) > **6d** (carbonyl containing substituent)). From these results it is evident that 3-methoxyphenyl is favoured over 4-methoxyphenyl in terms of rA_1 AR binding affinity.

For compounds **6e**, **6m**, **6n** and **6q**, 4-hydroxyphenyl was introduced at the R^2 position while maintaining almost all the same aryl groups mentioned earlier. Generally, these compounds displayed rA_1 AR affinity of 1 nM or smaller (except **6e**, aryl = $CONH_2$), with **6m** (aryl = -methylpyridine) being the best with rA_1K_i = 0.179 nM of these compounds. Comparing 4-methoxyphenyl and 4-hydroxyphenyl substitutions (**6a** vs **6e** and **6b** vs **6m**) showed that with the latter, rA_1 AR activity increased slightly. Looking at **6m** (aryl = methylpyridine) and **6n** (aryl = -methylbenzene), it appears that the introduction of a N-atom in compound **6m** had a positive influence in rA_1 AR affinity.

Replacing 4-hydroxyphenyl with 3-hydroxyphenyl at position R^2 was also studied (**6e** vs **6f** and **6m** vs **6j**), although a definite trend could not be observed with the limited data at hand. Comparison of **6f** (3-hydroxyphenyl) to its 4-methoxyphenyl substituted counterpart **6d** showed a four-fold decrease in rA_1 AR affinity, although rA_1 selectivity was maintained.

Comparison of **6a**, **6d**, **6e** and **6f** showed that the meta position is preferred to the para position whether OCH_3- or OH-group substitution is incorporated, and furthermore, it seems that a OCH_3-group is preferred to an OH-group.

Comparing **6l** and **6k** with 4-OCH_3 and 4-SCH_3 revealed that introducing a sulphur component increased binding affinity for compound **6k** (K_i = 8.82 nM) as compared to **6l** (21.2 nM).

Compounds, **6q**, **6r** and **6s** with the same (4-chlorophenyl)thiazole aryl substituent were also explored. All these compounds displayed rA_1 AR affinity but had no mentionable affinity for rA_{2A} ARs. Compound **6q** (R = 4-OCH_2CH_2OH) had the best affinity of these compouns with K_i = 0.383 nM. SARs of amino-3,5-dicyanopyridines against *r*A_1 AR are summarised in Fig. 3.

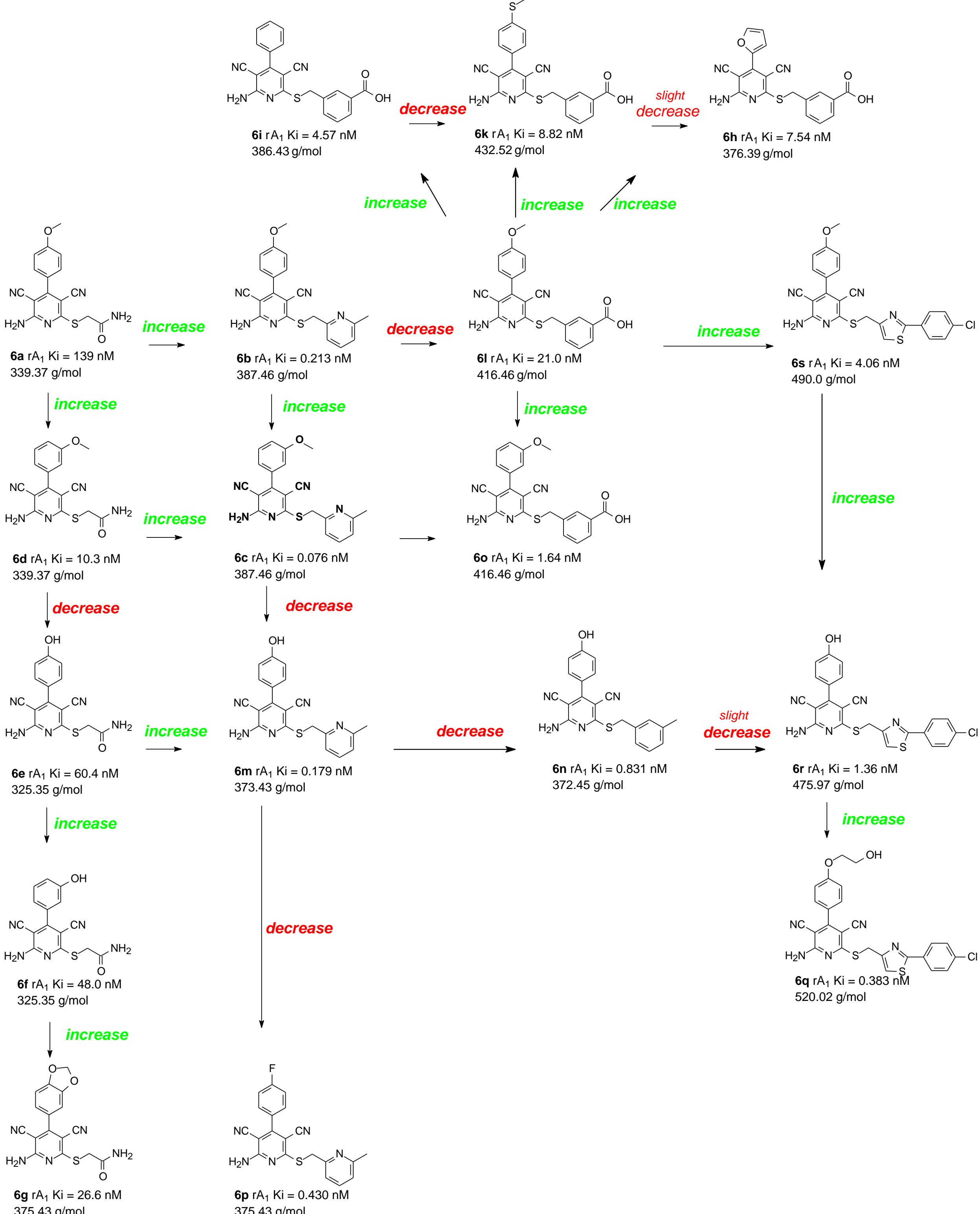

Fig. 3 Structure activity relationship of amino-3,5-dicyanopyridines against *r*A_1 AR

SAR for thieno[2,3-b]pyridines (target compounds)

Thieno[2,3-b]pyridine derivatives **7a–d** displayed poor affinity towards rA_1 ARs compared to their corresponding intermediate amino-3,5-dicyanopyridines (Fig. 4). These compounds all had a -$CONH_2$-group at the aryl position. The results indicate that ring closure from the intermediate open

ring to fused ring structures decreased activity towards both rA_1 and rA_{2A} ARs. This corresponds with a study by [7] in which intramolecular cyclisation of the 6-amino-3,5-dicyanopyridines, specifically BAY606583 (a potent A_{2B} receptor agonist) was evaluated. The study revealed that the bicyclic compound (thieno[2,3]pyridine derivative) that resulted after intramolecular cyclisation of BAY60 6586 bind none of the ARs suggesting that molecular stiffening decreases AR binding affinity.

decrease in rA_1 AR affinity

6a: R^2 = 4-methoxyphenyl (Ki = 139 nM)
6f: R^2 = 4-hydroxyphenyl (Ki = 48 nM)
6g: R^2 = benzo[d][1,3]dioxol-5-yl (Ki = 26.6 nM)

7a: R^2 = 4-methoxyphenyl (Ki = 139 nM)
7d: R^2 = 4-hydroxyphenyl (Ki = 48 nM)
7c: R^2 = benzo[d][1,3]dioxol-5-yl (Ki = 26.6 nM)

Fig. 4 The Structure activity relationships of amino-3,5-dicyanopyridines (**6a**, **6f**, **6g**) vs thieno[2,3]pyridines (**7a**, **7c**, **7d**)

GTP shift assays

The type of binding affinities that test compounds **6c**, **6d**, **6n**, **6o**, **6q** and **7c** displayed at the rA_1 AR were determined through GTP shift assays, as described in literature [57, 63, 64]. These test compounds were selected as they possessed the best rA_1 AR affinity among the investigated test compounds (Table 1). The theory of a GTP assay is that competition curve of an antagonist will be unaffected by GTP, thus resulting in a calculated GTP shift of approximately 1 [64]. Agonists' curves, on the other hand, will be shifted towards the right in the presence of GTP [65]. GTP shifts were calculated by dividing the rA*K*i values of compounds reported in the presence of GTP by the rAR*K*i values obtained in the absence of GTP and the results are summarised in Table 1. Compounds **6c**, **6d** and **6o** behaved as antagonists (interestingly, all these compounds contained a 3-OCH_3 group at position R^2), while **6n**, **6q** and **7c** behaved as agonists (Fig. 5). Contradictory to the present results, Guo et al. [53] found **6c** to be a partial agonist and not an antagonist. Notably, Louvel and co-workers [55] also found **6q** to be a full agonist in accordance with the present results.

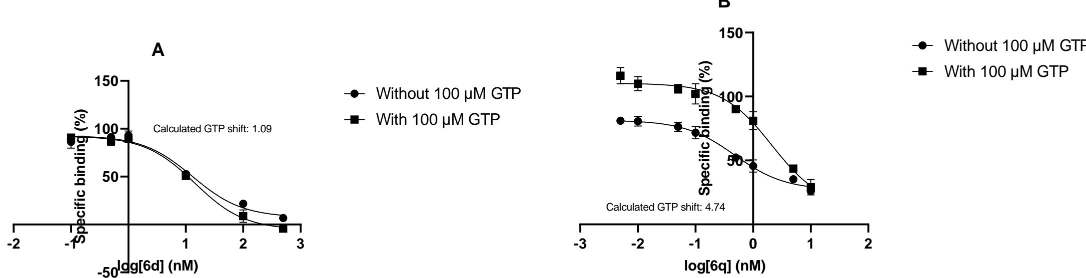

Fig. 5 The binding curves of test compounds **6d** and **6q** in the presence and absence of 100 µM GTP using [^{3}H]DPCPX as radioligand in rat whole brain membranes expressing adenosine A_1 receptors as representative cases for adenosine A_1 receptor antagonistic and agonistic activity. A. Calculated GTP shift: 1.09; B. Calculated GTP shift: 4.74.

***In silico* evaluation**

The physiochemical properties, pharmacokinetic profiles, drug-likeness and medicinal chemistry friendliness of compounds **6c**, **6d**, **6n**, **6o**, **6q** and **7c** were predicted through the free online web tool SwissADME (https://swissadme.ch). The prediction is based on the chemical structures of the compounds. The results are in the supplementary material.

The bioavailability radar (which takes in to account the physicochemical properties lipophilicity, size, polarity, solubility, flexibility and saturation) for compounds **6b**, **6d**, **6m**, **6o**, **6q** and **7c**. Almost all compounds fall within the optimal ranges of lipophilicity, size, solubility, and flexibility parameters except compound **6q** which exceeded the optimal size of the molecule (150-500 g/mol) since it has a molecular weight of 520.03 g/mol. All these compounds failed the saturation parameter since all have a lower fraction of carbon atoms in the sp3 hybridization (Csp3 > 0.25) and high polarity values (TPSA >130 Å^2). These compounds are considered to be too polar with low degree of saturation and consequently predicted not be orally bioavailable. The XLOGP3 (<5.0) value of compound **6q** slightly exceeded the limit (5.1) proving to be the most lipophilic.

In terms of water solubility (Log *S*), compound **6c**, **6m**, **6o** and **7c** are predicted to be moderately soluble to poorly soluble in water. Compound **6d** was predicted to be soluble to moderately soluble and compound **6q** was classified as poorly soluble. Poor water solubility of compound **6q** may be attributed to its high molecular weight and the presence of lipophilic halogen (Cl) as part of the

aryl substituent. Water solubility is the most important in terms of achieving desired drug concentration in systemic circulation for pharmacological response [66]. It must be understood that poorly water-soluble drugs have slow drug absorption leading to inadequate and variable bioavailability and gastrointestinal mucosal toxicity [66]. Solubility improvement techniques need to be employed for future formulation development especially for compound **6q** (capadenoson), since any drug to be absorbed must be present in an aqueous solution at the site of absorption.

The BOILED-Egg predictive model allows evaluation of passive gastrointestinal (GI) absorption and brain penetration (BBB). Compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c** are all predicted to have low GI absorption and no blood brain barrier (BBB) permeability, probably because of a high TPSA (>130), although some sources recommend TSPA <140Å^2 (e.g. **6c**) to be adequate for high probability of good intestinal permeability [67]. This may also be attributed to the high polarity of these compounds. Interestingly, a study by [59, 68] indicated that compound **6q** (capadenoson), showed hints of CNS effects in humans. Compound **6c** as well has been considered to possess high BBB permeability by [53, 60, 61] despite this prediction. The prediction of P-glycoprotein (P-gp) substrate indicates that only compound **6q** can be actively effluxed by P-glycoprotein (P-gp) while compounds **6c**, **6m**, **6o** and **7c** are not substrates of this efflux mechanism. The potential interaction of compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c** with cytochromes P450 (CYP) isoenzymes was also evaluated. This is important for determination of drug-drug interactions and adverse effects due to low drug clearance leading to accumulation of the drug [51, 52]. Generally, all the compounds are inhibitors of CYP isoforms (CYP1A2, CYP2C19, CYP2C9, CYP3A4) with a few exceptions, but they did not affect CYP2D6 except compound **6q**.

SwissADME also provides qualitative assessment of drug-likeness which predicts a molecule's chance to be classified as an oral drug candidate [69] by implementing different rule-based filters [70-74] (Table 4). Additionally, compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c** all had a bioavailability score of 0.55 (the probability that a compound will have > 10% bioavailability in rat or measurable Caco-2 permeability) [69].

The medical chemistry friendliness of compounds was assessed by identifying pan assay interference compounds (PAINS) [75] and structural alerts [76]. All compounds passed both PAINS and Brenk tests as no alerts were raised. This means that these compounds may not affect any bioassays [77] and generally have good pharmacokinetics properties with an acceptable toxic level [78]. Interestingly, only one compound (**6d)** passed the lead-likeness test, and hence, can be used as a lead compound in drug discovery processes. Compounds **6c**, **6m**, **6o**, **6q** and **7c** all had higher molecular weight (>350) as well as high log*P* values (XLOGP: >3.5). Structural optimisation for these chemical scaffolds is needed, most probably by decreasing size, polarity and/or lipophilicity.

Conclusion

The aim of this study was to investigate use of amino-3,5-dicyanopyridine and thieno[2,3-*b*]pyridine derivatives as potential AR agonists. A total of 23 test compounds were synthesised (**6a**–**s** and **7a**–**d**) and 7 of these were novel (**6d** and **6k**–**p**), while 4 compounds (**7a**–**d**) have been synthesised before but have never been tested for AR affinity.

Overall, amino-3,5-dicyanopyridine displayed superior activity towards rA_1 ARs compared to thieno[2,3]pyridines. The general poor activity of thieno[2,3-b]pyridines suggest that the intramolecular cyclisation results in molecular stiffening or rigidity which negatively affects binding to the receptors, perhaps, due to steric hindrance. On the R^2 substitution, it was observed that 3- and 4-methoxyphenyl groups favoured rA_1 AR binding compared to their 3- and 4-hydroxyphenyl counterparts. Looking at the aryl substitution, the methylpyridine substituent displayed the overall best rA_1 AR affinity. Novel compounds (**6d**, **6k**, **6l**, **6m**, **6n**, **6o** and **6p**) proved to be highly selective with low nanomolar rA_1 AR affinity (K_i values between 0.179 nM and 21.0 nM). The only thieno[2,3-*b*]pyridine derivative that displayed moderately good rA_1 AR activity (K_i = 61.9 nM) has been investigated as a TGF-β receptor kinase inhibitor for the treatment of tumors and now AR affinity may be included.

Compounds **6n**, **6q** and **7c** acted as potent, highly selective agonists at A_1 ARs; however, compounds **6c**, **6d** and **6o** (notably all containing a 3-OCH_3 group at position R^2) behaved as rA_1 antagonists.

The SwissADME profiles of the test compounds raised concern about their bioavailability; however, **6d** emerged as a promising lead compound. Further investigation to improve bioavailability through structure optimisation and/or prodrug approaches will be necessary.

The high affinity and selectivity for the rA_1 AR displayed by the amino-3,5-dicyanopyridine scaffold showed that, if correctly modified, it may produce highly potent AR ligands which can be used in development of treatment for epilepsy.

Experimental

Chemistry

Materials

Unless otherwise noted, all starting materials and solvents were purchased from commercial manufacturers (Sigma-Aldrich and AmBeed) and used without further purification. Thin layer chromatography (TLC) silica gel 60 F254 aluminium sheets from Merck was used to monitor reaction progress. Melting points were determined on a Buchi M-545 melting point apparatus. Melting points for compounds **6h**, **6i**, **6j**, **7c** and **7b** were obtained through differential scanning calorimetry (DSC) analysis using Mettler Toledo analyser. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 and 151 MHz respectively, using DMSO-d6 as solvent and TMS (tetramethylsilane- $Si(CH_3)_4$) as reference. Chemical shifts were reported in parts per million (ppm) in relation to the solvent peak (DMSO-d6: residual CH_3 at 2.50 ppm for ^{1}H NMR and 39.52 ppm for ^{13}C NMR). Spin multiplicities were indicated as follows: singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), triplet of doublets (td), double double doublet (ddd) and multiplet (m). Coupling constant (*J*) values were reported in Hertz (Hz). High-resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF-Q II mass spectrometer in atmospheric

chemical ionisation (APCI) mode. High-performance liquid chromatography (HPLC) analyses were done on Shimadzu Nexera-i LC-2040C 3D Plus HPLC system to determine the purity of test compounds.

Synthesis of test compounds

General procedure for the synthesis of **6a**–**6p**

2-((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)acetamide (6a)

Three drops of trimethylamine were added to a solution of malononitrile (0.629ml,10 mmol) in 20 mL of ethanol, while stirring with magnetic stirrer. The reaction mixture was cooled to 10°C and H_2S generated by reaction between FeS (12.081g) and HCl (90ml) was passed through the mixture for 24 hours to produce cyanothioacetamide (**1**). The reaction mixture was then stirred for 15–20 mins before adding 3-methoxybenzaldehyde (1.217ml, 10 mmol) while stirring at room temperature to produce **2a**. After obtaining a homogeneous mixture, more malononitrile (0.629ml, 10 mmol) was added and the mixture stirred until it became homogeneous again. It was then left to stand at room temperature for 12–14 hrs to obtain 2,6-diamino-4-methoxy-4H-thiopyran-3,5-dicarbonitrile (**4a**). The mixture was then diluted with an equal volume of dimethylformamide and 10% aqueous potassium hydroxide (5.6mL, 10 mmol) and left to stand for 24 hrs to produce potassium 6-amino-3,5-dicyano-4-methoxy-1,4-dihydropyridine-2-thiolate (**5a**). 2-bromoacetamide (10 mmol, 1.382 g) was added to the mixture and continuously stirred for 3 hrs, after which 10% aqueous potassium hydroxide (5.6 mL, 10 mmol) was added again. After 2–4 hrs, ice was added and the resulting precipitate was filtered off, washed with distilled H_2O, ethanol and hexane, dried (30°C) and recrystallised from methanol to yield the title compound **6a** as whitish powder (0.988g, 29.1%): Rf: 0.77 (DCM/PE/EtOAc 10:1:1); mp: 237.2 - 238.0 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.93 (s, 2H), 7.49 (d, *J* = 8.7 Hz, 2H), 7.46 (s, 1H), 7.19 (s, 1H), 7.12 (d, *J* = 8.7 Hz, 2H), 3.89 (s, 2H), 3.85 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C,-C̲O), 166.1 (C, C-6), 160.8 (C, C-2), 159.6 (C, C-4'), 158.0 (C, C-4), 130.1 (C, C1'), 125.7 (CH, C-2',C-6'), 115.3 (CH, C-3', C-5'), 115.3 (C, CN), 114.1 (C, CN), 93.3 (C, C-5), 85.9 (C, C-3), 55.3 (CH_3, OC̲H_3),

33.3 (CH_2, S-C̲H₂-); APCI-HRMS *m/z*: calculated for $C_{16}H_{14}N_5O_2S$ [M+H]+ 340.0863, found 340.0871; Purity (HPLC, λ = 280): 100%

2-amino-4-(4-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6b)

Prepared as for **6a** from 4-methoxybenzaldehyde (1.217ml, 10 mmol) and 2-(bromomethyl)-6-methylpyridine (1.862 g, 10 mmol) to yield **6b** which was recrystallised from methanol as white flakes (3.418g, 88.2%): Rf: 0.77 (PE:EtOAc 1:1); mp: 168.5 - 171.9 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.00 (s, 2H), 7.63 (t, *J* = 7.6 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 2H), 7.42 (d, *J* = 7.6 Hz, 1H), 7.12 (dd, *J* = 24.1, 8.1 Hz, 3H), 4.56 (s, 2H), 3.84 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.1 (C, C-6), 160.8 (C, C-2), 159.6 (C, C-4'), 158.1 (C, C-4), 157.7 (C, C-2''), 155.73 (C, C-6''), 137.0 (CH, C-4''), 130.1 (C, C-1'), 125.8 (CH, C-2', C-6'), 121.8 (CH, C-5''), 120.6 (CH, C-3''), 115.4 (CH, C-3', C-5'), 115.4 (C, CN), 114.0 (C, CN), 93.2 (C, C-5), 85.9(C. C-3), 55.3 (CH_3, $O\underline{C}H_3$), 35.3 (CH_2, $-S\underline{C}H_2-$), 23.9 (CH_3, -methylpyridine); APCI- HRMS *m/z*: calculated for $C_{21}H_{18}N_5OS$ [M+H]+ 388.1227, found 388.1208; Purity (HPLC, λ = 254): 100%

2-amino-4-(3-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6c)

Prepared as for **6a** from malononitrile (0.315ml, 5mmol), 3-methoxybenzaldehyde (5 mmol, 0.608ml) and 2-(bromomethyl)-6-methylpyridine (0.933g, 5 mmol) to yield **6c** which was recrystallised from methanol as white solid (0.505g, 26.1%): Rf: 0.64 (DCM/PE/EtOAc 10:1:1); mp: 175.5 - 176.7 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.05 (s, 2H), 7.63 (t, *J* = 7.7 Hz, 1H), 7.50 – 7.40 (m, 2H), 7.17 – 7.08 (m, 3H), 7.07 – 7.05 (m, 1H), 4.57 (s, 2H), 3.80 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.1 (C, C-6), 159.5 (C, C-2), 159.0 (C, C-3'), 158.1 (C, C-4), 157.7 (C, C-2''), 155.7 (C, C-6''), 137.0 (C, C-1'), 135.1 (CH, C-4''), 129.9 (CH, C-5'), 121.8 (CH, C-3''), 120.6 (CH, C-5''), 120.4 (CH, C-6'), 115.8 (CH, C-4'), 115.1 (CH, C-2'), 115.0 (C, CN), 114.0 (C, CN), 93.2 (C, C-5), 86.0 (C, C-3), 55.3 (CH_3, $O\underline{C}H_3$), 35.3 (CH_2, $-S\underline{C}H_2-$), 23.9 (CH_3,

methylpyridine). APCI-HRMS *m/z*: calculated for $C_{21}H_{18}N_5OS$ [M+H]+ 388.1227, found 388.1208; Purity (HPLC, λ = 254): 100%

2-((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)acetamide (6d)

Prepared as for **6c** from 3-methoxybenzaldehyde (5 mmol, 0.608ml) and 2-bromoacetamide (0.691g, 5 mmol) to yield **6d** which was recrystallised from methanol as yellowish powder (0.537g, 31.6%): Rf: 0.78 (DCM/PE/EtOAc 10:1:1); mp: 232.3 - 237.0 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.98 (s, 2H), 7.53 – 7.42 (m, 2H), 7.20 (s, 1H), 7.14 – 7.09 (m, 2H), 7.08 – 7.04 (m, 1H), 3.89 (s, 2H), 3.81 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C, CO), 166.0 (C, C-6), 159.4 (C-C2), 159.0 (C, C-3'), 158.1 (C, C-4), 135.1 (C, C-1'), 129.9 (CH, C-5'), 120.4 (CH, C-6'), 115.8 (C, C-4'), 115.1 (C, CN), 115.0 (CH, C-2'), 114.0, (C, CN) 93.3 (C, C-5), 86.0 (C, C-5), 55.3 (CH_3, O<u>C</u>H_3), 33.3 (CH_2, -S-<u>C</u>H_2-); APCI-HRMS *m/z*: calculated for $C_{16}H_{14}N_5O_2S$ [M+H]+ 340.0863, found 340.0844; Purity (HPLC, λ = 254): 100%

2-((6-amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-yl)thio)acetamide (6e)

Prepared as for **6c** from 4-hydroxybenzaldehyde (0.614g, 5 mmol) and 2-bromoacetamide (0.692g, 5 mmol) to yield compound **6e** which was recrystallised from methanol as cream white powder (0.650g, 40.0%): Rf: 0.15 (EtAOc only); mp: 267.6 - 268.0 °C; ^{1}H NMR (600 MHz, DMSO) δ 10.03 (s, 1H), 7.89 (s, 2H), 7.46 (s, 1H), 7.40 – 7.34 (m, 2H), 7.19 (s, 1H), 6.95 – 6.89 (m, 2H), 3.88 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 168.9 (C, CO), 166.1 (C, C-6), 159.7 (C, C-2), 159.4 (C, C-4), 158.3 (C, C-4'), 130.1 (C, C-1'), 124.1 (CH, C-2', C-6'), 115.4 (CH, C-3', C-5'), 115.4 (C, CN, 115.4 (C, CN), 93.2 (C, C-5), 85.8 (C, C-3), 33.3 (CH_2, -S<u>C</u>H_2-); APCI-HRMS *m/z*: calculated for $C_{15}H_{12}N_5O_2S$ [M+H]+ 326.0706, found 326.0688; Purity (HPLC, λ = 254): 100%

2-((6-amino-3,5-dicyano-4-(3-hydroxyphenyl)pyridin-2-yl)thio)acetamide (6f)

Prepared as for **6c** from 3-hydroxybenzaldehyde (0.612g, 5 mmol) and 2-bromoacetamide (0.690g, 5 mmol) to yield **6f** which was recrystallised from ethanol as light brown solid (0.735g, 45.2%): Rf: 0.28 (DCM/MeOH: 10:1); mp: 248.1 - 248.4 °C; ^{1}H NMR (600 MHz, DMSO) δ 9.83 (s, 1H), 7.95 (d, *J* = 6.9 Hz, 2H), 7.47 (s, 1H), 7.35 (t, *J* = 7.9 Hz, 1H), 7.20 (s, 1H), 6.95 (ddd, *J* = 8.2, 2.4,

0.8 Hz, 1H), 6.92 – 6.88 (m, 1H), 6.88 – 6.85 (m, 1H), 3.89 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C, CO), 166.1 (C, C-6), 159.5 (C, C-2), 158.3 (C, C-4), 157.3 (C, C-3'), 135.0 (C, C-1'), 129.9 (CH, C-5'), 118.8 (CH, C-6'), 117.2 (CH, C-4'), 115.1 (CH, C-2'), 115.0 (C, CN), 115.0 (C, CN), 93.2 (C, C-5), 85.9 (C, C-3), 33.3 (CH_2, -S$\underline{C}H_2$-); APCI-HRMS *m/z*: calculated for $C_{15}H_{12}N_5O_2S$ [M+H]+ 326.0706, found 326.0689; Purity (HPLC, λ = 254): 100%

2-((6-amino-4-(benzo[d][1,3]dioxol-5-yl)-3,5-dicyanopyridin-2-yl)thio)acetamide (6g)

Prepared as for **6c** from piperonaldehyde (0.752g, 5mmol) and 2-bromoacetamide (0.690g, 5 mmol) to yield **6g** which was recrystallised from methanol as light orange solid (0.682g, 38.6%): Rf: 0.40 (DCM/MeOH: 10:1); mp: 244.5 - 245.1 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.93 (s, 2H), 7.46 (s, 1H), 7.19 (s, 1H), 7.14 (d, *J* = 1.8 Hz, 1H), 7.10 (d, *J* = 8.0 Hz, 1H), 7.02 (dd, *J* = 8.0, 1.8 Hz, 1H), 6.15 (s, 2H), 3.88 (s, 2H).; ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C, CO), 166.0 (C, C-6), 159.5 (C, C-2), 157.9 (C, C-4), 148.9 (C, C-3',C-4'), 147.3 (C, C-1'), 127.2 (C, C-6'), 122.9 (CH, C-2'), 115.2 (CH, C-5'), 108.8, (C, CN) 108.5 (C, CN), 101.7 (CH_2, C at dioxol), 93.4 (C, C-5), 86.1 (C, C-3), 33.3 (CH_2, -S$\underline{C}H_2$-); APCI-HRMS *m/z*: calculated for $C_{16}H_{12}N_5O_3S$ [M+H]+ 354.0655, found 354.0635; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzoic acid (6h)

Prepared as for **6c** from furan-2-carbaldehyde (0.414g, 5mmol) and 3-(bromomethyl)benzoic acid (1.081g, 5 mmol) to yield **6h** which was recrystallised from acetone as cream white powder (0.341g, 18.1%):Rf: 0.38 (DCM:MeOH 10:1); mp: 279.59°C; ^{1}H NMR (600 MHz, DMSO) δ 8.10 – 8.03 (m, 2H), 7.86 (d, *J* = 6.3 Hz, 2H), 7.57 (d, *J* = 7.0 Hz, 1H), 7.37 (d, *J* = 3.6 Hz, 1H), 7.30 (t, *J* = 7.6 Hz, 1H), 6.81 (dd, *J* = 3.6, 1.7 Hz, 1H), 4.50 (s, 2H).; ^{13}C NMR (151 MHz, DMSO) δ 167.4 (C, COOH), 160.1 (C, C-6), 146.5 (C, C-2), 145.1 (C, C-4, C-2'), 143.7 9CH, C-5', 136.5 (C-3'), 127.7 (C, C-4'', C-1''), 116.3 (CH, C-5'',C-6'', C-2''), 115.7 (C, CN), 115.7 C, CN), 112.8 (CH, C-3', C-4'), 89.2 (C, C-5), 81.6 (C, C-3), 33.4 (CH_2, -S$\underline{C}H_2$); APCI-HRMS *m/z*: calculated for $C_{19}H_{13}N_4O_3S$ [M+H]+ 377.0703, found 377.0687; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-phenylpyridin-2-yl)thio)methyl)benzoic acid (6i)

Prepared as for **6c** from benzaldehyde (0.460ml, 5mmol) and 3-(bromomethyl)benzoic acid (1.083g, 5 mmol) to yield **6i** which was recrystallised from hexane as white fluffy solid (0.601g, 31.1%): Rf: 0.89 (DCM:MeOH 10:1); mp: 200°C; ^{1}H NMR (600 MHz, DMSO) δ 8.01 (s, 2H), 7.78 (d, *J* = 7.6 Hz, 1H), 7.58 – 7.49 (m, 5H), 7.44 (d, *J* = 7.5 Hz, 1H), 7.22 (t, *J* = 7.6 Hz, 1H), 4.51 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.4 (C-COOH), 159.5 (C, C-6), 158.3 (C, C-2), 135.7 (C, C-4), 133.9 (C, C-3"), 130.3 (C, C-1'), 130.2 (CH, C-4"), 128.6 (C, C-1"), 128.3 (CH, C-3', C-5', C-4', C2") 128.0 (CH, C-5", C-6"), 127.1 (CH, C-2', C-4'), 115.1 (C, CN), 115.1 (C-CN), 93.2 (C, C-5), 85.9 (C, C-3), 33.6 (C, -SCH2); APCI-HRMS *m/z*: calculated for $C_{21}H_{15}N_4O_2S$ [M+H]+ 354.0655, found 354.0635; Purity (HPLC, λ = 254): 100%

2-amino-4-(3-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6j)

Prepared as for **6c** from 3-hydroxybenzaldehyde (0.610g, 5mmol) and 2-(bromomethyl)-6-methylpyridine (0.933g, 5 mmol) to yield **6j** which was recrystallised from methanol as light yellow powder (0.580g, 31.1%): Rf: 0.73 (DCM/MeOH 10:1); mp: 231.22°C; ^{1}H NMR (600 MHz, DMSO) δ 9.82 (s, 1H), 8.04 (s, 2H), 7.63 (t, *J* = 7.7 Hz, 1H), 7.43 (d, *J* = 7.7 Hz, 1H), 7.34 (t, *J* = 7.9 Hz, 1H), 7.15 (d, *J* = 7.7 Hz, 1H), 6.94 (ddd, *J* = 8.2, 2.4, 0.7 Hz, 1H), 6.91 – 6.83 (m, 2H), 4.56 (s, 2H), 2.46 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.1 (C, C-6), 159.5 (C, C-2), 158.4 (C, C-4), 157.7 (C, C-2"), 157.3 (C, C-6"), 155.7 (C, C-3'), 137.0 (C, C-1'), 135.0 (CH, C-4"), 129.8 (CH, C-5'), 121.8 (CH, C-3"), 120.6 (CH, C-5"), 118.8 (C, C-6'), 117.2 (CH,C-4'), 115.1 (CH, C-2'), 115.0 (C, CN), 115.0 (C, CN), 93.1, (C, C-5) 85.8 (C, C-3), 35.4 (CH_2, -SCH_2-), 23.9 (CH_3, Methylpyridine); APCI-HRMS *m/z*: calculated for $C_{20}H_{16}N_5OS$ [M+H]+ 374.1070, found 354.1063; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(4-(methylthio)phenyl)pyridin-2-yl)thio)methyl)benzoic acid (6k)

Prepared as for **6c** from 4-(methylthio)benzaldehyde (0.664ml, 5mmol) and 3-(bromomethyl)benzoic acid (1.080g, 5 mmol) to yield **6k** which was recrystallised from methanol as yellowish powder (0.580g, 31.1%): Rf: 0.69 (DCM/PE/EtOAc 10:1:1); mp: 193.2 - 193.3 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.05 (s, 1H), 7.83 (d, *J* = 7.6 Hz, 1H), 7.73 (d, *J* = 7.5 Hz, 1H), 7.47 (dd, *J* = 6.1, 4.2 Hz, 2H), 7.44 – 7.35 (m, 3H), 4.57 (s, 2H), 2.53 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.01 (C, <u>C</u>OOH), 163.8 (C, C-6), 159.6 (C, C-2), 157.9 (C, C-4), 141.7 (C, C-3", C-4'), 130.1 (C, C-4'), 129.8 (C, C-4"), 129.1 (C, C-1"), 128.3 (CH, C-2"), 128.2 (CH, C-5", C-6"), 125.2 (CH, C-2', C-3', C-5', C-6'), 115.4 (C, CN, CN), 93.1 (C, C-5), 85.9 (C, C-3), 32.9 (CH_2, -S<u>C</u>H_2-), 14.0 (CH_3, SCH_3). APCI-HRMS *m/z*: calculated for $C_{22}H_{17}N_4O_2S_2$ [M+H]+ 3433.0787, found 433.0784; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (6l)

Prepared as for **6c** from 4-methoxybenzaldehyde (0.608ml, 5mmol) and 3-(bromomethyl)benzoic acid (1.080g, 5 mmol) to yield **6l** which was recrystallised from methanol as white solid (0.311g, 14.9%): Rf: 0.97 (DCM/PE/EtOAc 10:1:1); mp: 226.5 - 226.6 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.07 (s, 1H), 7.87 (d, *J* = 7.2 Hz, 1H), 7.57 (d, *J* = 6.7 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 2H), 7.28 (t, *J* = 7.6 Hz, 1H), 7.08 (d, *J* = 8.8 Hz, 2H), 4.50 (s, 2H), 3.82 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.3 (C, <u>C</u>OOH), 160.8 (C, C-6), 159.7 (C, C-2), 158.1 (C, C-4'), 136.6 (C, C-4), 130.4, (C, C-3") 130.3 (CH, C-4"), 128.4 (C, C-1", C-1'), (128.4 (CH, C-2', C-6'), 127.7 (CH, C-2"), 125.8 (CH, C-5", C-6"), 115.6 (CH, C-3', C-5'), 115.5 (C, CN), 114.1, (C, CN), 93.2 (C, C-5), 85.9 (C, C-3), 55.4 (CH_3, O<u>C</u>H_3), 33.3 (CH_2, -S<u>C</u>H_2-); APCI-HRMS *m/z*: calculated for $C_{22}H_{17}N_4O_3S$ [M+H]+ 417.1016, found 417.1009; Purity (HPLC, λ = 254): 100%

2-amino-4-(4-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6m)

Prepared as for **6c** from 4-hydroxybenzaldehyde (0.611g, 5mmol) and 2-(bromomethyl)-6-methylpyridine (0.932g, 5 mmol) to yield **6m** which was recrystallised from methanol as creamy white powder (1.611g, 86.3%): Rf: 0.68 (PE:EtOAC 1:1); mp: 190.8 - 191.7 °C; ^{1}H NMR (600 MHz, DMSO) δ 10.09 (s, 1H), 7.63 (t, *J* = 7.7 Hz, 1H), 7.43 (d, *J* = 7.6 Hz, 1H), 7.41 – 7.33 (m, 2H), 7.14 (d, *J* = 7.7 Hz, 1H), 6.94 – 6.87 (m, 2H), 4.54 (s, 2H), 2.45 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.2 (C, C-6), 159.8 (C, C-2), 159.5 (C, C-4), 158.4 (C, C-4'), 157.8 (C, C-2"), 155.9 (C, C-6"), 137.1 (CH, C-4"), 130.3 (C, C-1'), 124.1 (CH, C-2', C-6'), 121.9 (CH, C-3"), 120.8 (CH, C-5"), 115.7 (CH, C-3', C-5'), 115.6 (C, CN), 115.4 (C, CN), 93.1 (C, C-5), 85.8 (C, C-3), 35.4 (CH_2, -S<u>C</u>H_2-), 24.0 (CH_3, <u>Methyl</u>pyridine); APCI-HRMS *m/z*: calculated for $C_{20}H_{16}N_5OS$ [M+H]+ 374.1070, found 374.1061; Purity (HPLC, λ = 254): 100%

2-amino-4-(4-hydroxyphenyl)-6-((3-methylbenzyl)thio)pyridine-3,5-dicarbonitrile (6n)

Prepared as for **6c** from 4-hydroxybenzaldehyde (0.612g, 5mmol) and 1-(bromomethyl)-3-methylbenzene (0.678ml, 5 mmol) to yield **6n** which was recrystallised from methanol as light yellowish powder (0.532g, 28.6%): Rf: 0.75 (PE/EtOAC 1:1); mp: 224.9 - 226.1 °C;^{1}H NMR (600 MHz, DMSO) δ 10.06 (s, 1H), 7.39 – 7.33 (m, 2H), 7.33 – 7.26 (m, 2H), 7.20 (t, *J* = 7.6 Hz, 1H), 7.06 (d, *J* = 7.5 Hz, 1H), 6.93 – 6.85 (m, 2H), 4.45 (s, 2H), 2.28 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.3 (C, C-6), 159.7 (C, C-2), 159.4 (C, C-4), 158.4 (C, C-4'), 137.6 (C, C-1"), 137.3 (C, C-3"), 130.3 (C, C-1'), 129.9 (CH, C-2', C-6'), 128.3 (CH, C-5"), 127.9 (CH, C-4"), 126.4 (CH, C-2"), 124.2 (CH, C-6"), 115.6 (CH,C-3', C-5'), 115.6 (C, CN), 115.4 (C, CN), 93.1 (C, C-5), 85.7 (C, C-3), 33.2 (CH_2, -S-<u>C</u>H_2-), 20.9 (CH_3, <u>Methyl</u>benzyl); APCI-HRMS *m/z*: calculated for $C_{21}H_{17}N_4OS$ [M+H]+ 373.1118, found 373.1111; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (6o)

Prepared as for **6c** from 3-methoxybenzaldehyde (0.608ml, 5mmol) and 3-(bromomethyl)benzoic acid (1.083g, 5 mmol) to yield **6o** which was recrystallised from methanol as cream white powder

(0.490g, 23.5%):Rf: 0.70 (DCM/PE/EtOAc 10:1:1); mp: 240.0 - 241.1°C; ^{1}H NMR (600 MHz, DMSO) δ 8.04 (s, 1H), 7.83 (d, *J* = 7.7 Hz, 1H), 7.69 (d, *J* = 7.6 Hz, 1H), 7.48 – 7.42 (m, 1H), 7.37 (t, *J* = 7.7 Hz, 1H), 7.14 – 7.04 (m, 3H), 4.57 (s, 2H), 3.79 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.0 (C, COOH), 159.5 (C, C-6), 159.0 (C, C-2), 158.2 (C, C-3'), 137.5 (C, C-4), 135.2 (C, C-1', C-3"), 132.5 (C,C-4"), 130.1 (C, C-5', C-1"), 130.0 (CH, C-2"), 128.2 (CH, C-5", C6"), 120.5 (CH, C-6'), 115.9 (CH, C-4'), 115.2 (C, CN), 115.2 (C, CN), 114.0 (CH, C-2'), 93.3 (C, C-5), 86.1 (C, C-3), 55.3 (CH_3, O$\underline{C}H_3$), 32.9 (CH_2, -S$\underline{C}H_2$-); APCI-HRMS *m/z*: calculated for $C_{22}H_{16}N_4O_3S$ [M+H]+ 417.1016, found 417.1012; Purity (HPLC, λ = 254): 100%

2-amino-4-(4-fluorophenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6p)

Prepared as for **6c** from 4-fluorobenzaldehyde (0.536ml, 5mmol) and 2-(bromomethyl)-6-methylpyridine (0.932g, 5 mmol) to yield **6o** which was recrystallised from methanol as white powder (0.222g, 11.8%): Rf: 0.84 (PE:EtOAC 1:1); mp: 216.8 - 216.9 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.33 (s, 2H), 7.62 (dt, *J* = 8.9, 6.5 Hz, 3H), 7.48 – 7.36 (m, 3H), 7.14 (d, *J* = 7.7 Hz, 1H), 4.55 (s, 2H), 2.45 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.2 C, C-6), 164.0 (C, C-2), 162.3 (C, C-4), 159.5 (C, C-2"), 157.8 (C, C-6"), 157.5 (CH, C-5"), 155.8 (CH, C-3"), 137.1 (CH, C-4"), 131.1 (d, *J* = 8.8 Hz ,C, C-1'), 131.1 (C, C-6'), 130.3 (d, *J* = 3.0 Hz, CH, C-2', C-6'), 121.9 (C, CN), 120.8 (C, CN), 115.8 (d, *J* = 22.0 Hz, C, C-4'), 115.2 (d, *J* = 11.2 Hz, CH, C-3', C-5'), 93.3 (C, C-5), 86.1 (C, C-5), 35.4 (CH_2, -$\underline{S}CH_2$-), 24.0 (CH_3, Methylpyridine); APCI-HRMS *m/z*: calculated for $C_{20}H_{15}FN_5S$ [M+H]+ 376.1027, found 376..1039; Purity (HPLC, λ = 254): 100%

General procedure for the synthesis of **6q–6s and 7a:**

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-(2-hydroxyethoxy)phenyl)pyridine-3,5-dicarbonitrile (6q)

Cyanothioacetamide (0.503g, 5mmol) was dissolved in 10ml ethanol. The reaction mixture was then stirred for 15–20 mins before adding 4-(2-hydroxyethoxy)benzaldehyde (0.696ml, 5mmol) while stirring at room temperature. After obtaining a homogeneous mixture, malononitrile

(0.315ml, 5mmol) was added and the mixture stirred until it became homogeneous again, then left to stand at room temperature for 12–14 hrs. The mixture was then diluted with an equal volume of dimethylformamide and 10% aqueous potassium hydroxide (2.8ml, 5mmol) and left to stand for 24 hrs. 4-(Chloromethyl)-2-(4-chlorophenyl)thiazole (1.221g, 5mmol) was added to the mixture and continuously stirred for 3 hrs, after which 10% aqueous potassium hydroxide (5.6 mL, 10 mmol) was added again. After 2–4 hrs, ice was added and the resulting precipitate was filtered off, washed with distilled H_2O, ethanol and hexane, dried (30°C) and recrystallised from methanol to yield the title compound **6q** as light brown powder (1.355g, 52.1%): Rf: 0.82 (DCM/PE/EtOAc 10:1:1); mp: 162.1 - 163.5 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.97 – 7.92 (m, 2H), 7.89 (s, 1H), 7.59 – 7.53 (m, 2H), 7.50 – 7.42 (m, 2H), 7.15 – 7.02 (m, 2H), 4.86 (t, *J* = 5.5 Hz, 1H), 4.64 (s, 2H), 4.08 (t, *J* = 5.0 Hz, 2H), 3.74 (dd, *J* = 10.1, 5.2 Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 165.8 (C, C-2''), 165.6 (C, C-6), 160.3 (C, C-2), 159.7 (C, C-4'), 158.1 (C, C-4), 152.4 (C, C-4''), 134.8 (C, C-1'''), 131.6 (C, C-4'''), 130.1 (C, C-1'), 129.2 (CH, C-3''', C5'''), 127.7 (CH, C-2', C-6'), 125.7 (CH, C-2''', C-6'''), 118.7 (CH, C-5''), 115.3 (CH, C-3', C-5'), 115.3 (C, CN), 114.5 (C, CN), 93.4 (C, C-5), 85.9 (C, C-3), 69.7(CH_2, O$\underline{C}H_2CH_2$OH) , 59.4 (CH_2, O$CH_2\underline{C}H_2$OH), 29.2 (CH_2, -S$\underline{C}H_2$-); APCI-HRMS *m/z*: calculated for $C_{25}H_{19}ClN_5O_2S_2$ [M+H]+ 520.0663, found 520.0651; Purity (HPLC, λ = 254): 100%

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-hydroxyphenyl)pyridine-3,5-dicarbonitrile (6r)

Prepared as for **6q** from 4-hydroxybenzaldehyde (0.612g, 5mmol) and 4-(chloromethyl)-2-(4-chlorophenyl)thiazole (1.220g, 5 mmol) to yield **6r** which was recrystallised from methanol as light pink powder (1.203g, 50.6%): Rf: 0.81(DCM/PE/EtOAc 10:1:1); mp: 233.8 - 234.6 °C; ^{1}H NMR (600 MHz, DMSO) δ 10.06 (s, 1H), 7.97 – 7.92 (m, 2H), 7.90 (s, 1H), 7.58 – 7.52 (m, 2H), 7.39 – 7.33 (m, 2H), 6.92 – 6.87 (m, 2H), 4.63 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 165.9 (C, C-2''), 165.6 (C, C-6), 159.8 (C, C-2), 159.4 (C, C-4), 158.4 (C, C-4'), 152.5 (C, C-4''), 134.8 (C, C-1'''), 131.6 (C, C-4'''), 130.2 (C, C-1'), 129.3 (CH, C-2', C-6'), 127.8 (CH, C-2''', C-6'''), 124.1

(CH, C-3''', C-5'''), 118.8 (CH, C-3', C-5'), 115.6 (CH, C-5''), 115.5 (C, CN), 115.4 (C, CN), 93.3 (C, C-5), 85.8 (C, C-3), 29.3 (CH_2, -S<u>C</u>H_2-); APCI-HRMS *m/z*: calculated for $C_{23}H_{15}ClN_5OS_2$ [M+H]+ 476.0401, found 476.0386; Purity (HPLC, λ = 254): 100%

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (6s)

Prepared as for **6q** from 4-methoxybenzaldehyde (0.608ml, 5mmol) and 4-(chloromethyl)-2-(4-chlorophenyl)thiazole (1.222g, 5 mmol) to yield **6s** which was recrystallised from methanol as cream white solid (0.850g, 34.9%): Rf: 0.72 (DCM/PE/EtOAc 10:1:1); mp: 238.2 - 240.4 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.06 (s, 2H), 7.96 – 7.92 (m, 2H), 7.89 (s, 1H), 7.58 – 7.53 (m, 2H), 7.50 – 7.46 (m, 2H), 7.13 – 7.08 (m, 2H), 4.64 (s, 2H), 3.83 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 165.8 (C, C-2''), 165.6 (C, C-6), 160.8 (C, C-2), 159.7 (C, C-4'), 158.0 (C, C-4), 152.4 (C, C-4''), 134.8 (C, C-1'''), 131.6 (C, C-4'''), 130.1 (C, C-1'), 129.2 (CH, C-2', C-6'), 127.7 (CH, C-'''3, C-5'''), 125.7 (CH, C-2''', C-6'''), 118.7 (CH, C-5''), 115.4 (CH, C-3', C-5'), 115.3 (C, CN), 114.0 (C, CN), 93.4 (C, C-5), 85.9 (C, C-3), 55.3 (CH_3, O<u>C</u>H_3), 29.3 (CH_2, -S<u>C</u>H_2-); APCI-HRMS *m/z*: calculated for $C_{24}H_{17}ClN_5OS_2$ [M+H]+ 490.0558, found 490.0538; Purity (HPLC, λ = 254): 100%

3,6-diamino-5-cyano-4-(4-fluorophenyl)thieno[2,3-b]pyridine-2-carboxamide (7a)

Prepared as for **6q** from 4-fluorobenzaldehyde (0.536ml, 5mmol) and 2-bromoacetamide (0.693g, 5 mmol) to yield compound **7a** which was recrystallised from methanol as yellow to greenish solid (0.687g, 42.0%): Rf: 0.77 (DCM/PE/EtOAc 10:1:1); mp: 257.5 – 260.7 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.72 – 7.53 (m, 2H), 7.52 – 7.39 (m, 2H), 7.33 (s, 2H), 6.99 (s, 2H), 5.63 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.7 (C, CO), 163.6 (C, C-6), 163.4 (C, between position 1 & 7 of thienopyridine), 162.0 (C, C-4), 158.3 (C, C-3), 151.3 (C, C-2), 146.1 (C, C-1'), 130.60 (d, *J* = 8.5 Hz, C-2',C-6'), 129.91 (d, *J* = 3.1 Hz), C-3', C-5'), 116.22 – 115.68 ((m), C-4'), 114.2 (C, between position 3 & 4 of thienopyridine), 93.3 (C, CN), 90.4 (C-C5); APCI-HRMS *m/z*: calculated for $C_{15}H_{11}FN_5OS$ [M+H]+ 328.0663, found 328.0652; Purity (HPLC, λ = 254): 100%

General procedure for synthesis of **7b** and **7c**

3,6-diamino-5-cyano-4-(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (7b)

Prepared by dissolving 2-((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)acetamid (**6a**) (0.203g, 0.598mmol) in 10ml dimethylformamide. Two or three drops of 10% aqueous potassium hydroxide was then added to the solution. The reaction mixture was maintained at room temperature for 24 hrs. Product was precipitated by adding ice. The resulting precipitate was filtered off, washed with distilled H_2O, ethanol and hexane, dried (30°C) and recrystallised from methanol to yield **7b** as yellow powder (0.054g, 31.8%): Rf: 0.75 (DCM/PE/EtOAc 10:1:1); mp: 263.9 – 264.9 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.42 (d, *J* = 8.4 Hz, 2H), 7.30 (s, 2H), 7.14 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 2H), 5.70 (s, 2H), 3.85 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.8 (C, CO), 163.4 (C, C-6), 160.2 (C, C-4'), 158.5 (C, between position 1 & 7 of thienopyridine), 152.3 (C, C-4), 146.3 (C, C-3), 129.7 (CH, C-2', C-6'), 125.5 (C, C-2), 116.0 (C, C-1'), 114.4 (C, between position 3 & 4 of thienopyridine), 114.3 (CH, C-3',C-5'), 92.9 (C, CN), 90.5 (C, C-5), 55.3 (CH_3, O$\underline{C}H_3$); APCI-HRMS *m/z*: calculated for $C_{16}H_{14}N_5O_2S$ [M+H]+ 340.0863, found 340.0853; Purity (HPLC, λ = 254): 93.8%

3,6-diamino-4-(benzo[d][1,3]dioxol-5-yl)-5-cyanothieno[2,3-b]pyridine-2-carboxamide (7c)

Prepared as for **7b** from 2-((6-amino-4-(benzo[d][1,3]dioxol-5-yl)-3,5-dicyanopyridin-2-yl)thio)acetamide (**6g**) (0.134g, 0.379mmol) to yield **7c** which was washed with distilled H_2O and hexane and dried without further purification to yield an orange solid (0.034g, 19.3%): Rf: 0.56 (DCM/PE/EtOAc 10:1:1); mp: 296.82°C; ^{1}H NMR (600 MHz, DMSO) δ 7.28 (s, 1H), 7.15 – 7.03 (m, 1H), 7.03 – 6.91 (m, 1H), 6.06 (dd, *J* = 118.5, 114.5 Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.8 (C, CO), 158.4 (C, C-6), 152.0 (C, between position 1 & 7 of thienopyridine), 148.5 (C, C-4), 147.5 (C, C3', C4'), 146.3 (C, C-3), 126.8 (C, C-1'), 122.0 (C, C-2), 115.9 (C, between position 3 & 4 of thienopyridine), 114.4 (CH, C-6'), 108.8 (CH, C-2'), 108.7 (C, CN), 101.7 (CH, C-5'), 92.8 (CH_2, at dioxol), 90.5 (C, C-5); APCI-HRMS *m/z*: calculated for $C_{16}H_{12}N_5O_3S$ [M+H]+ 354.0655, found 354.0647; Purity (HPLC, λ = 254): 62.6%

3,6-diamino-5-cyano-4-(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (7d)

Prepared by refluxing a solution of 2-((6-amino-3,5-dicyano-4-(3-hydroxyphenyl)pyridin-2-yl)thio)acetamide (**6f**) (0.201g, 0.518mmol) and potassium hydroxide (0.080g, 1.426mmol) in ethanol (10ml) for 6 hrs. After cooling, ice-cold water was added to the reaction mixture to precipitate the product. The resulting precipitate was filtered off, washed with distilled H_2O, ethanol and hexane, dried (30°C) and recrystallised from methanol to yield **7d** as greenish solid (0.163g, 66.4%): Rf: 0.15 (DCM/PE/EtOAc 10:1:1); mp: 285.12 °C; ^{1}H NMR (600 MHz, DMSO) δ 9.97 (s, 1H), 7.39 (t, *J* = 7.7 Hz, 1H), 7.31 (s, 2H), 6.98 (s, 3H), 6.90 – 6.76 (m, 2H), 5.70 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.7 (C,<u>C</u>O), 163.3 (C, C-6), 158.4 (C, C, between position 1 & 7 of thienopyridine), 157.6 (C, C-3'), 152.2 (C, C-4), 146.0 (C, C-1'), 134.8 (C, C-3), 130.4 (CH, C-5'), 118.3 (C, C-2), 116.9 (C, C between position 3 & 4 of thienopyridine), 115.8 (CH, C-6'), 114.7 (CH, C-4'), 113.9 (CH, C-2'), 93.0 (C, CN), 89.9 (C, C-5); APCI-HRMS *m/z*: calculated for $C_{15}H_{12}N_5O_2S$ [M+H]+ 326.0706, found 326.0690; Purity (HPLC, λ = 254): 100%

Biology

***In vitro* evaluation**

Materials

All reagents were commercially available and purchased from various manufacturers. Radioligands [^{3}H]DPCPX (120 Ci/mmol) and [^{3}H]NECA (27.1 Ci/mmol) were obtained from PerkinElmer. Adenosine deaminase from bovine spleen (157 units/mg, 5.9 mg/ml, or 130 units/mg, 6.8mg/ml), CPA, DPCPX, istradefylline, caffeine and anhydrous $MgCl_2$ were all obtained from Sigma-Aldrich. Radioactivity was counted by a PerkinElmer Tri-CARB 2810 TR liquid scintillation analyser.

Ethics

The collection of tissue samples for the A_1 and A_{2A} AR radioligand binding assays were approved by the Health Sciences Ethics Office for Research, Training and Support, North-West University

(NWU-00418-21-A5) and were performed in accordance with the guidelines of the South African National Standard (SANS) document (The care and use of animals for scientific purposes).

Tissue samples

Male Sprague–Dawley rats whole brain membranes (excluding cerebellum and brain stem) and rat striatal membranes were used for the A_1 and A_{2A} AR radioligand binding assays, respectively. Protein concentration of the rat brain tissues was determined according to the Bradford protein assay, using bovine serum albumin as reference standard [79].

Adenosine A_1 and A_{2A} receptor radioligand binding assays

The degree of binding affinity the test compounds showed toward A_1 and A_{2A} ARs were determined through radioligand binding assays, as previously described in literature [56, 63, 79, 80]. The A_1 AR radioligand binding assay used rat whole brain membranes (expressing A_1 ARs) and 0.1 nM 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) as radioligand while the A_{2A} AR radioligand binding assay used rat striatal membranes (expressing A_{2A} ARs) and 4 nM [^{3}H]5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA) as radioligand. In A_{2A} AR radioligand binding assays, 50 nM CPA was also added to reduce the binding of [^{3}H]NECA to adenosine A_1 receptors and 10 mM $MgCl_2$ was also included to increase radioligand binding and decrease non-specific binding. Membrane suspension equivalent to 120 µg rat whole brain membrane proteins was used for both A_1 and A_{2A} binding assays. Adenosine deaminase was included in both A_1 and A_{2A} binding assay to inactivate any remaining endogenous adenosine.

Non-specific binding of [^{3}H]DPCPX and [^{3}H]NECA for the radioligand binding assays were defined as binding in the presence of 100 µM CPA or 10µM DPCPX.. Specific binding was defined as the total binding minus the non-specific binding.

Guanosine triphosphate shift assays

The type of binding affinity at the rat A_1 AR displayed by test compounds was determined via a guanosine triphosphate (GTP) shift assay, as described in literature [57, 63, 64, 81]. A GTP shift assay follows similar method as A_1 AR radioligand binding assay, but additionally 100 µM GTP

was added. GTP is thought to act by uncoupling the receptors from their G-proteins which causes agonists of the receptor to lose binding affinity [82]. Non-specific binding was defined as binding in the presence of 10 μM DPCPX.

Statistical data analyses

All statistical data analyses were carried out with Microsoft Excel and GraphPad Prism Software. Sigmoidal dose response curves, from which IC_{50} values were calculated, were obtained by plotting the specific binding against the logarithm of the test compounds' concentrations. Subsequently, the IC_{50} values were used to calculate the Ki values for the competitive inhibition of [^{3}H]DPCPX (K_d = 0.36 nM) against rat whole brain membranes and [^{3}H]NECA (K_d = 15.3 nM) against rat striatal membranes by the test compounds by means of the Cheng–Prusoff equation. All incubations were carried out in triplicate and the inhibition constant (K_i) values are expressed as the mean ± standard error of mean (SEM). GTP shifts were calculated by dividing the Ki values of compounds reported in the presence of GTP by the Ki values obtained in the absence of GTP.

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Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

1. Sheth S, Brito R, Mukherjea D, Rybak LP, Ramkumar V. Adenosine receptors: expression, function and regulation. Int. J. Mol. Sci. 2014;15(2):2024-52.

2. Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nat. Rev. Drug. Discov. 2006;5(3):247-64.

3. Abbracchio MP, Cattabeni F. Brain adenosine receptors as targets for therapeutic intervention in neurodegenerative diseases. Ann. N. Y. Acad. Sci. 1999;890(1):79-92.

4. Chen JF, Eltzschig HK, Fredholm BB. Adenosine receptors as drug targets--what are the challenges? Nat. Rev. Drug. Discov. 2013;12(4):265-86.

5. Effendi WI, Nagano T, Kobayashi K, Nishimura Y. Focusing on adenosine receptors as a potential targeted therapy in human diseases. Cells. 2020;9(3):1-36.

6. Boison D. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. Neuroscientist. 2005;11(1):25-36.

7. Betti M. Design,Synthesis and Pharmacological evaluation of new adenosine receptor ligands. 2016;Florence:University of Florence (Thesis -PhD).

8. Klotz KN. Adenosine receptors and their ligands. Naunyn Schmiedebergs Arch. Pharmacol. 2000;362:382-91.

9. Cunha RA. How does adenosine control neuronal dysfunction and neurodegeneration? J. Neurochem. 2016;139(6):1019-55.

10. Benarroch EE. Adenosine and its receptors: multiple modulatory functions and potential therapeutic targets for neurologic disease. Neurology. 2008;70(3):231-6.

11. Wardas J. Neuroprotective role of adenosine in the CNS. Pol. J. Pharmacol. 2002;54(4):313-26.

12. Stone TW, Ceruti S, Abbracchio MP. Adenosine receptors and neurological disease: neuroprotection and neurodegeneration. Handb. Exp. Pharmacol. 2009;193:535-87.

13. Ribeiro J, Sebastiao A, De Mendonça A. Adenosine receptors in the nervous system: pathophysiological implications. Prog. Neurobiol. 2002;68(6):377-92.

14. Masino SA, Kawamura MJ, Ruskin DN. Adenosine receptors and epilepsy: current evidence and future potential. Int. Rev. Neurobiol. 2014;119:233-55.

15. Epilepsy: a public health imperative. World Health Organization; 2019.

16. Sills, G.J. Mechanisms of action of antiepileptic drugs. In: Rugg-Gunn, F.J. & Stapley H.B., editors. Epilepsy 2017.From Bench to Bedside. A Practical Guide to Epilepsy, 2017. p. 395-303.

17. Juge N, Gray JA, Omote H, Miyaji T, Inoue T, Hara C et al. Metabolic control of vesicular glutamate transport and release. Neuron. 2010;68(1):99-112.

18. Pati S, Alexopoulos AV. Pharmacoresistant epilepsy: from pathogenesis to current and emerging therapies. Cleve. Clin. J. Med. 2010;77(7):457-567.

19. Klaft ZJ, Hollnagel JO, Salar S, Caliskan G, Schulz SB, Schneider UC et al. Adenosine A1 receptor-mediated suppression of carbamazepine-resistant seizure-like events in human neocortical slices. Epilepsia. 2016;57(5):746-56.

20. Rombo DM, Ribeiro JA, Sebastião AM. Role of adenosine receptors in epileptic seizures. In: Borea P, Varani K, Gessi S, Merighi S, Vincenzi F, editors. The Adenosine Receptors. 2018. p. 309-50.

21. Domingos CIA. Probing the synaptic target of a new putative antiepileptic drug: Modulation of the excitatory transmission in the hippocampus: University of Lisboa (PhD Thesis); 2016.

22. Fabera P, Parizkova M, Uttl L, Vondrakova K, Kubova H, Tsenov G et al. Adenosine A1 Receptor Agonist 2-chloro-N6-cyclopentyladenosine and Hippocampal Excitability During Brain Development in Rats. Front. Pharmacol. 2019;10:656.

23. Spanoghe J, Larsen LE, Craey E, Manzella S, Van Dycke A, Boon P et al. The Signaling Pathways Involved in the Anticonvulsive Effects of the Adenosine A1 Receptor. Int. J. Mol. Sci. 2021;22(1):320.

24. Tomé ÂR, Silva H, Cunha RA. Role of the purinergic neuromodulation system in epilepsy. Open Neurosci. J. 2010;4(1).

25. Gouder N, Fritschy JM, Boison D. Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. Epilepsia. 2003;44(7):877-85.

26. Müller CE. A1 adenosine receptors and their ligands: overview and recent developments. Il farmaco. 2001;56(1-2):77-80.

27. Dal Ben D, Lambertucci C, Buccioni M, Marti Navia A, Marucci G, Spinaci A et al. Non-Nucleoside Agonists of the Adenosine Receptors: An Overview. Pharmaceuticals (Basel). 2019;12(4):1-22.

28. Gao ZG, Jacobson KA. Emerging adenosine receptor agonists: an update. Expert Opin. Emerg. Drugs. 2011;16(4):597-602.

29. Jacobson KA, Van Galen PJ, Williams M. Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. J. Med. Chem. 1992;35(3):407-22.

30. Knutsen LJS, Lau J, Sheardown MJ, Eskesen K, Thomsen C, Weis JU et al. Anticonvulsant Actions of Novel and Reference Adenosine Agonists. In: Belardinelli L, Pelleg A, editors. Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology. Norwell, MA USA: Kluwer Academic Publishers; 1995. p. 479-87.

31. Jacobson KA, Tosh DK, Jain S, Gao ZG. Historical and Current Adenosine Receptor Agonists in Preclinical and Clinical Development. Front. Cell. Neurosci. 2019;13(124):1-17.

32. Masino SA, Kawamura Jr M, Ruskin DN. Adenosine receptors and epilepsy: current evidence and future potential. Int. Rev. Neurobiol. 2014;119:233-55.

33. Alinaghizadeh F, Zahedifar M, Seifi M, Sheibani H. Cascade synthesis of thieno [2, 3-b] pyridines by using intramolecular cyclization reactions of 3-cyano-2-(organylmethylthio) pyridines. J. Braz. Chem. Soc. 2016;27(4):663-9.

34. Litvinov VP, Dotsenko VV, Krivokolysko SG. Thienopyridines: synthesis, properties, and biological activity. Russ. Chem. Bull. 2005;54(4):864-904.

35. Al-Trawneh SA, El-Abadelah MM, Zahra JA, Al-Taweel SA, Zani F, Incerti M et al. Synthesis and biological evaluation of tetracyclic thienopyridones as antibacterial and antitumor agents. Bioorg. Med. Chem. 2011;19(8):2541-8.
36. Amorim R, de Meneses MDF, Borges JC, da Silva Pinheiro LC, Caldas LA, Cirne-Santos CC et al. Thieno [2, 3-b] pyridine derivatives: a new class of antiviral drugs against Mayaro virus. Arch. Virol. 2017;162(6):1577-87.
37. Bernardino AM, Pinheiro LC, Ferreira VF, Azevedo AR, Carneiro J, Souza TM et al. Synthesis and antiviral activity of new 4-(phenylamino) thieno [2, 3-b] pyridine derivatives. Heterocycl. Commun. 2004;10(6):407-10.
38. Adachi I, YamamoriI T, Hiramatsu Y, SakaiI K, Mihara S-I, KawakamiI M et al. Studies on dihydropyridines. III. Synthesis of 4, 7-dihydrothieno [2, 3-b]-pyridines with vasodilator and antihypertensive activities. Chem. Pharm. Bull. 1988;36(11):4389-402.
39. Bahekar RH, Jain MR, Jadav PA, Prajapati VM, Patel DN, Gupta AA et al. Synthesis and antidiabetic activity of 2, 5-disubstituted-3-imidazol-2-yl-pyrrolo [2, 3-b] pyridines and thieno [2, 3-b] pyridines. Bioorg. Med. Chem. 2007;15(21):6782-95.
40. Liu H, Li Y, Wang X-Y, Wang B, He H-Y, Liu J-Y et al. Synthesis, preliminary structure–activity relationships, and in vitro biological evaluation of 6-aryl-3-amino-thieno [2, 3-b] pyridine derivatives as potential anti-inflammatory agents. Bioorganic Med. Chem. Lett. 2013;23(8):2349-52.
41. Ouf SA, Gaber HM. New fused pyridines: Synthesis of certain pyridothienopyrimidine derivatives as anti-dermatophytic agents. Afinidad: Revista de química teórica y aplicada. 2005;62(518):337-45.
42. Masch A, Nasereddin A, Alder A, Bird MJ, Schweda SI, Preu L et al. Structure-activity relationships in a series of antiplasmodial thieno[2,3-b]pyridines. Malar. J. 2019;18(89):1-10.

43. Hassan AY, Sarg MT, Said MM, El-Sebaey SA. Utility of thieno[2,3-b] pyridine derivatives in the synthesis of some condensed heterocyclic compounds with expected biological activity. Univers. Org. Chem. 2013;1(1):1-15.
44. Saku O, Saki M, Kurokawa M, Ikeda K, Takizawa T, Uesaka N. Synthetic studies on selective adenosine A2A receptor antagonists: Synthesis and structure–activity relationships of novel benzofuran derivatives. Bioorganic Med. Chem. Lett. 2010;20(3):1090-3.
45. Dyachenko IV, Dyachenko VD, Dorovatovskii PV, Khrustalev VN, Nenajdenko VG. Multicomponent Synthesis of 4-Alkyl(Aryl, Hetaryl)-2-alkoxycarbonyl(aroyl, carbamoyl)- 3,6-diamino-5-cyanothieno[2,3-b]pyridines. Russ. J. Org. Chem. 2018;54(10):1435-45.
46. Artemov V, Ivanov V, Koshkarov A, Shestopalov A, Litvinov V. Synthesis of pyrido [3′, 2′: 4, 5] thieno [2, 3-c] cinnolines—A new heterocyclic system. Chem. Heterocycl. Compd. 1998;34(1):96-101.
47. Gad-Elkareem MA, Elneairy MA, Taha AM. Reactions with 3, 6-diaminothieno [2, 3-b]-pyridines: Synthesis and characterization of several new fused pyridine heterocycles. Heteroat. Chem. 2007;18(4):405-13.
48. Liu H, Zaplishnyy V, Mikhaylichenko L. Facilitating Students' Review of the Chemistry of Nitrogen-Containing Heterocyclic Compounds and Their Characterization through Multistep Synthesis of Thieno [2, 3-b] Pyridine Derivatives. J. Chem. Educ. 2016;93(10):1785-7.
49. Mabkhot YN, Aladdi SS, Al-Showiman S, Al-Majid A, Barakat A, Ghabbour HA et al. Synthesis and characterization of novel thieno-fused bicyclic compounds through new enaminone containing thieno [2, 3-b] pyridine scaffold. J. Chem. 2015;2015.
50. Li Z, Khaliq M, Zhou Z, Post CB, Kuhn RJ, Cushman M. Design, synthesis, and biological evaluation of antiviral agents targeting flavivirus envelope proteins. J. Med. Chem. 2008;51(15):4660-71.

51. Janse van Rensburg HD, Legoabe LJ, Terre'Blanche G. C3 amino-substituted chalcone derivative with selective adenosine rA1 receptor affinity in the micromolar range. Chem. Pap. 2021;75(4):1581-605.

52. Viesser RV, Ducati LC, Tormena CF, Autschbach J. The halogen effect on the 13 C NMR chemical shift in substituted benzenes. Phys. Chem. Chem. Phys. 2018;20(16):11247-59.

53. Guo M, Gao ZG, Tyler R, Stodden T, Li Y, Ramsey J et al. Preclinical Evaluation of the First Adenosine A1 Receptor Partial Agonist Radioligand for Positron Emission Tomography Imaging. J. Med. Chem. 2018;61(22):9966-75.

54. Betti M, Catarzi D, Varano F, Falsini M, Varani K, Vincenzi F et al. Modifications on the Amino-3,5-dicyanopyridine Core To Obtain Multifaceted Adenosine Receptor Ligands with Antineuropathic Activity. J. Med. Chem. 2019;62(15):6894-912.

55. Louvel J, Guo D, Soethoudt M, Mocking TA, Lenselink EB, Mulder-Krieger T et al. Structure-kinetics relationships of Capadenoson derivatives as adenosine A1 receptor agonists. Eur. J. Med. Chem. 2015;101:681-91.

56. Bruns RF, Fergus JH, Badger EW, Bristol JA, Santay LA, Hartman JD et al. Binding of the A 1-selective adenosine antagonist 8-cyclopentyl-1, 3-dipropylxanthine to rat brain membranes. Naunyn-Schmiedeb. Arch. Pharmacol. 1987;335(1):59-63.

57. Lohse MJ, Klotz K-N, Lindenborn-Fotinos J, Reddington M, Schwabe U, Olsson RA. 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX)—a selective high affinity antagonist radioligand for A 1 adenosine receptors. Naunyn-Schmiedeb. Arch. Pharmacol. 1987;336(2):204-10.

58. Shimada J, Koike N, Nonaka H, Shiozaki S, Yanagawa K, Kanda T et al. Adenosine A2A antagonists with potent anti-cataleptic activity. Bioorganic Med. Chem. Lett. 1997;7(18):2349-52.

59. Robinson SJ, Petzer JP, Terre'Blanche G, Petzer A, Van der Walt MM, Bergh JJ et al. 2-Aminopyrimidines as dual adenosine A1/A2A antagonists. Eur. J. Med. Chem. 2015;104:177-88.

60. Van der Walt M, Terre'Blanche G. Selected C8 two-chain linkers enhance the adenosine A1/A2A receptor affinity and selectivity of caffeine. Eur. J. Med. Chem. 2017;125:652-6.

61. Grahner B, Winiwarter S, Lanzner W, Mueller CE. Synthesis and structure-activity relationships of deazaxanthines: analogs of potent A1-and A2-adenosine receptor antagonists. J. Med. Chem. 1994;37(10):1526-34.

62. Müller CE, Geis U, Hipp J, Schobert U, Frobenius W, Pawłowski M et al. Synthesis and structure− activity relationships of 3, 7-dimethyl-1-propargylxanthine derivatives, A2A-selective adenosine receptor antagonists. J. Med. Chem. 1997;40(26):4396-405.

63. Van der Walt MM, Terre'Blanche G, Petzer A, Petzer JP. The adenosine receptor affinities and monoamine oxidase B inhibitory properties of sulfanylphthalimide analogues. Bioorg. Chem. 2015;59:117-23.

64. Van der Werten EM, Hartog-Witte HR, Roelen HC, Künzel JKvFD, Pirovano IM, Mathôt RA et al. 8-Substituted adenosine and theophylline-7-riboside analogues as potential partial agonists for the adenosine A1 receptor. Eur. J. Pharmacol.: Mol. Pharmacol. 1995;290(3):189-99.

65. Schumacher B, Scholle S, Hölzl J, Khudeir N, Hess S, Müller CE. Lignans isolated from valerian: identification and characterization of a new olivil derivative with partial agonistic activity at A1 adenosine receptors. J. Nat. Prod. 2002;65(10):1479-85.

66. Savjani KT, Gajjar AK, Savjani JK. Drug solubility: importance and enhancement techniques. Int. Sch. Res. Notices. 2012;2012.

67. Zakeri MP, Tajerzadeh H, Eslamboulchilar Z, Barzegar S, Valizadeh H. The relation between molecular properties of drugs and their transport across the intestinal membrane. 2006.

68. Meibom D, Albrecht-Kupper B, Diedrichs N, Hubsch W, Kast R, Kramer T et al. Neladenoson Bialanate Hydrochloride: A Prodrug of a Partial Adenosine A1 Receptor Agonist for the Chronic Treatment of Heart Diseases. ChemMedChem. 2017;12:728-37.

69. Daina A, Michielin O, Zoete V. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep. 2017;7(1):1-13.

70. Egan WJ, Merz KM, Baldwin JJ. Prediction of drug absorption using multivariate statistics. J. Med. Chem. 2000;43(21):3867-77.

71. Ghose AK, Viswanadhan VN, Wendoloski JJ. A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. J. Comb. Chem. 1999;1(1):55-68.

72. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 1997;23(1-3):3-25.

73. Muegge I, Heald SL, Brittelli D. Simple selection criteria for drug-like chemical matter. J. Med. Chem. 2001;44(12):1841-6.

74. Veber DF, Johnson SR, Cheng H-Y, Smith BR, Ward KW, Kopple KD. Molecular properties that influence the oral bioavailability of drug candidates. J. Med. Chem. 2002;45(12):2615-23.

75. Baell JB, Holloway GA. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. J. Med. Chem. 2010;53(7):2719-40.

76. Brenk R, Schipani A, James D, Krasowski A, Gilbert IH, Frearson J et al. Lessons learnt from assembling screening libraries for drug discovery for neglected diseases. ChemMedChem: Chemistry Enabling Drug Discovery. 2008;3(3):435-44.

77. Bolz SN, Adasme MF, Schroeder M. Toward an understanding of pan-assay interference compounds and promiscuity: A structural perspective on binding modes. J. Chem. Inf. Model. 2021;61(5):2248-62.

78. Prabha B, Ezhilarasi M. Synthesis, Spectral Characterization, in Vitro and in Silico Studies of Benzodioxin Pyrazoline derivatives. 2020.

79. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72(1-2):248-54.
80. Bruns RF, Lu GH, Pugsley TA. Characterization of the A2 adenosine receptor labeled by [3H] NECA in rat striatal membranes. Mol. Pharmacol. 1986;29(4):331-46.
81. Lohse M, Lenschow V, Schwabe U. Two affinity states of Ri adenosine receptors in brain membranes. Analysis of guanine nucleotide and temperature effects on radioligand binding. Mol. Pharmacol. 1984;26(1):1-9.
82. Kull B, Svenningsson P, Hall H, Fredholm BB. GTP differentially affects antagonist radioligand binding to adenosine A1 and A2A receptors in human brain. Neuropharmacology. 2000;39(12):2374-80.

CHAPTER 4

4. CONCLUSION

Despite the availability of different classes of antiepileptic drugs (AEDs), only 70% of patients benefit from AEDs, while about 30% remain medically refractory to AEDs due to these drugs inefficacy in controlling seizures. This significant one third of epileptic patients who do not experience satisfactory seizure control with present treatments has been an important drive in the search for alternative epilepsy treatment.

Due to their widespread physiological functions in the human body, adenosine receptors (ARs) play a role in various pathologic conditions such as neurodegenerative disorders including epilepsy. In particular, A_1 ARs have been implicated in excitatory transmission in the brain. Activation of these receptors is considered to be an inhibitory biological endogenous mechanism, which decreases excitability, limits the duration of seizures and mediates seizure arrest. Therefore, a new field of adenosine-based therapies has been investigated including adenosine itself, AR agonists and antagonists and adenosine kinase inhibitors. The aim of this research study was to design, synthesise, and evaluate amino-3,5-dicyanopyridines and thieno[2,3-*b*]pyridine derivatives as potent and selective A_1 AR agonists for the potential treatment of drug-resistant epilepsy.

It was hypothesized that since 3,5-dicyanopyridine derivatives, which serve as intermediates in the synthesis of thieno[2,3-b]pyridine derivatives, exhibit affinity for ARs, a suitably substituted thieno[2,3-b]pyridine core can also lead to derivatives which exhibit potent AR affinity, based on their chemical similarity. This was also inspired by the fact that other bicyclic scaffolds such as benzofurans, tetralones and indanones have previously shown potency towards ARs.

In an effort to evaluate the significance of intramolecular cyclisation in relation to AR binding, this study focused on the design, synthesis and evaluation of thieno[2,3-b]pyridine derivatives based on amino-3,5-dicyanopyridine derivatives (intermediate compounds) which have displayed good affinity at A_1 AR. The synthesis of the target compounds, thieno[2,3-b]pyridine derivatives, and intermediates was carried out through multicomponent condensation of malononitrile with hydrogen sulfide, a corresponding aldehyde, and a suitable halide, in the presence of triethylamine. The results are presented as a research article to be submitted for publication in an academic journal (**Chapter 3**).

In total, 23 test compounds were synthesised, 4 target compounds (thieno[2,3]pyridine derivatives: **7a–d**) and 19 intermediate compounds (amino-3,5-dicyanopyridine derivatives: **6a–s**). 7 of the intermediates were novel (**6d**, **6k–p**). All synthesised compounds were characterised via proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy, high-

resolution mass spectrometry (HRMS), melting point (mp) determination via derivative thermogravimetry (DTG) and differential scanning calorimetry (DSC), and hot stage microscopy (HSM). Purities of the test compounds were determined via high-performance liquid chromatography (HPLC). *In vitro* radioligand binding assays were performed on all test compounds to assess affinity and/or activity at rA_1 and rA_{2A} ARs. Additionally, *in silico* evaluation of test compound was done using the SwissADME web tool.

Details of the results obtained during compound characterisation, purity determination, radioligand binding assays and *in silico* evaluation, can be found in the research supplementary material, which also contains a summary of the (unsuccessful) ring closure attempts made to synthesise thieno[2,3]pyridine derivatives.

The challenges experienced during synthesis is that the last stage of the multicomponent reaction system did not go to completion to yield thieno[2,3-b]pyridine derivatives for almost all reactions except the one that yielded compound **7a**. Instead, the method produced intermediate compounds amino-3,5-dicyanopyridine derivatives. Modifications of various literature techniques were employed in order to optimise reaction conditions for intramolecular cyclisation of the amino-3,5-dicyanopyridines (see supplementary material). These attempts yielded compound **7b–d**, thieno[2,3]pyridines. From observation, only compounds with a carbonyl (C=O) group at the aryl position managed to go to completion to target compounds thieno[2,3-b]pyridine derivatives. This was in line with available literature which implies that a carbonyl component has a great influence on intramolecular cyclisation of the 3,5-amino-dicyanopyridines to produce thieno[2,3-b]pyridines. A follow-up study will be necessary to investigate structure and reaction optimisations for synthesis of this scaffold.

Assessment of structure-activity relationships (SAR) of all test compounds, governing AR-ligand interactions at rA_1 and rA_{2A} receptors was done. There was a significant loss of binding affinity for both rA_1 and rA_{2A} receptors from open ring structures (amino-3,5-dicyanopyridine derivatives) to closed ring structures (thieno[2,3]pyridine derivatives). This correlates with Betti (2016) who evaluated intramolecular cyclisation of the 6-amino-3,5-dicyanopyridines, specifically BAY606583 (A_{2B} receptor agonist) and the resulting bicyclic compound, a thieno[2,3]pyridine derivative, which displayed poor affinity towards all ARs. The poor activity of thieno[2,3-b]pyridines could be attributed to the molecular rigidity that occurs during cyclisation. In this study the only thieno[2,3-*b*]pyridine derivative that displayed relatively good rA_1 AR activity (K_i = 61.9 nM) was compound **7c** (R^2 = [1.3]dioxol-5-yl). The good binding affinity of this compound (<100 nM) against *r*A_1 ARs is indication that it is possible to design and develop thieno[2,3-*b*]pyridine derivatives as AR ligands. The rest of the thieno[2,3-*b*]pyridine derivatives exhibited poor rA_1 AR binding affinities as follows: **7a** (R^2 = -F; K_i = 1008 nM, **7b** (R^2 = 4-OCH_3; K_i = 556 nM) and **7d** (R^2 = 4-OH; K_i = 305 nM).

For amino-3,5-dicyanopyridines (**6a–6s**), the overall best results were obtained in the rA_1 AR binding assays where most of the compounds showed high affinity at this receptor ($rA_1 K_i$ values of <10 nM). Compound **6c** was the most active with $rA_1 K_i$ value of 0.076 nM. The latter compound together with **6b** displayed better rA_{2A} affinity than other test compounds with $rA_{2A} K_i$ values of 48.3 nM (**6c**) and 48.0 nM (**6b**), respectively, but remain selective for the rA_1 AR. Generally, better rA_1 AR binding affinities were achieved with a 3-methoxyphenyl group at position 4(R^2) compared to a 4-methoxyphenyl substituent, but both of these methoxy substituents were favoured over their 3- & 4-hydroxyphenyl counterparts and other substituents that were explored at the same position (furan, fluorine, benzene, .4-methylthiophenyl), except for compound **6r** and **6s** where opposite trend was observed. Looking at position 6 (aryl substitution at the methylthio linker), it was observed that the methylpyridine group is preferred for rA_1 AR affinity over (in decreasing order) 4-chlorophenylthiazole, methylbenzene, benzoic acid and carbonyl substituents being the least favoured. Novel compounds (**6d**, **6k**, **6l**, **6m**, **6n**, **6o** and **6p**) proved to be highly selective with low nanomolar rA_1 AR affinity ($rA_1 K_i$ values between 0.179 and 21.0nM). The GTP shift assay indicated compounds **6n**, **6q** and **7c** as potent, highly selective agonists at A_1 ARs; however, compounds **6c**, **6d** and **6o** (notably all containing a 3-OCH_3 group at position R^2) behaved as rA_1 antagonists.

3,5-Dicyanopyridine scaffold

Best selective rA_1 affinity
6c (Ki = 0.076 nM)
R^2 = 3-methoxyphenyl
aryl = methylpyridine

Thieno[2,3-b]pyridine scaffold

Best rA_1 affinity
7c (Ki = 61.9 nM)
R^2 = benzo[d][1,3]dioxol-5-yl
aryl = -$CONH_2$

Figure 4-1: Best selective rA_1 ligands of the 3,5-amino-dicyanopyridines and thieno[2,3-b]pyridines

In silico evaluation of the test compounds highlighted bioavailability issues for both oral and blood brain barrier permeability due to the high polarity and degree of saturation of test compounds. No compound raised structural alerts when screened for potentially problematic substructures and generally all test compounds performed well on the drug-likeness test (i.e. chance of being

considered as an oral drug candidate). Interestingly only one compound (**6d**) passed the lead-likeness test, and hence, can used as a lead compound in drug discovery processes. The rest of the compounds had high molecular weight (>350) as well as high log*P* values (XLOGP: >3.5). Structures optimisation for these chemical scaffolds is needed most probably by decreasing size and polarity and/or lipophilicity.

In conclusion, although the number of thieno[2,3-b]pyridine derivatives synthesised was not adequate for comprehensive evaluation, the good binding of compound **7c** towards rA_1 AR (K_i = 61.9 nM) inspires further exploration of this scaffold as an AR ligand. A follow-up study aimed particularly at optimizing the synthesis method for thieno[2,3-b]pyridine derivatives is recommended.

ANNEXURE A: ORIGINAL ARTICLE SUPPLEMENTARY MATERIAL

EQUATIONS

Eq. 1

$$\% \text{ yield} = \frac{\text{Actual mass}}{\text{Theoretical mass}} \times 100$$

Eq. 2

Specific binding = total binding – average binding

Eq. 3

$$\text{Specific binding \%} = \frac{\text{Specific binding}}{\text{Average binding}} \times 100$$

Eq. 4

Cheng-Prusoff equation for the A_1 radioligand binding assay (using $[^3H]$DPCPX as radioligand)

$$K_i = \frac{\text{IC50}}{1+([RL]/K_d)}$$

Ki = inhibition constant
IC50 = half maximal inhibitory concentration
[RL] = radioligand concentration (0.1nM)
Kd = Equation dissociation constant of radioligand

Eq. 5

Selective index (**SI**) = ratio of the A_1 and A_{2A} AR *K*i values of test compounds.

Eq. 6.

$$\text{GTP shifts} = \frac{K\text{i values in the presence of GTP}}{K\text{i values in the absence of GTP}}$$

UNSUCCESSFUL CYCLISATION SYNTHETIC METHODS

A.

Scheme S1: Reflux a solution of amino-3,5-dicyanopyrine derivative (0.71mmol) and KOH (1.42 mmol) in absolute ethanol (10ml) for 3 hrs. After cooling, precipitate the product by adding ice-cold water

Attempted modifications:

- Increased reflux time gradually up to 6 hrs try and bring the reaction to completion
- Attempted different reflux temperatures (60-85°C)
- Increased KOH concentration gradually up to 2 mmol

Intermediates attempted for cyclisation: **6b, 6c, 6h, 6i, 6j, 6k, 6m, 6n, 6p, 6q, 6r** and **6s**.

(Betti *et al.*, 2018; Gad-Elkareem *et al.*, 2007)

B.

Scheme S2: Add 2-3 drops of 10% aqueous KOH to a solution of amino-3,5-dicyanopyrine derivative (1 mmol) in DMF. Leave the reaction at room temperature for 2 hrs. Precipitate the product by adding water.

Attempted modifications:

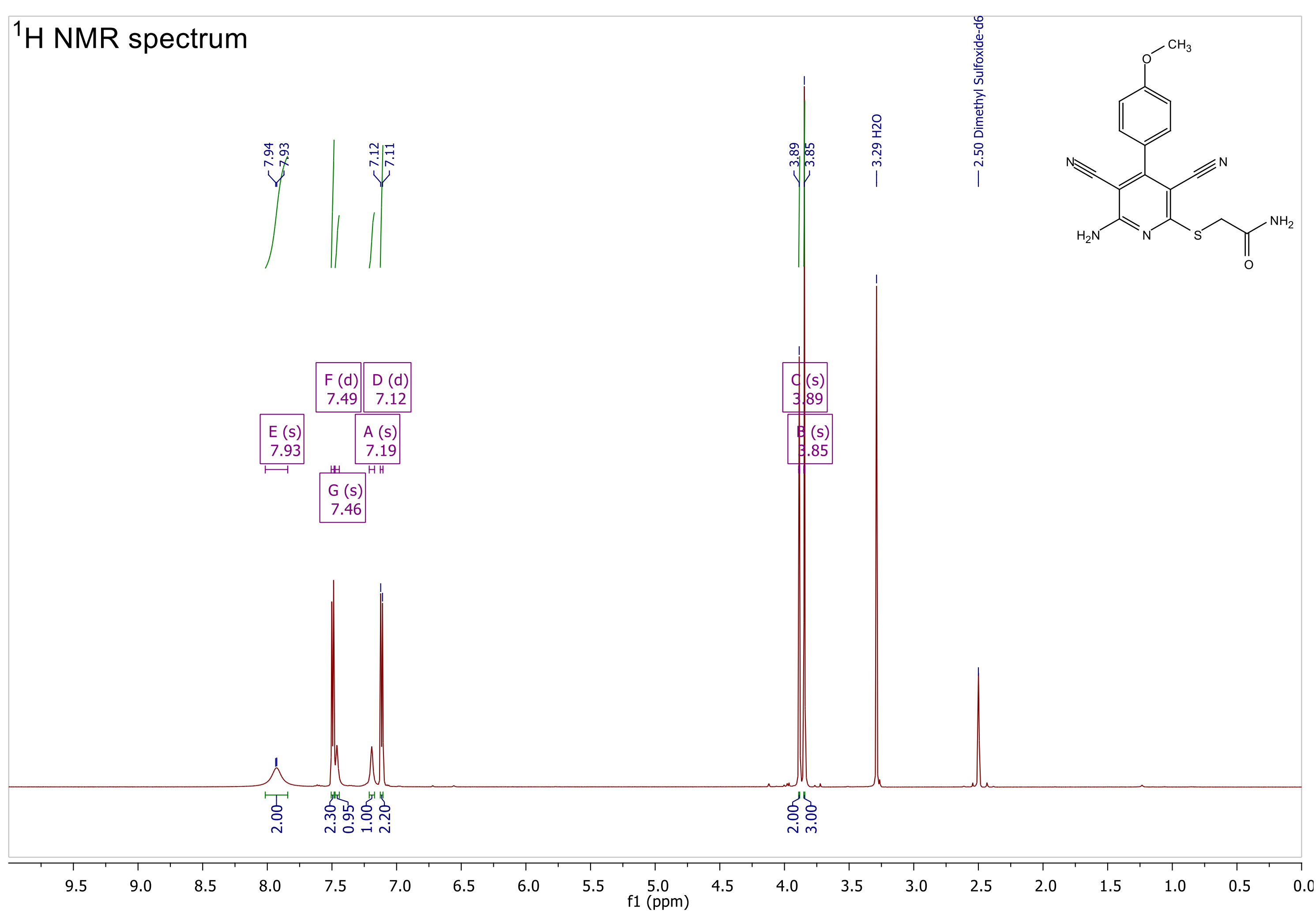
- Increased reaction standing time up to 12 hrs
- Increased the KOH concentration up to 20%

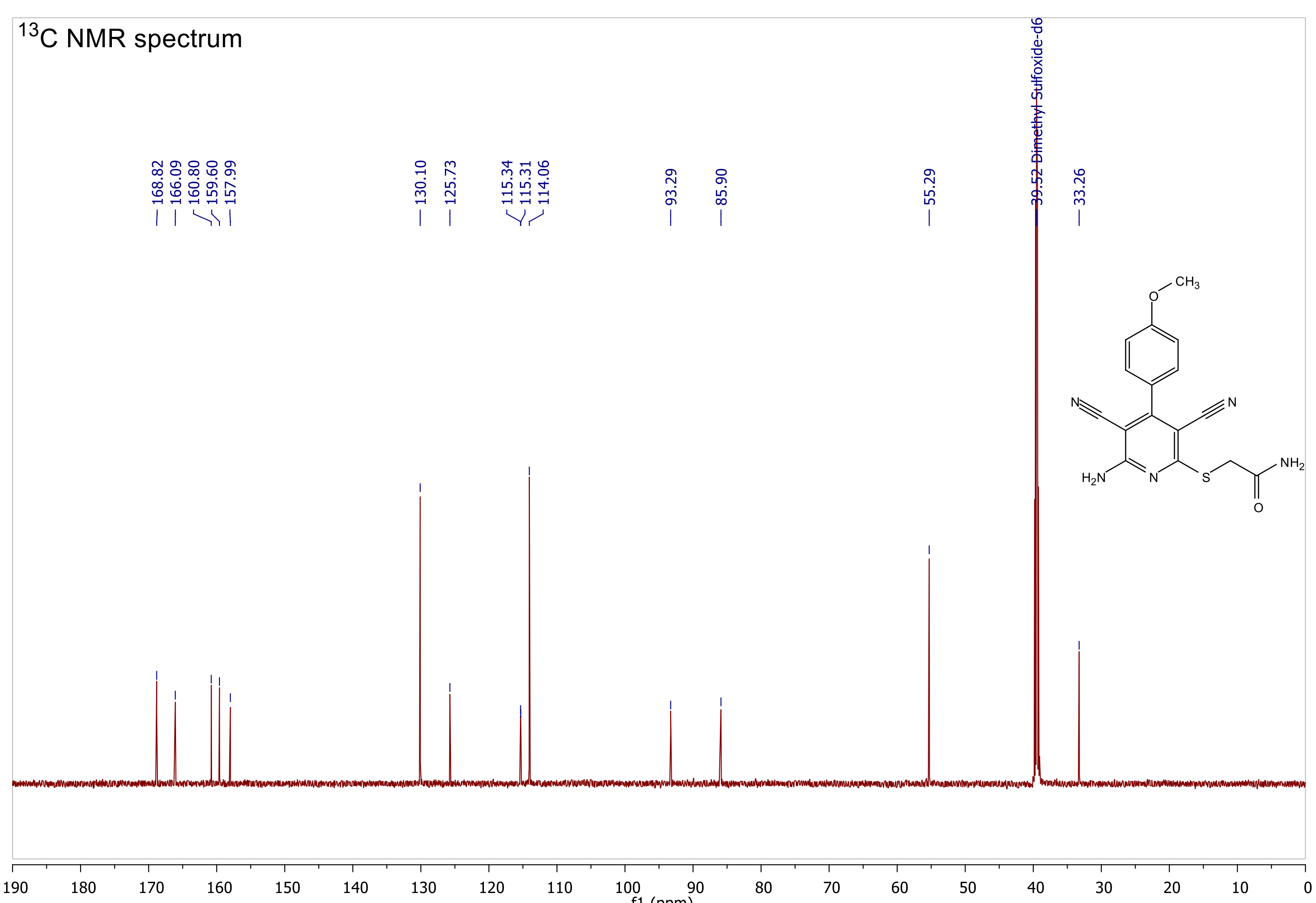
Intermediates attempted for cyclisation: **6b, 6c, 6e, 6h, 6i, 6j, 6k.6m, 6n, 6p, 6q, 6r** and **6s**.

(Artemov *et al.*, 1998)

1. Nuclear Magnetic Resonance (NMR) SPECTRA of test compounds

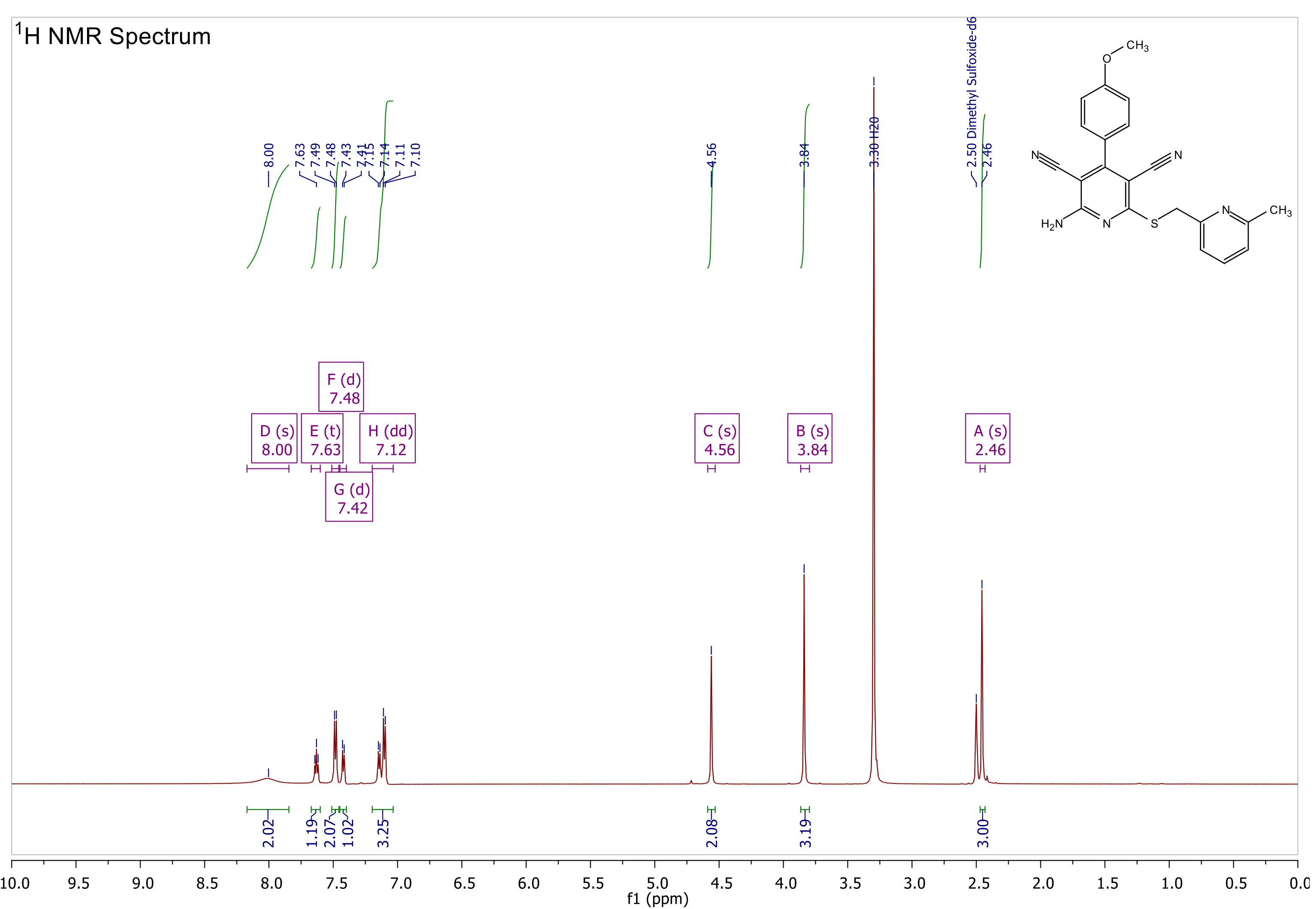
2-((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)acetamide (6a)

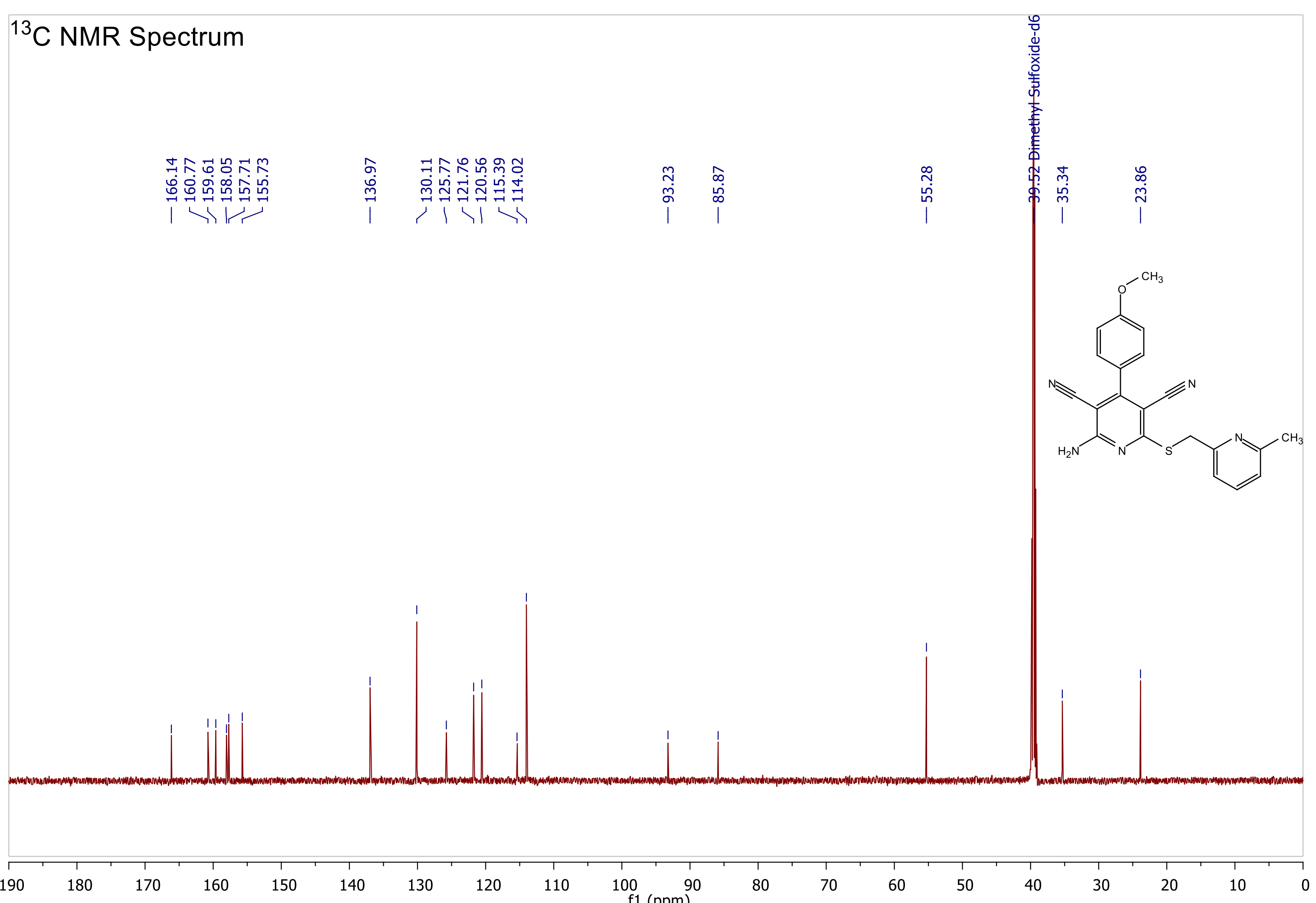




13C NMR spectrum
168.82
166.09
160.80
159.60
157.99
130.10
125.73
115.34
115.31
114.06
93.29
85.90
55.29
39.52 Dimethyl Sulfoxide-d6
33.26
CH3
O
N
N
H2N
N
S
NH2
O
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

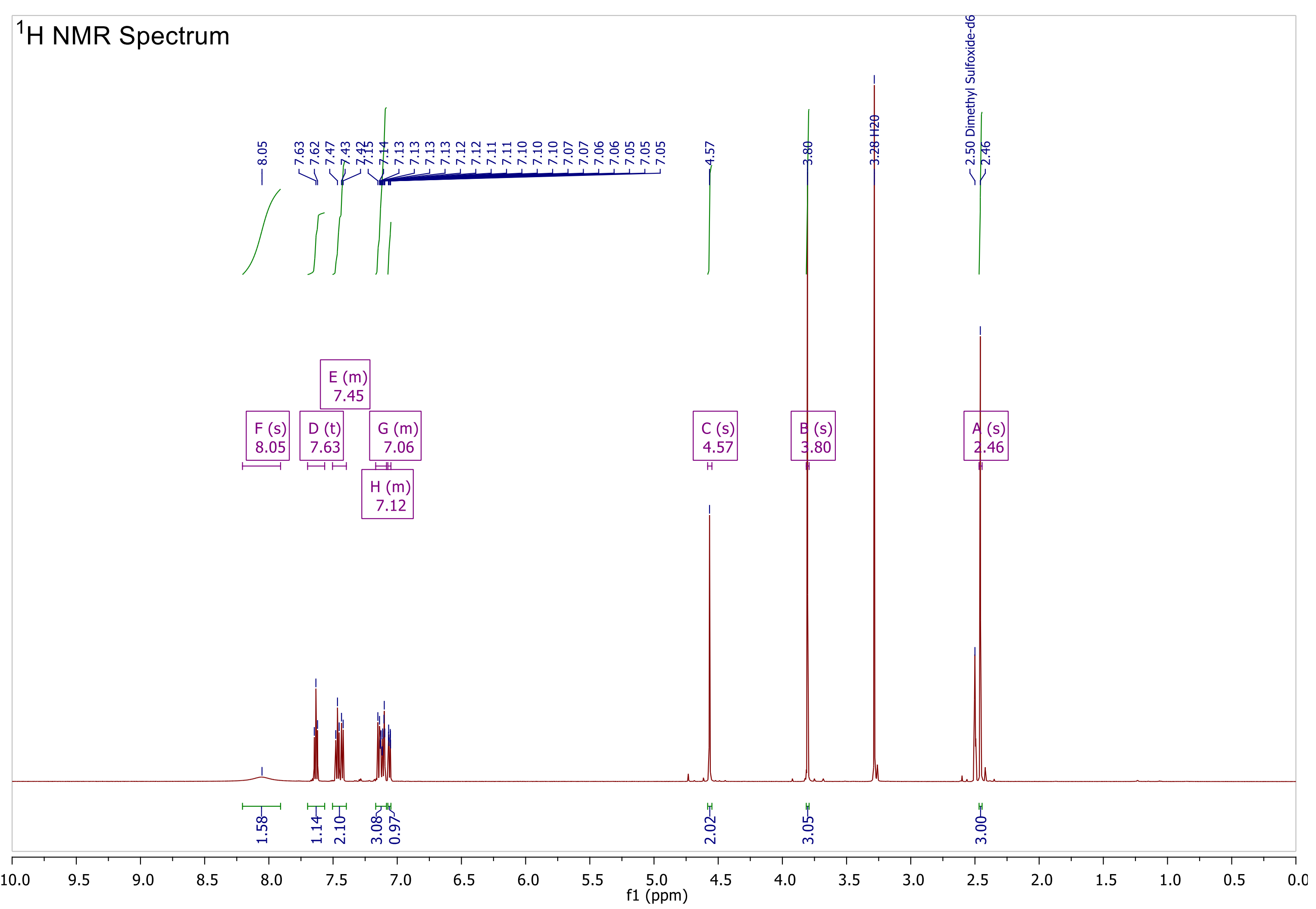
2-amino-4-(4-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6b**)

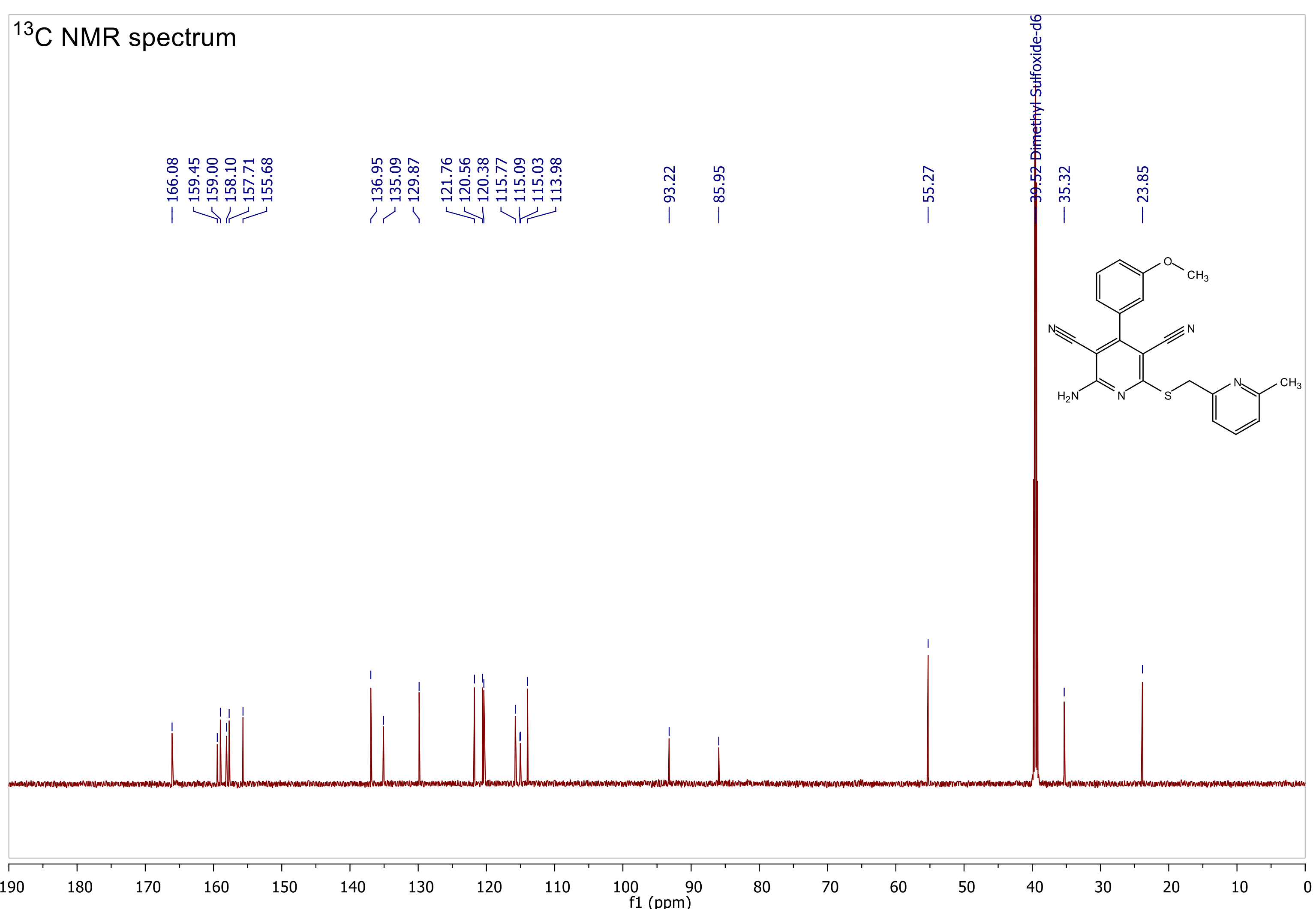




13C NMR Spectrum
166.14
160.77
159.61
158.05
157.71
155.73
136.97
130.11
125.77
121.76
120.56
115.39
114.02
93.23
85.87
55.28
39.52 Dimethyl Sulfoxide-d6
35.34
23.86
CH3
O
N
N
H2N
N
S
N
CH3
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

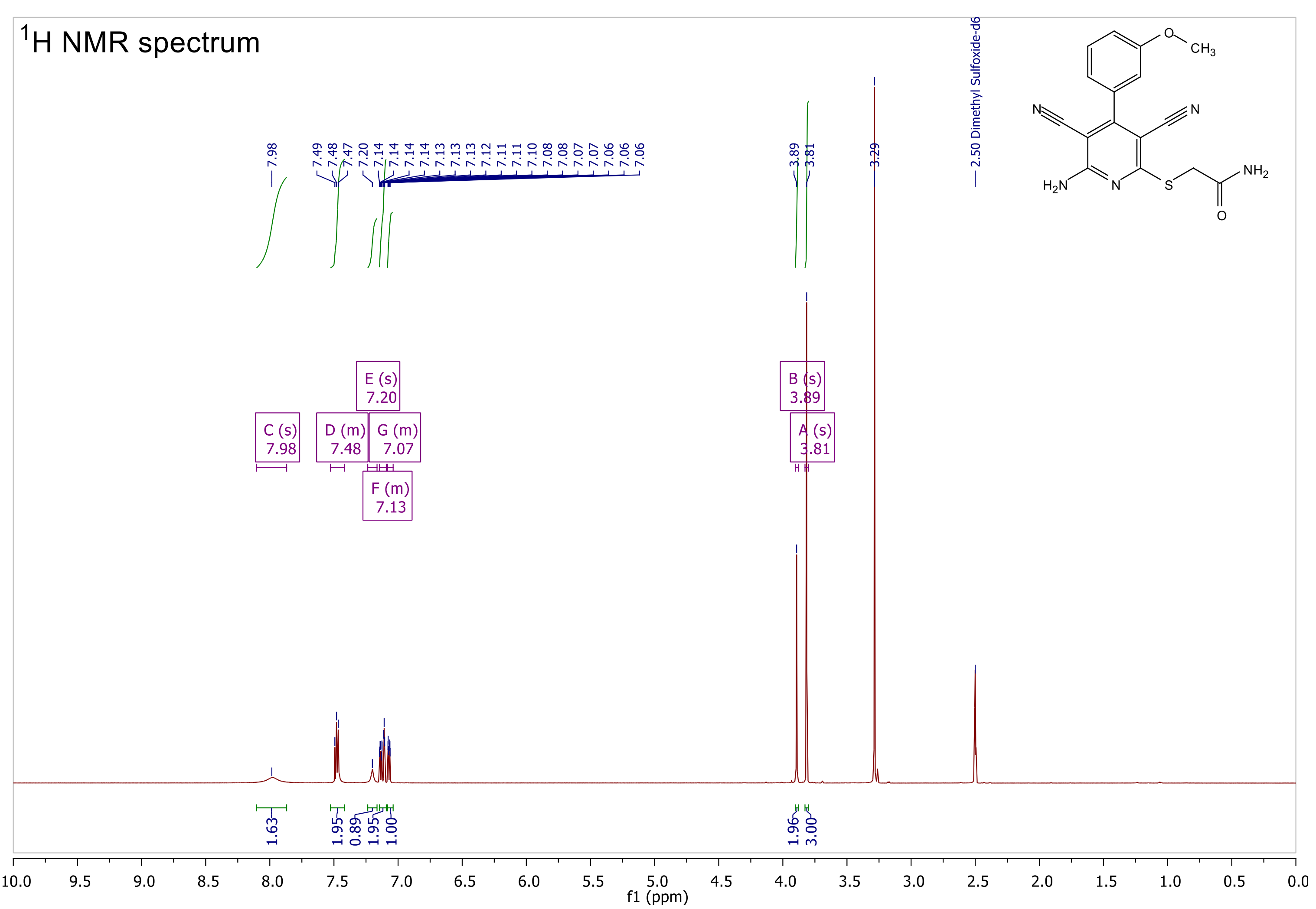
2-amino-4-(3-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6c**)

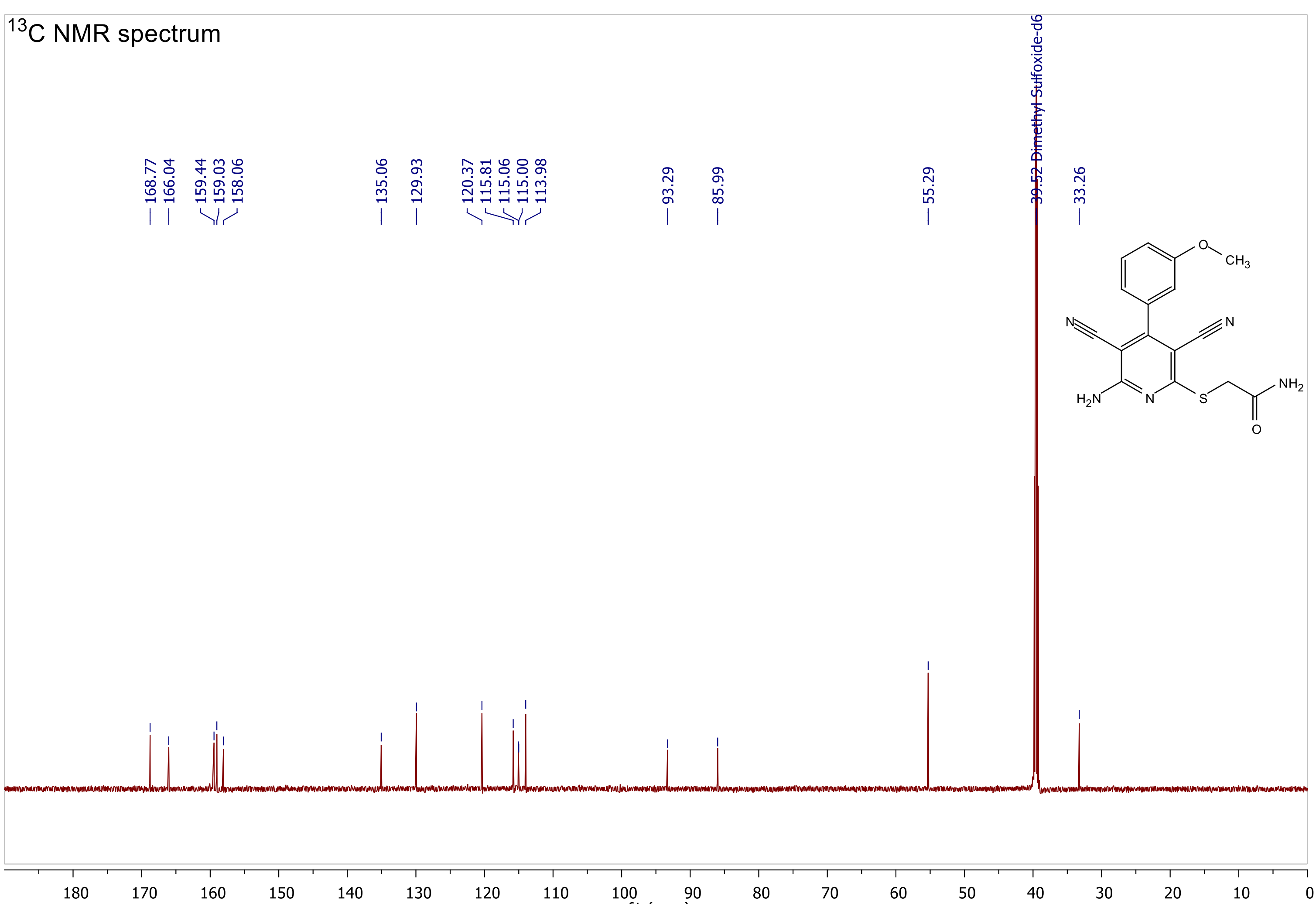




13C NMR spectrum
166.08
159.45
159.00
158.10
157.71
155.68
136.95
135.09
129.87
121.76
120.56
120.38
115.77
115.09
115.03
113.98
93.22
85.95
55.27
39.52 Dimethyl Sulfoxide-d6
35.32
23.85
O
CH3
N
N
H2N
N
S
N
CH3
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

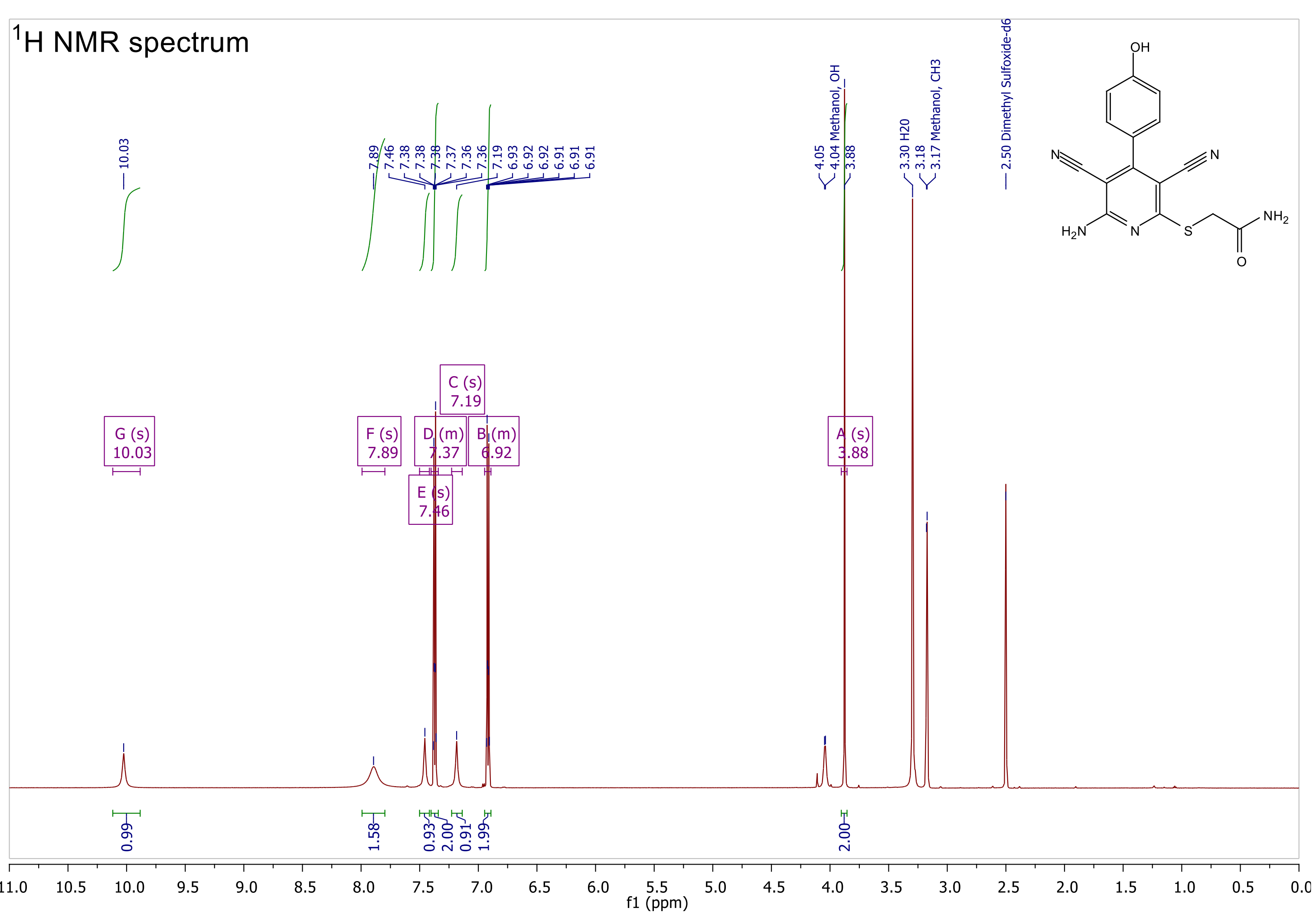
2-((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)acetamide(**6d**)

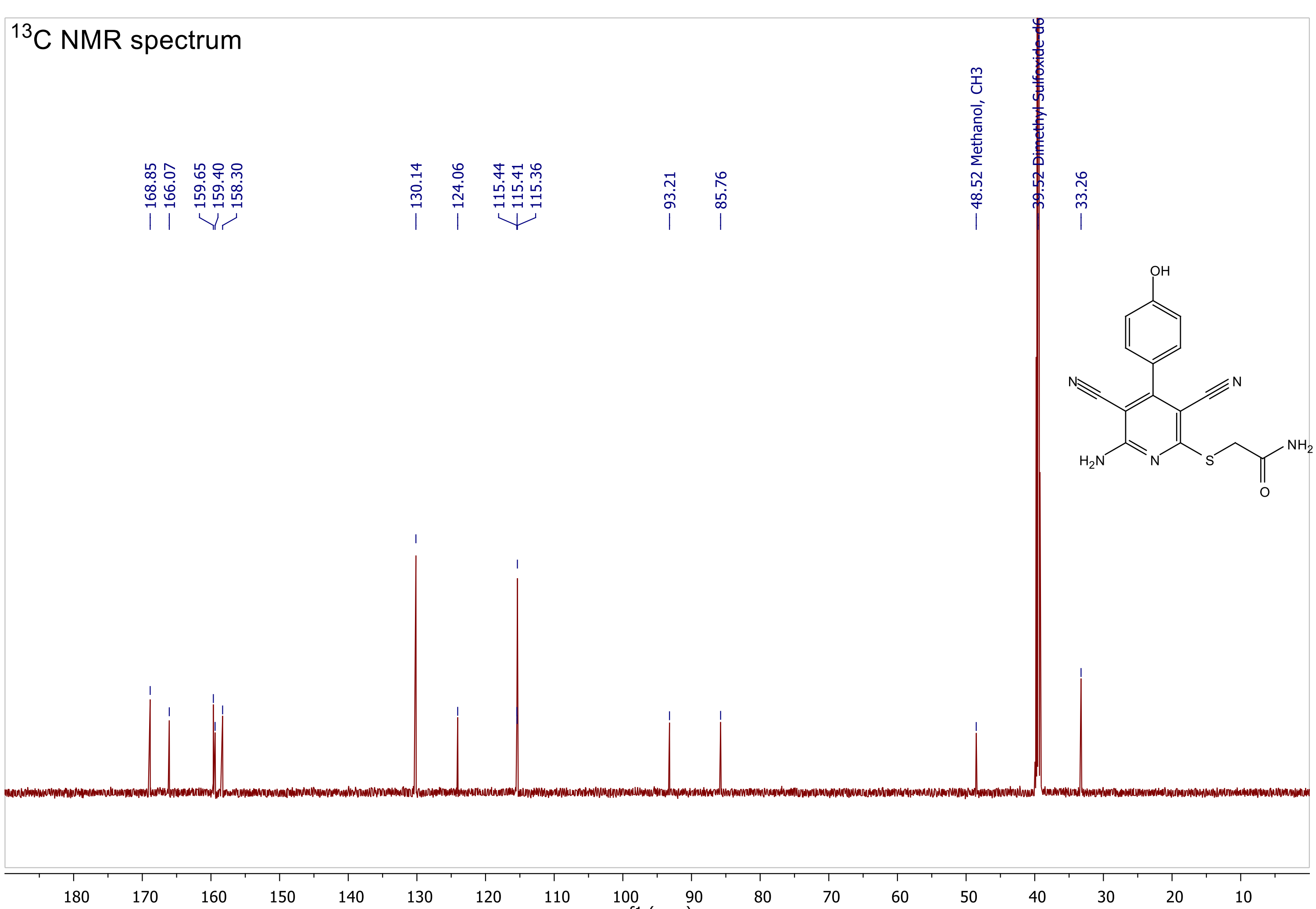




13C NMR spectrum
168.77
166.04
159.44
159.03
158.06
135.06
129.93
120.37
115.81
115.06
115.00
113.98
93.29
85.99
55.29
39.52 Dimethyl Sulfoxide-d6
33.26
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

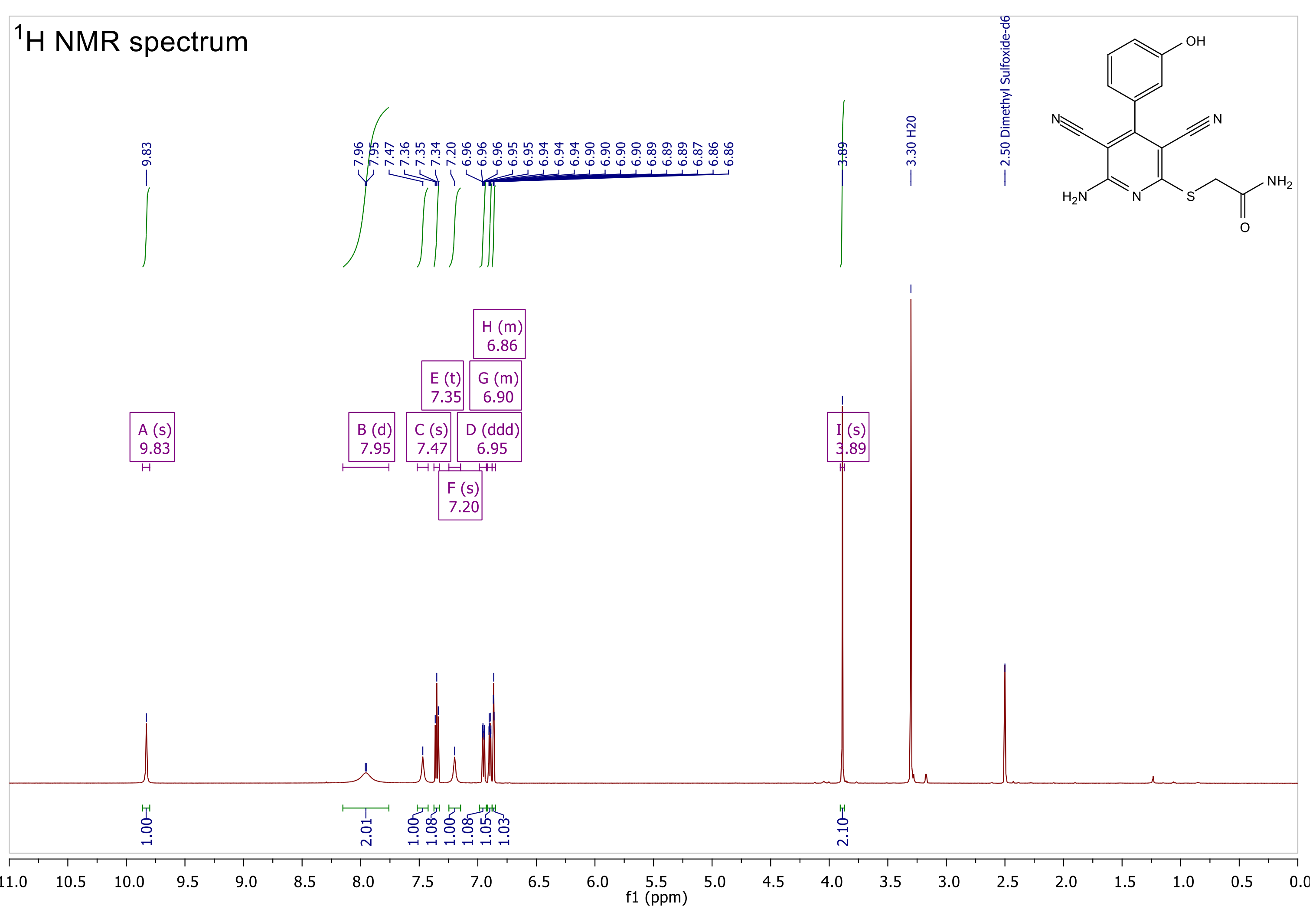
2-((6-amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-yl)thio)acetamide (**6e**)

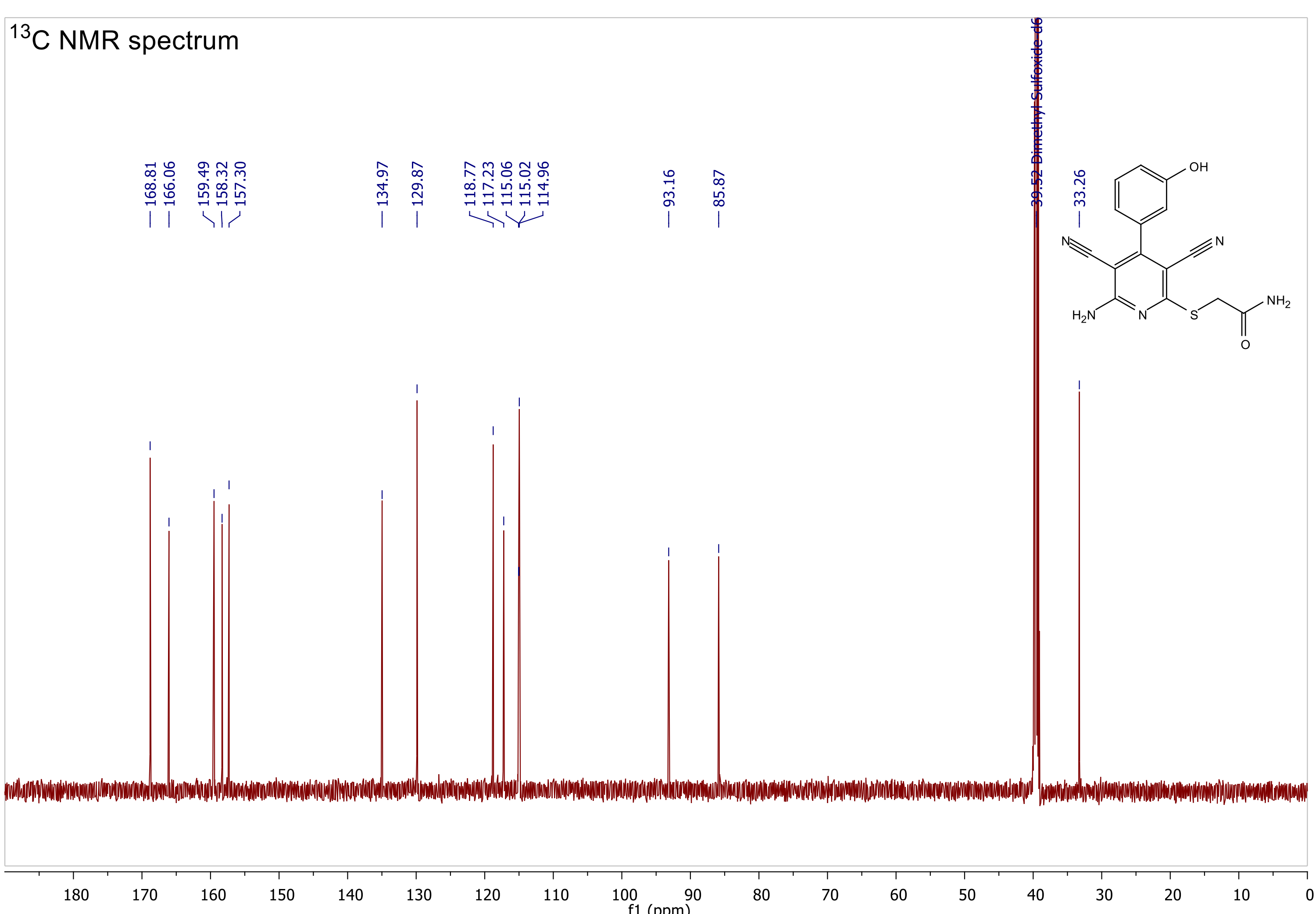




13C NMR spectrum
168.85
166.07
159.65
159.40
158.30
130.14
124.06
115.44
115.41
115.36
93.21
85.76
48.52 Methanol, CH3
39.52 Dimethyl Sulfoxide-d6
33.26
OH
N
N
NH2
H2N
N
S
O
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

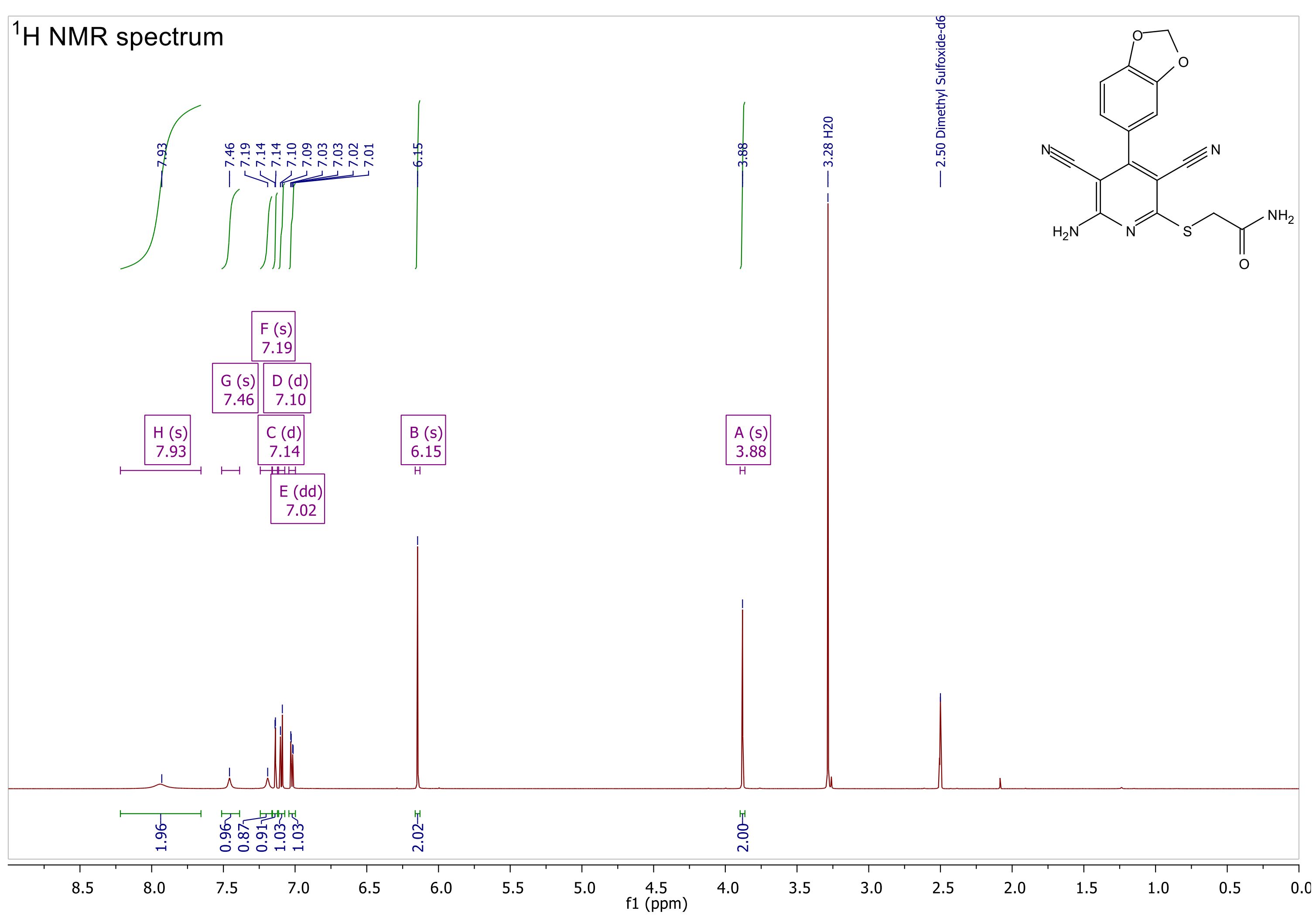
2-((6-amino-3,5-dicyano-4-(3-hydroxyphenyl)pyridin-2-yl)thio)acetamide (**6f**)

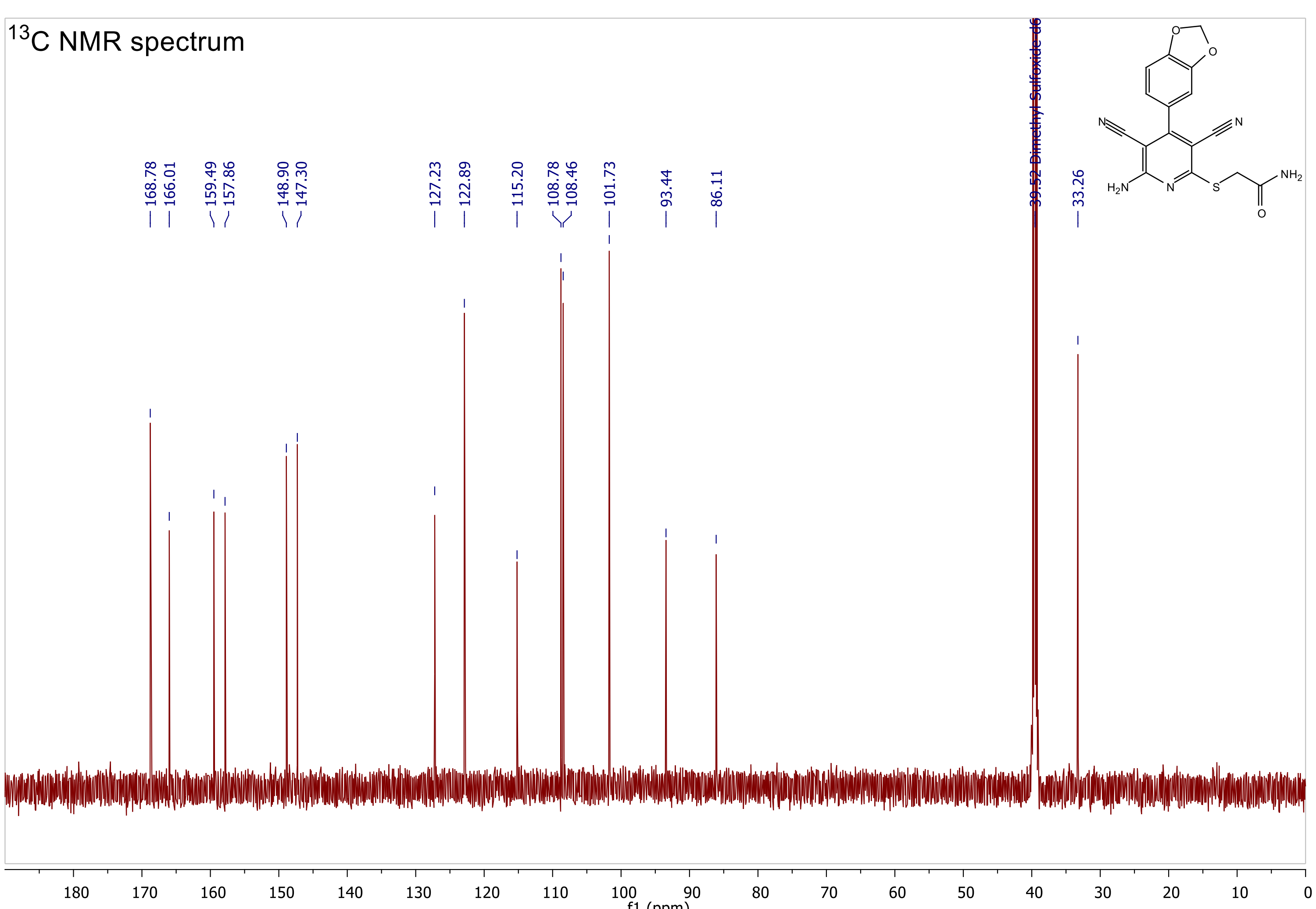




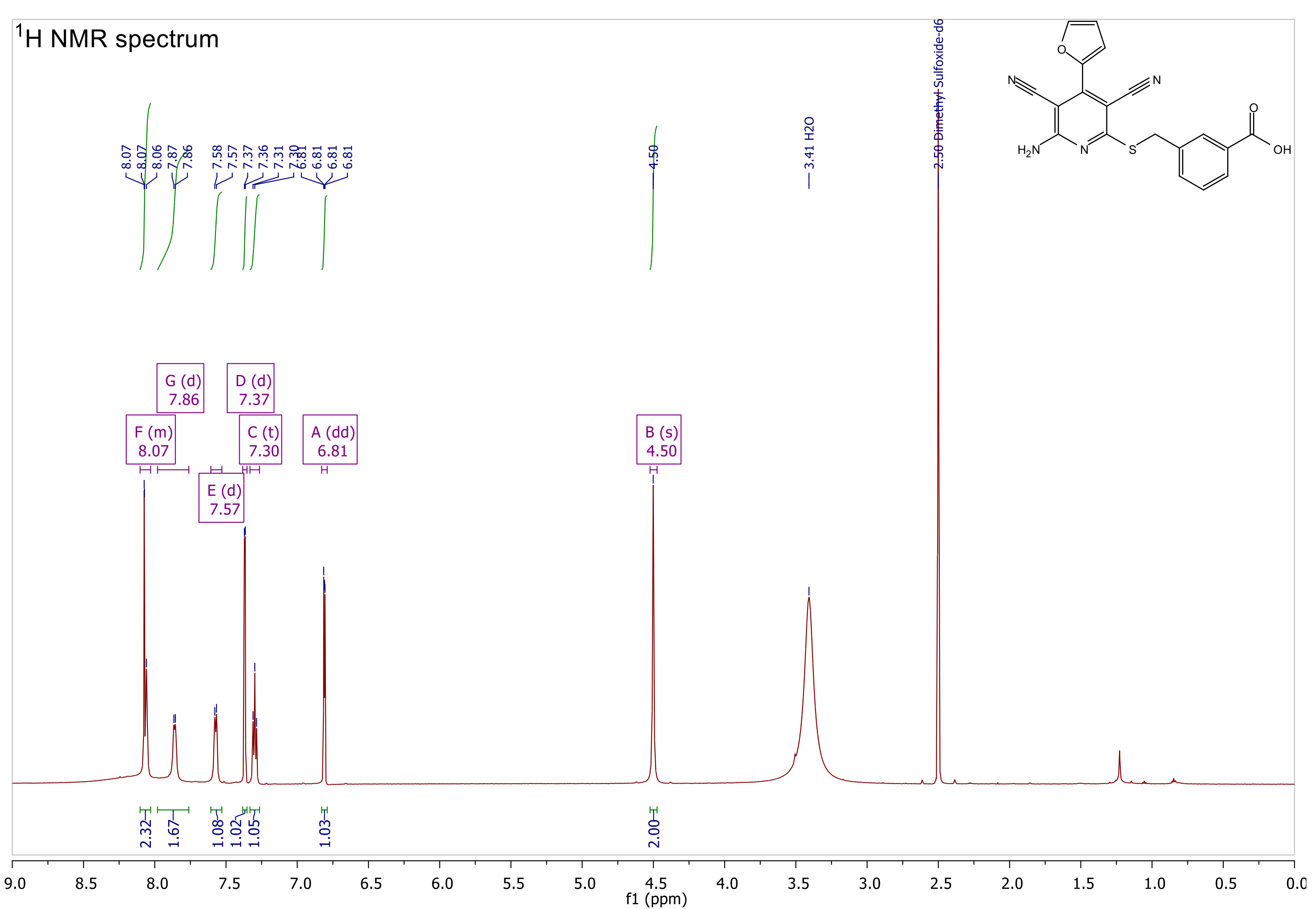
13C NMR spectrum
168.81
166.06
159.49
158.32
157.30
134.97
129.87
118.77
117.23
115.06
115.02
114.96
93.16
85.87
39.52 Dimethyl Sulfoxide-d6
33.26
OH
N
N
H2N
N
S
NH2
O
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

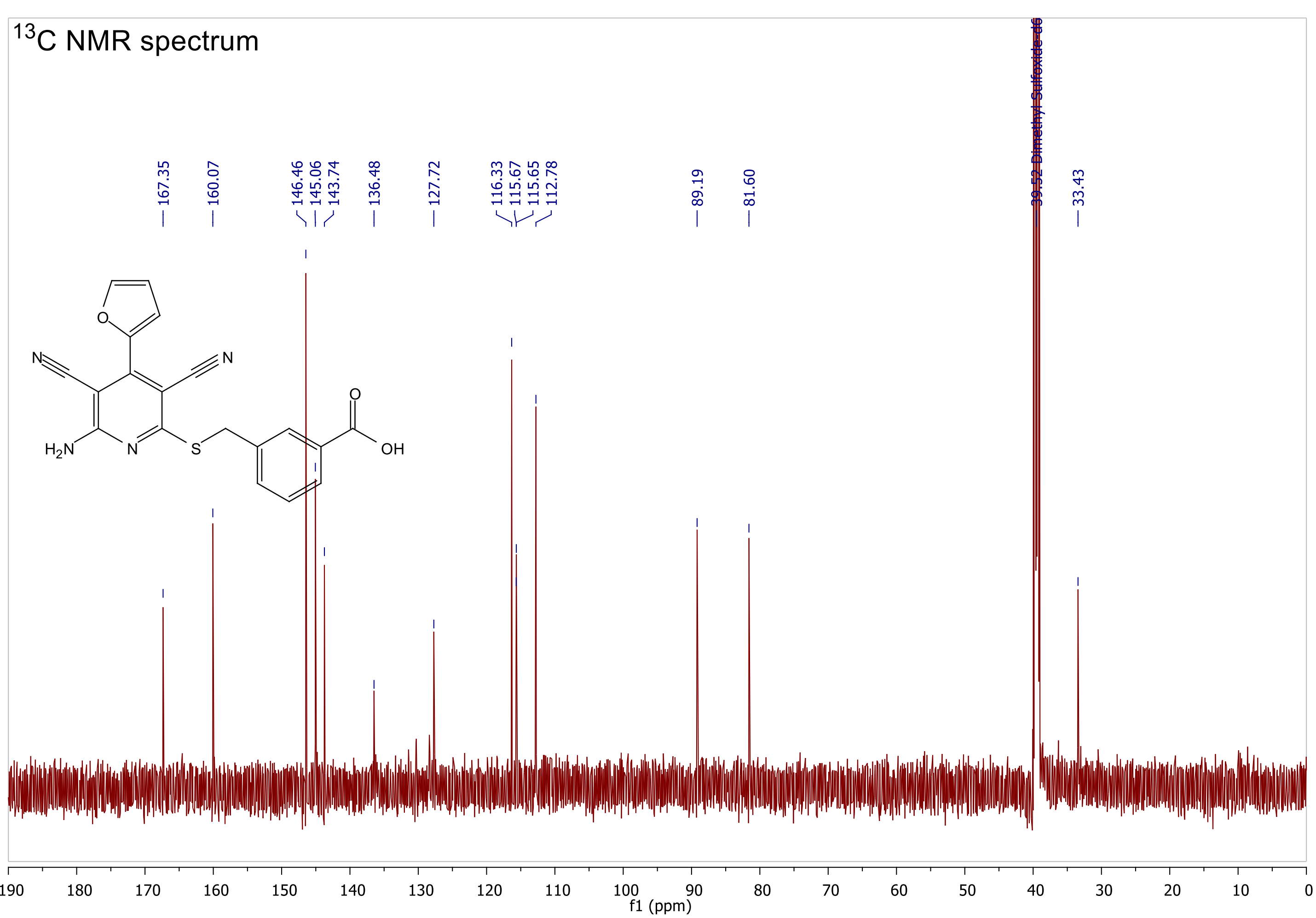
2-((6-amino-4-(benzo[d][1,3]dioxol-5-yl)-3,5-dicyanopyridin-2-yl)thio)acetamide (**6g**)




13C NMR spectrum
168.78
166.01
159.49
157.86
148.90
147.30
127.23
122.89
115.20
108.78
108.46
101.73
93.44
86.11
39.52 Dimethyl Sulfoxide-d6
33.26
O
O
N
N
H2N
N
S
NH2
O
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

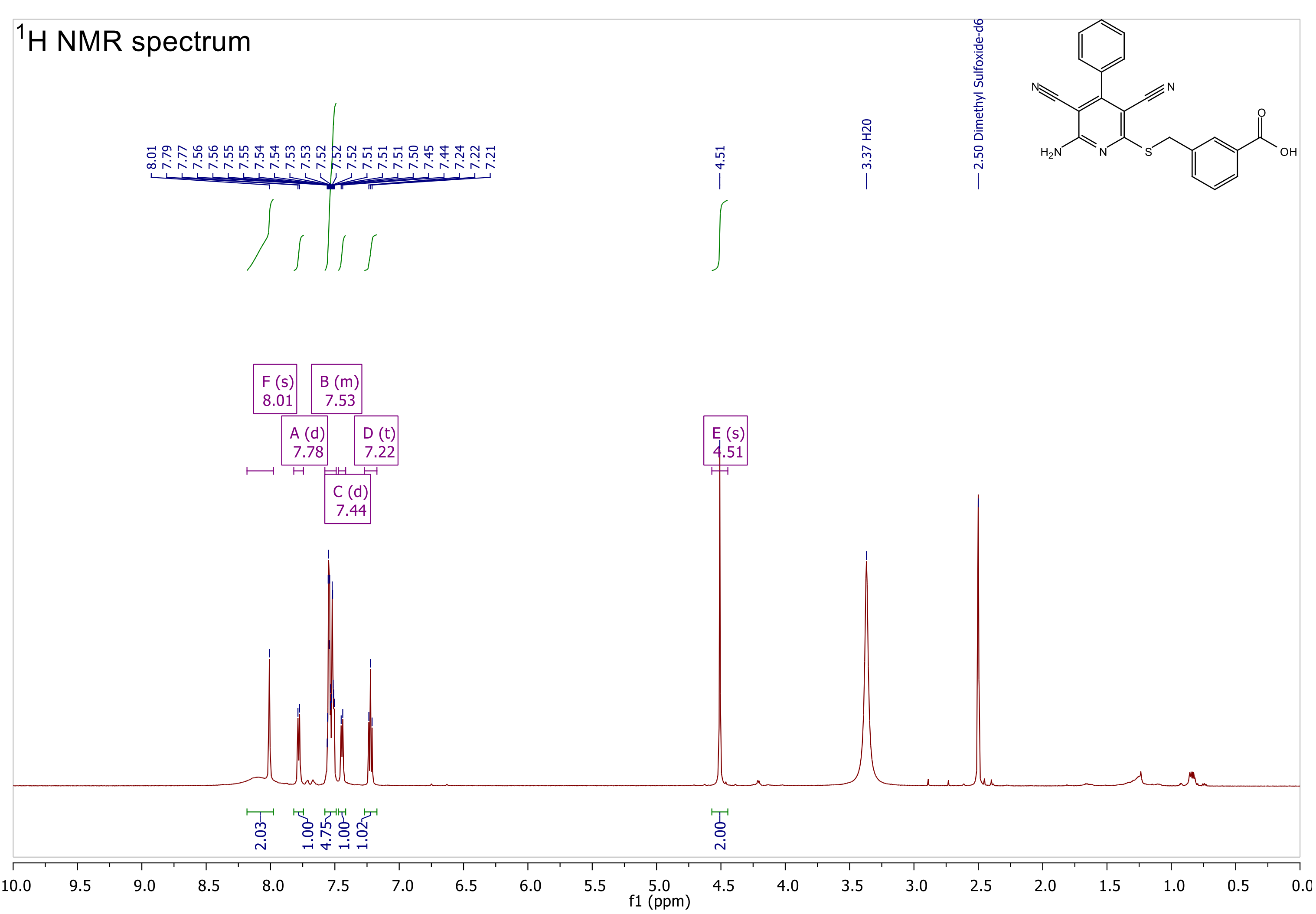
3-(((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzoic acid (**6h**)

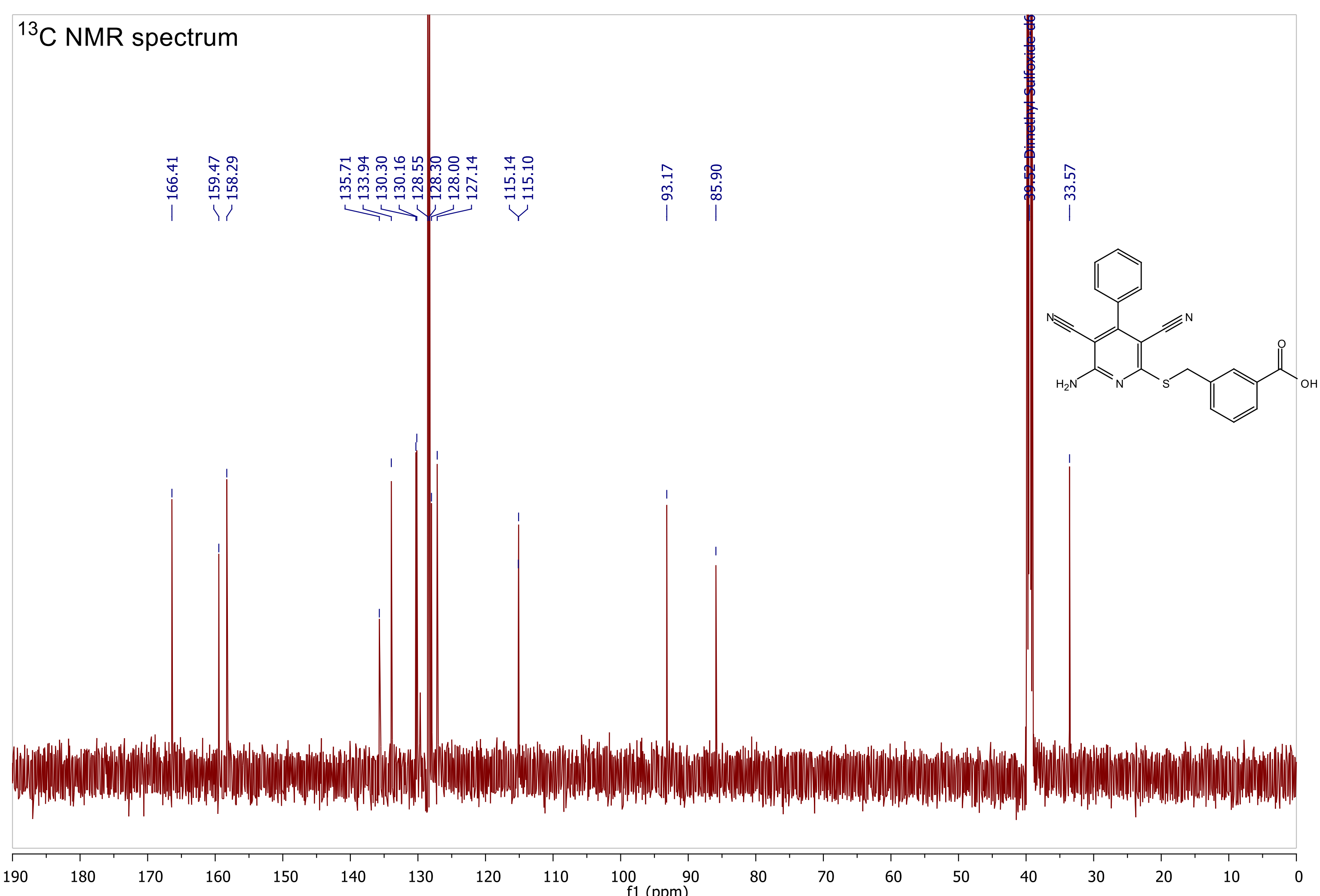




13C NMR spectrum
167.35
160.07
146.46
145.06
143.74
136.48
127.72
116.33
115.67
115.65
112.78
89.19
81.60
39.52 Dimethyl Sulfoxide-d6
33.43
O
N
N
O
OH
H2N
N
S
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

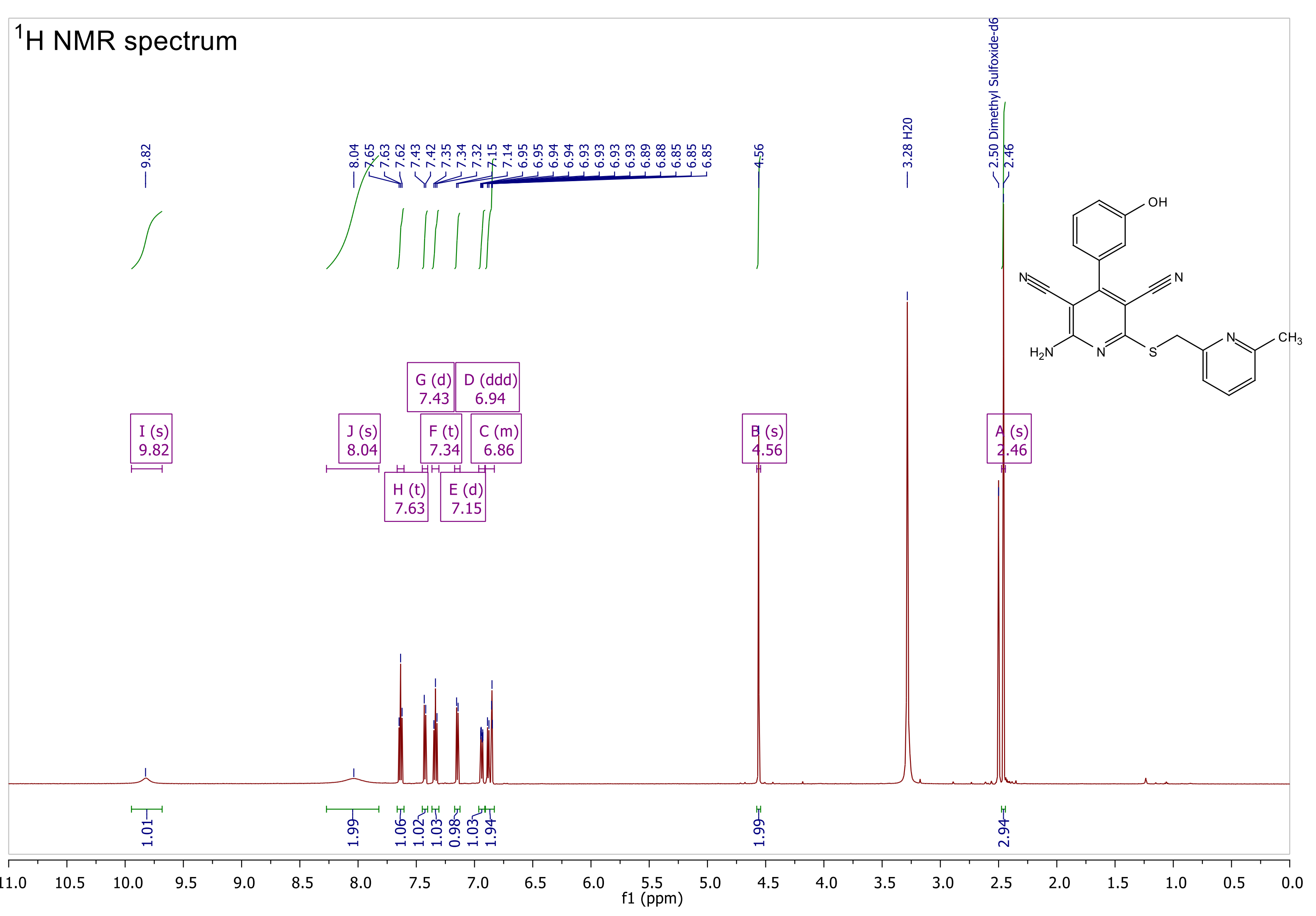
3-(((6-amino-3,5-dicyano-4-phenylpyridin-2-yl)thio)methyl)benzoic acid (**6i**)

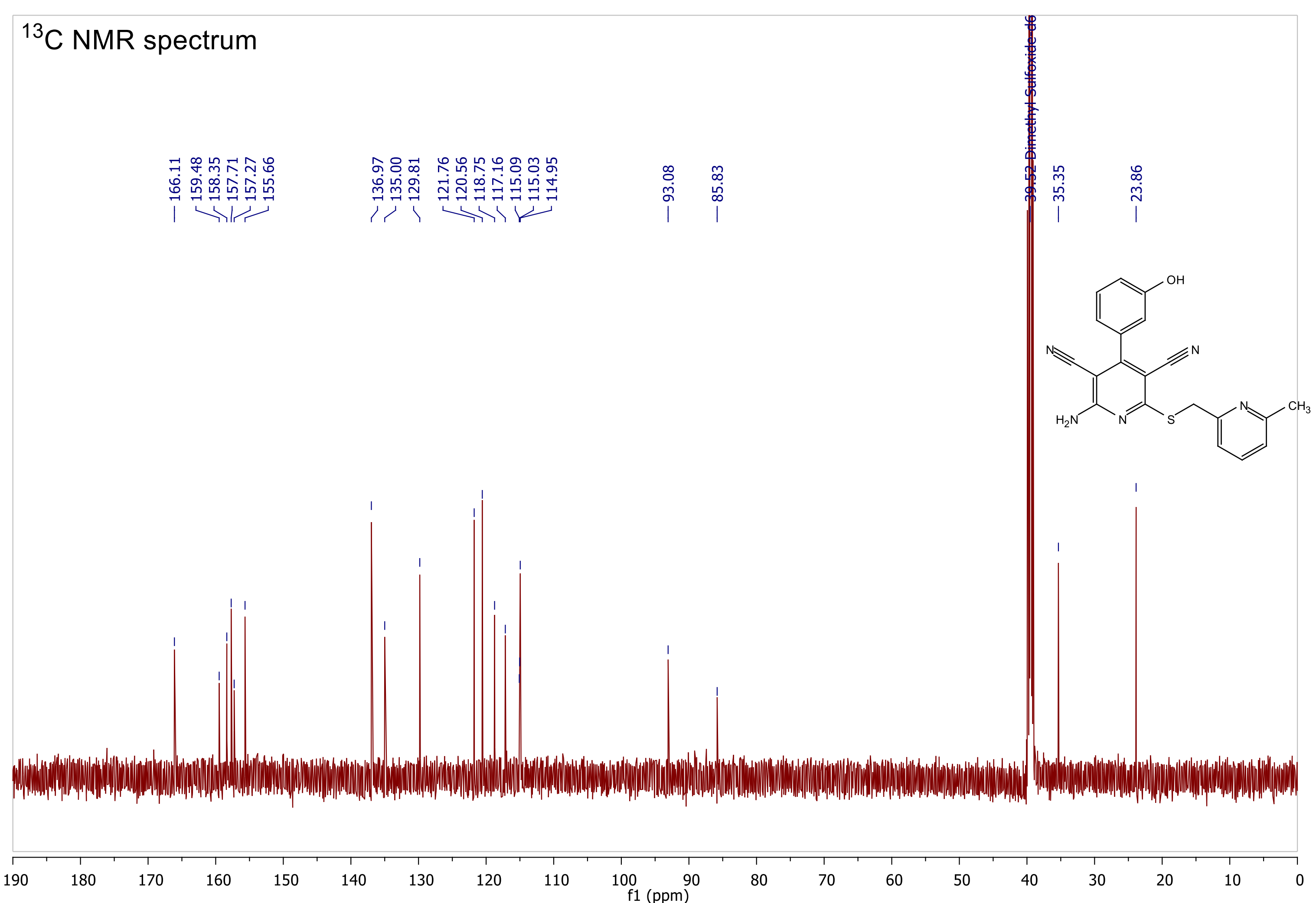




^{13}C NMR spectrum
166.41
159.47
158.29
135.71
133.94
130.30
130.16
128.55
128.30
128.00
127.14
115.14
115.10
93.17
85.90
39.52 Dimethyl Sulfoxide-d6
33.57
N
N
H2N
N
S
O
OH
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

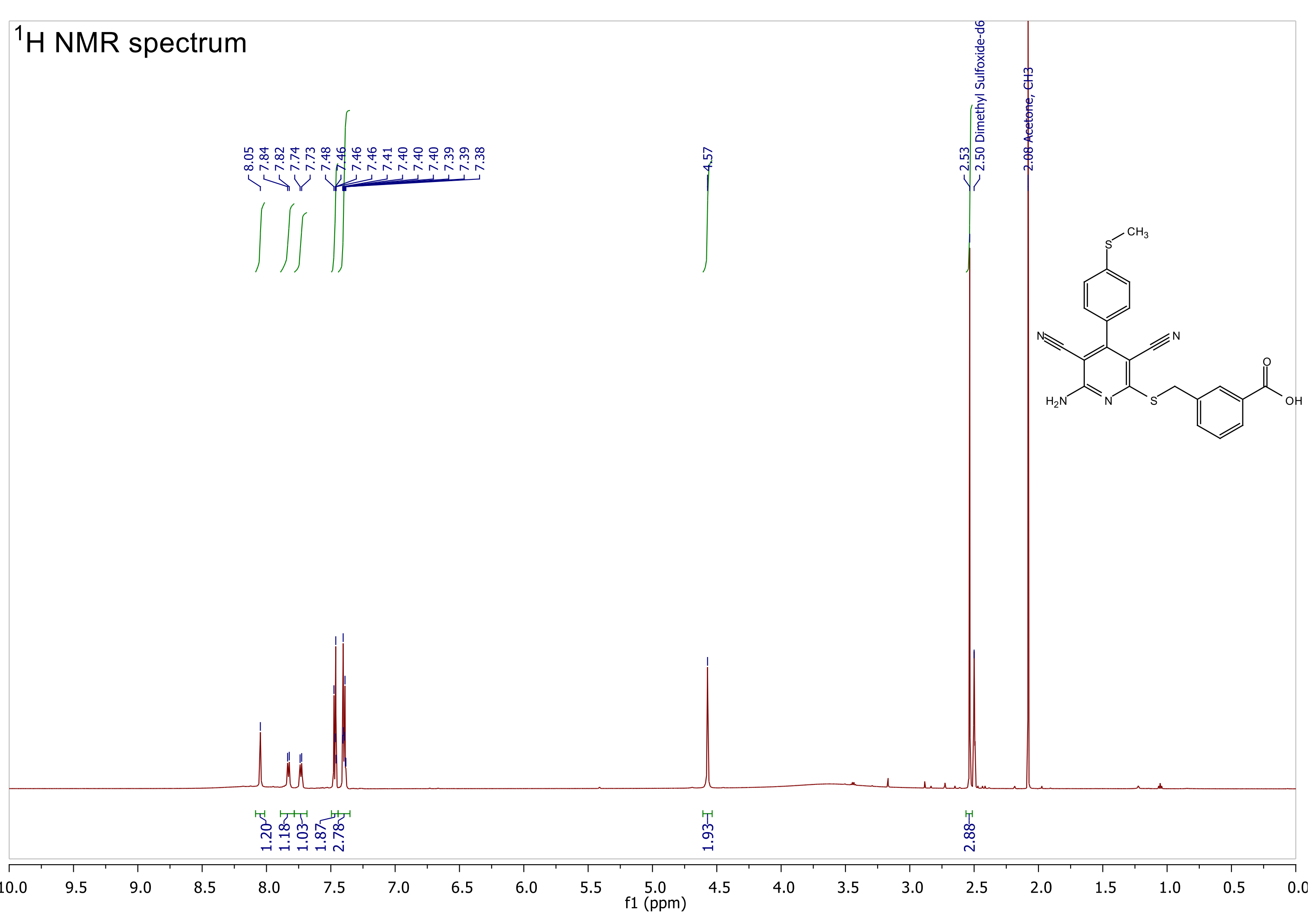
2-amino-4-(3-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6j**)

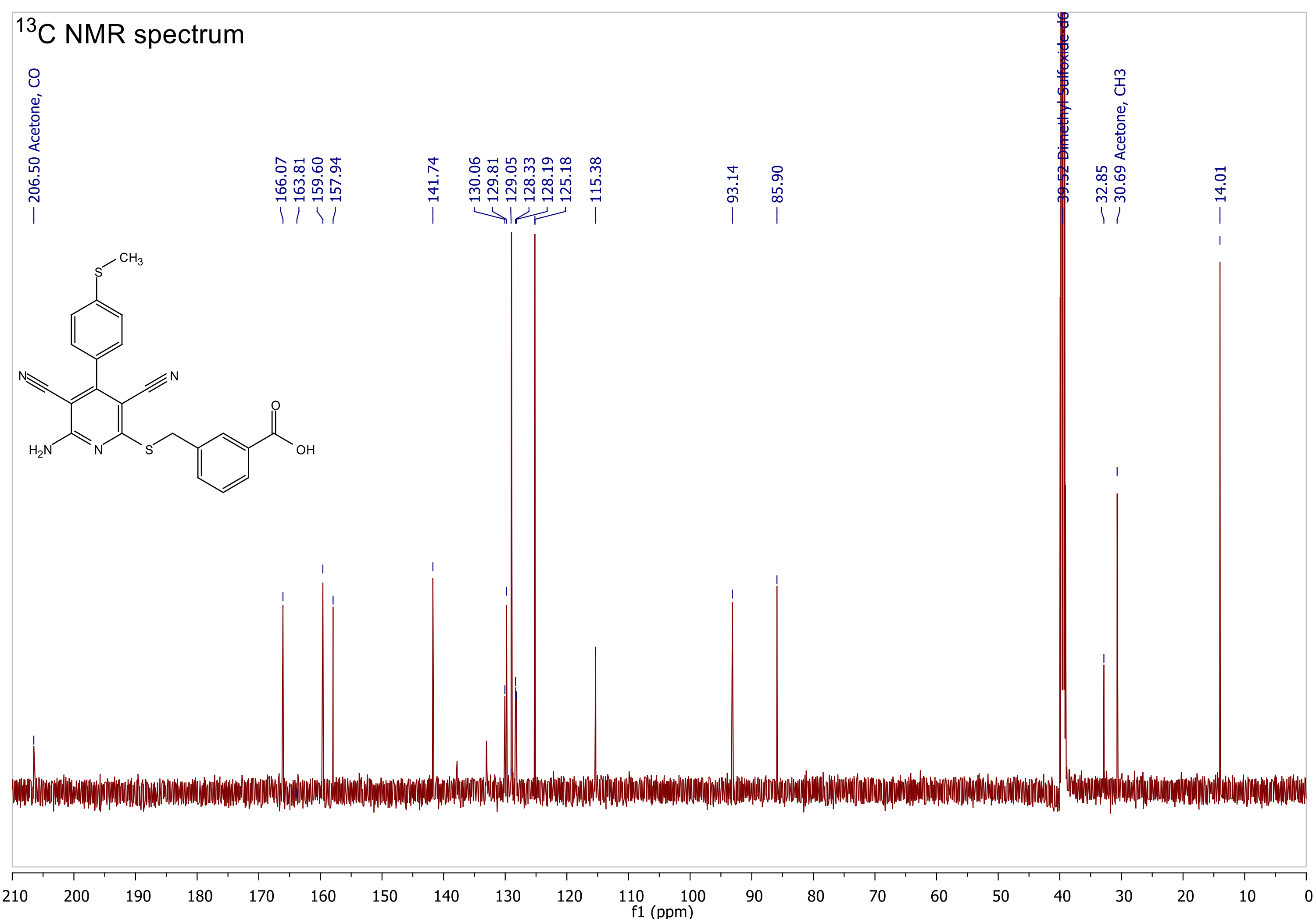




13C NMR spectrum
166.11
159.48
158.35
157.71
157.27
155.66
136.97
135.00
129.81
121.76
120.56
118.75
117.16
115.09
115.03
114.95
93.08
85.83
39.52 Dimethyl Sulfoxide-d6
35.35
23.86
OH
N
N
H2N
N
S
N
CH3
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

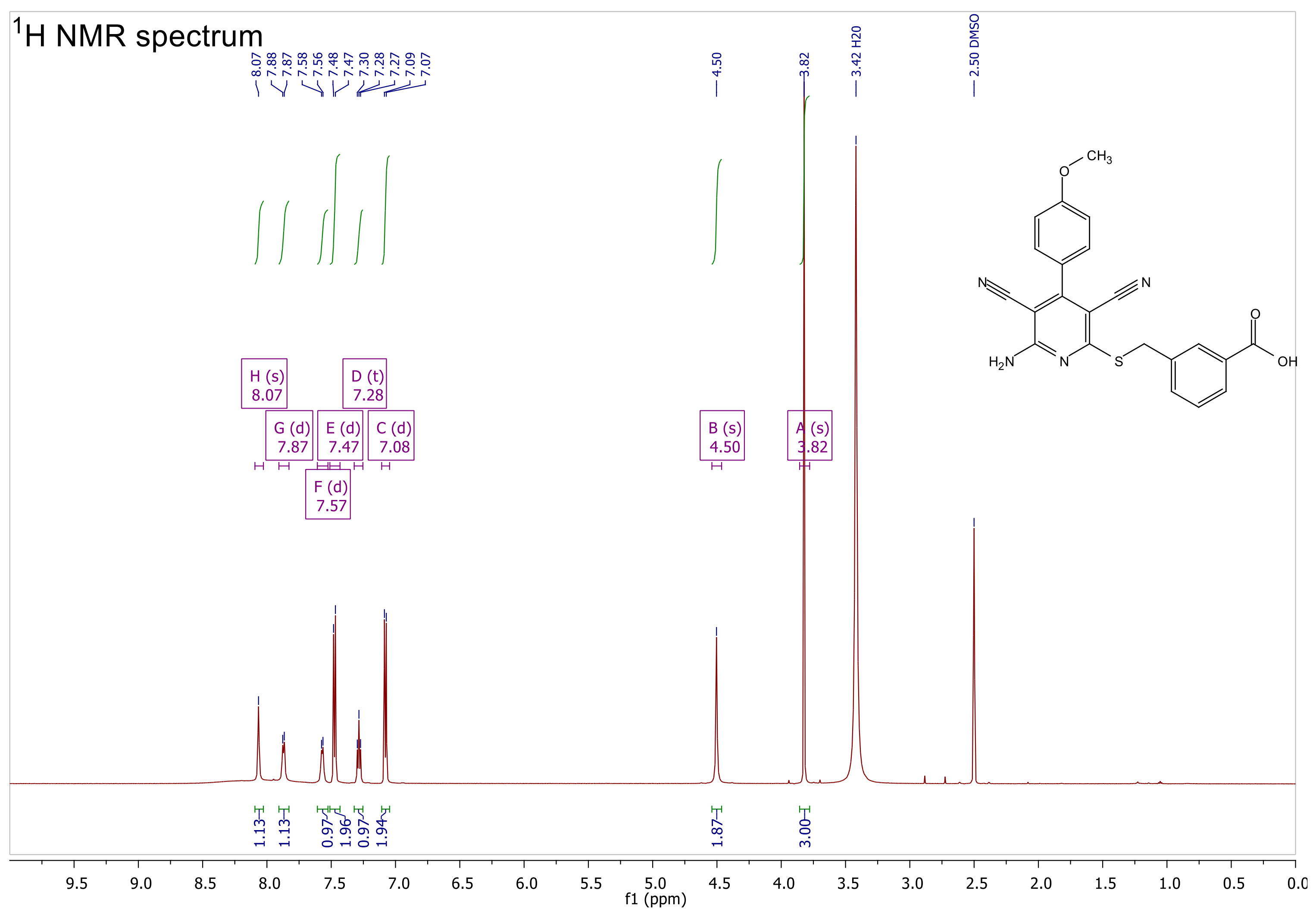
3-(((6-amino-3,5-dicyano-4-(4-(methylthio)phenyl)pyridin-2-yl)thio)methyl)benzoic acid (**6k**)





13C NMR spectrum
206.50 Acetone, CO
166.07
163.81
159.60
157.94
141.74
130.06
129.81
129.05
128.33
128.19
125.18
115.38
93.14
85.90
39.52 Dimethyl Sulfoxide-d6
32.85
30.69 Acetone, CH3
14.01
CH3
S
N
N
O
OH
H2N
N
S
f1 (ppm)

3-(((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (**6l**)



^{13}C NMR spectrum

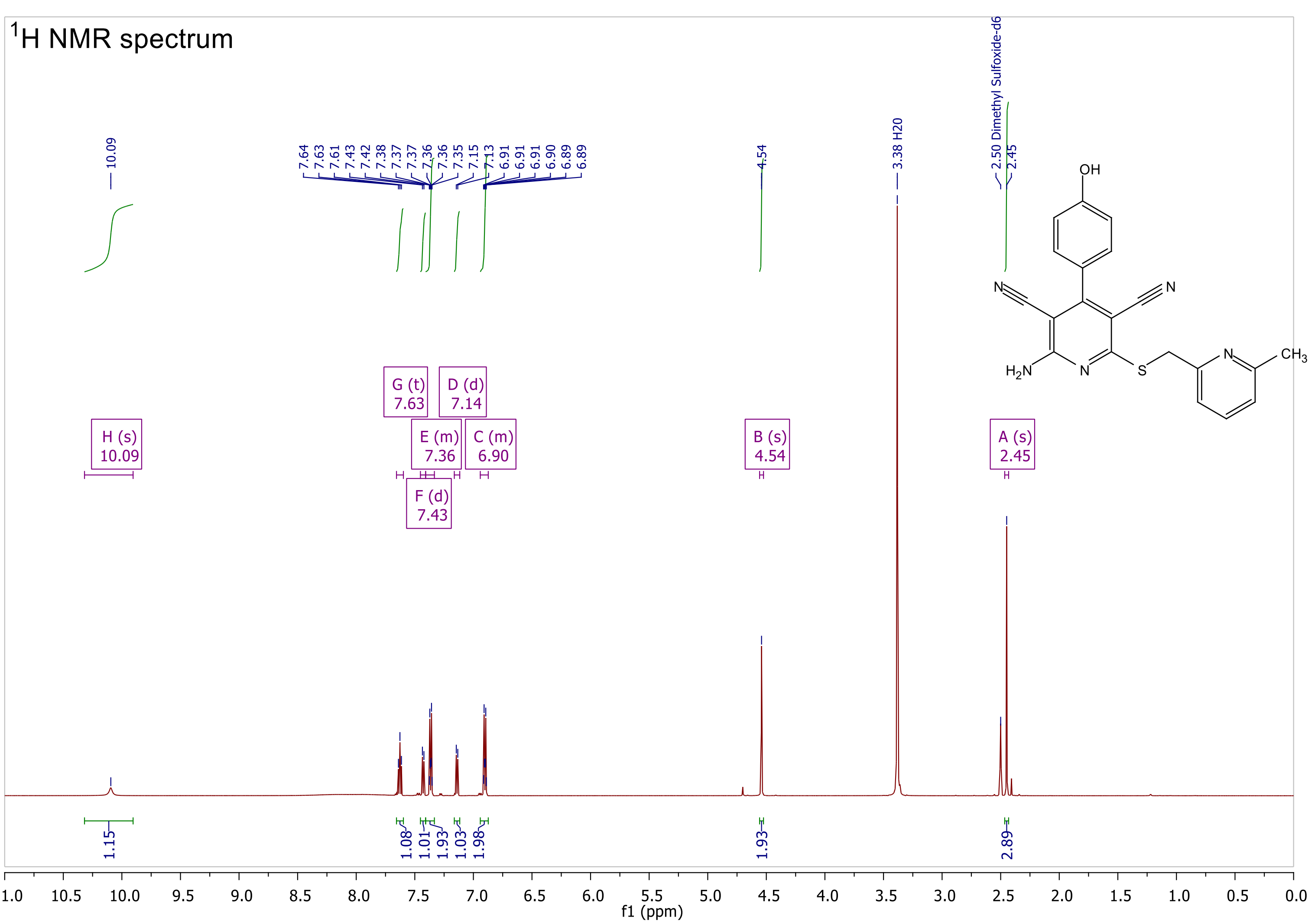
166.28, 160.81, 159.67, 158.11, 136.57, 130.37, 130.26, 128.39, 127.65, 125.84, 115.57, 115.54, 114.07, 93.21, 85.89, 55.37, 39.52 DMSO, 33.33

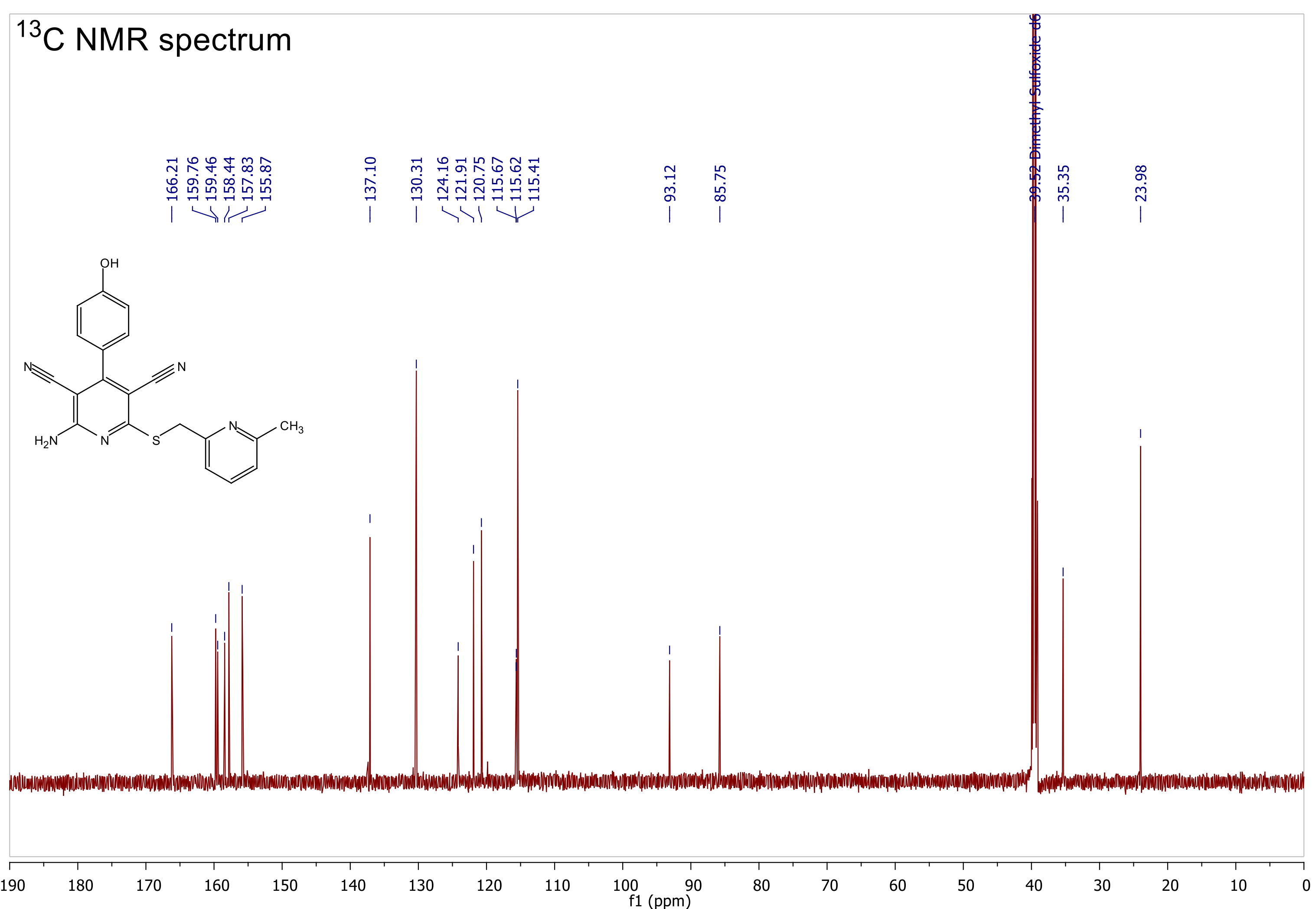
H_2N, N, N, N, S, O, CH_3, O, OH

190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0

f1 (ppm)

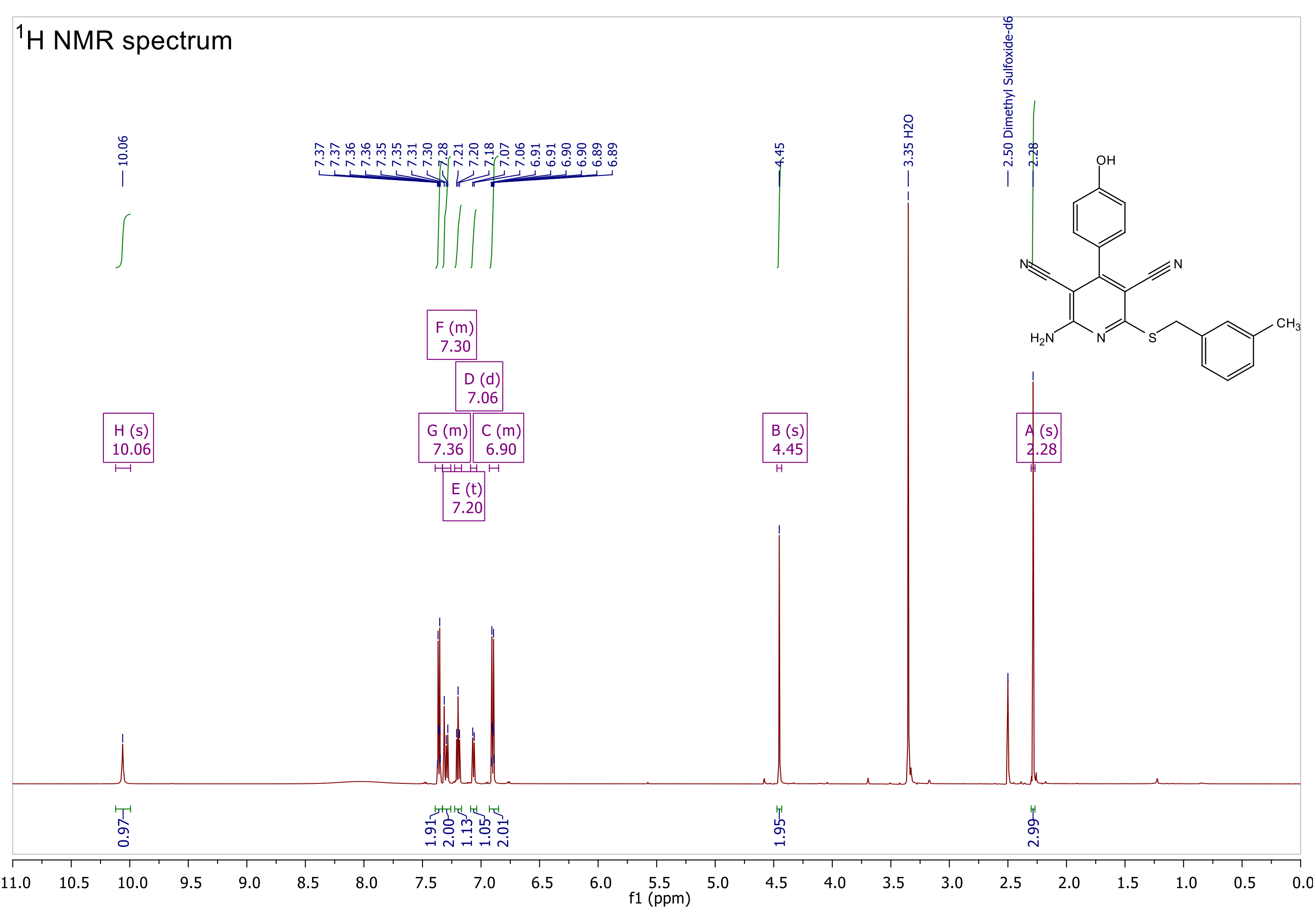
2-amino-4-(4-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6m**)

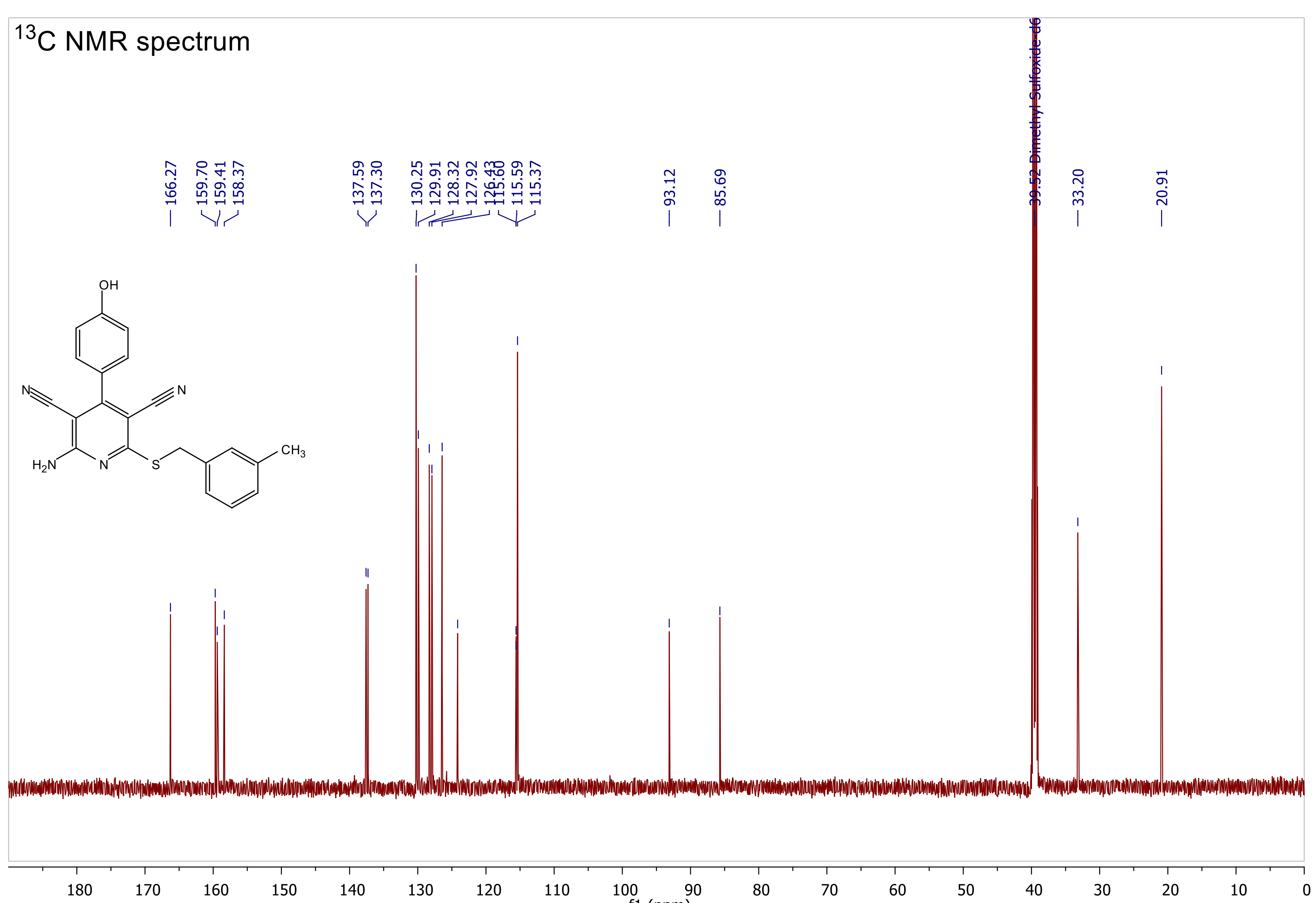




^{13}C NMR spectrum
166.21
159.76
159.46
158.44
157.83
155.87
137.10
130.31
124.16
121.91
120.75
115.67
115.62
115.41
93.12
85.75
39.52 Dimethyl Sulfoxide-d6
35.35
23.98
OH
N
N
H_2N
N
S
N
CH_3
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

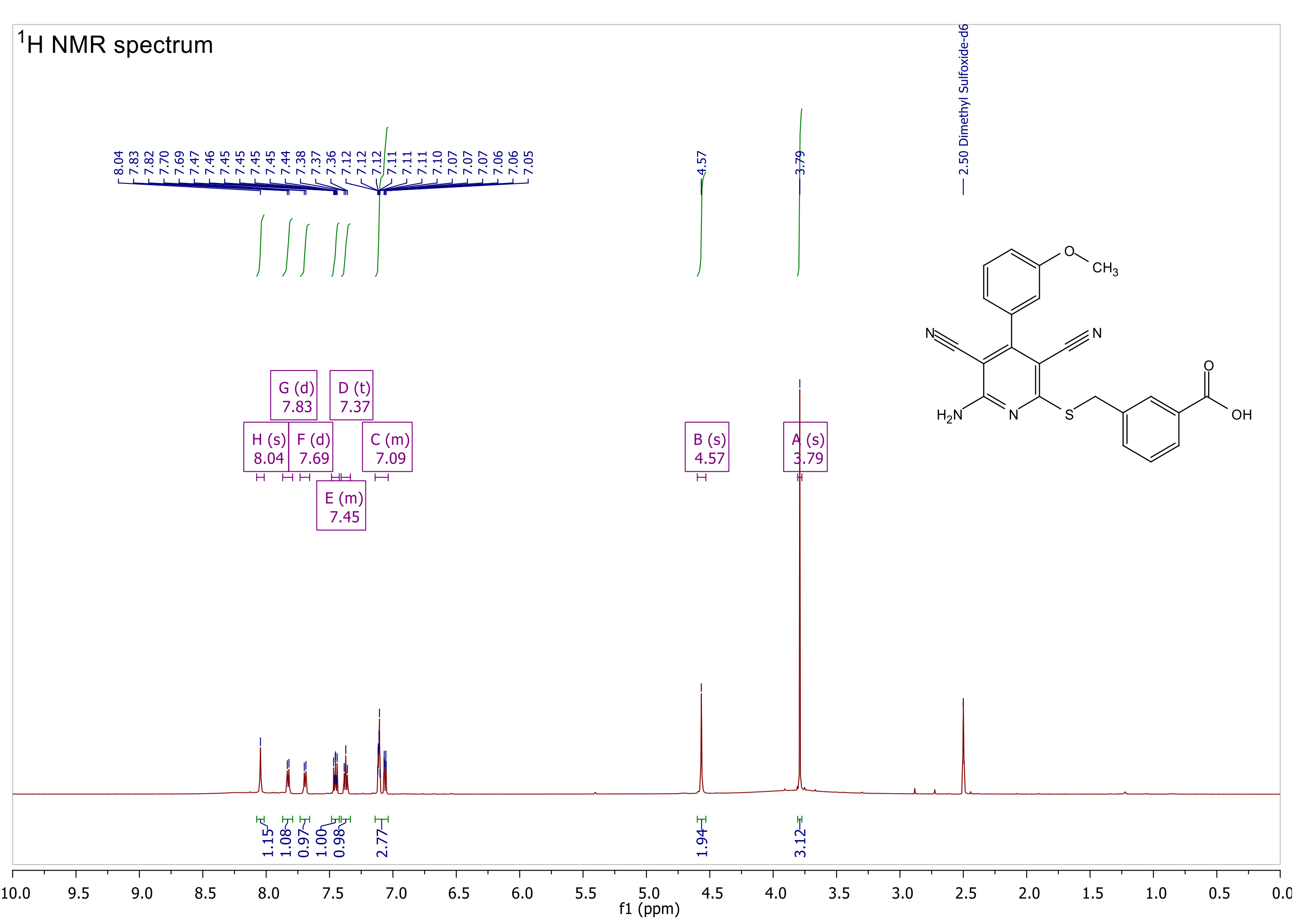
2-amino-4-(4-hydroxyphenyl)-6-((3-methylbenzyl)thio)pyridine-3,5-dicarbonitrile (**6n**)

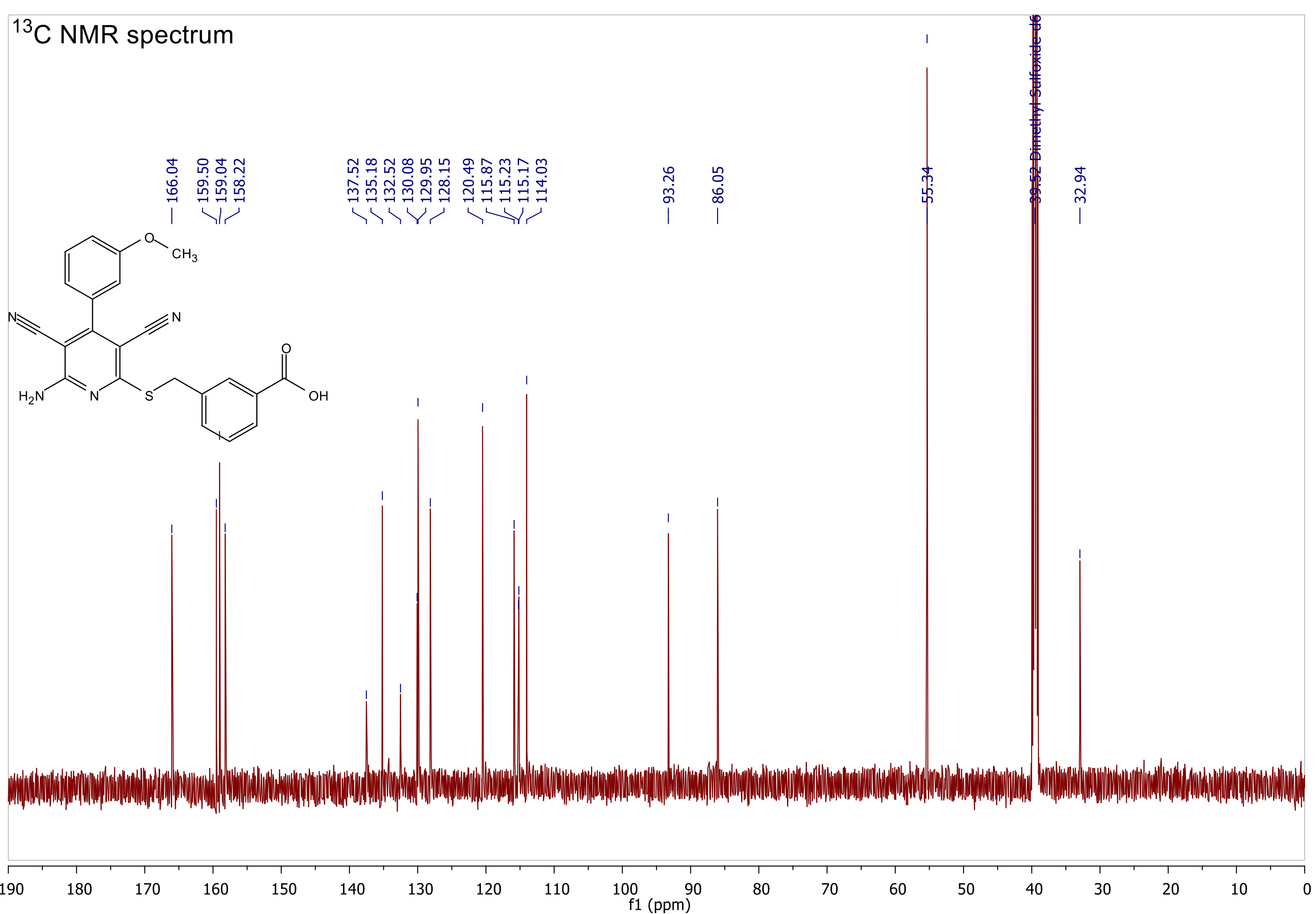




^{13}C NMR spectrum
166.27
159.70
159.41
158.37
137.59
137.30
130.25
129.91
128.32
127.92
126.43
115.60
115.59
115.37
93.12
85.69
39.52 Dimethyl Sulfoxide-d6
33.20
20.91
OH
N
N
H_2N
N
S
CH_3
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

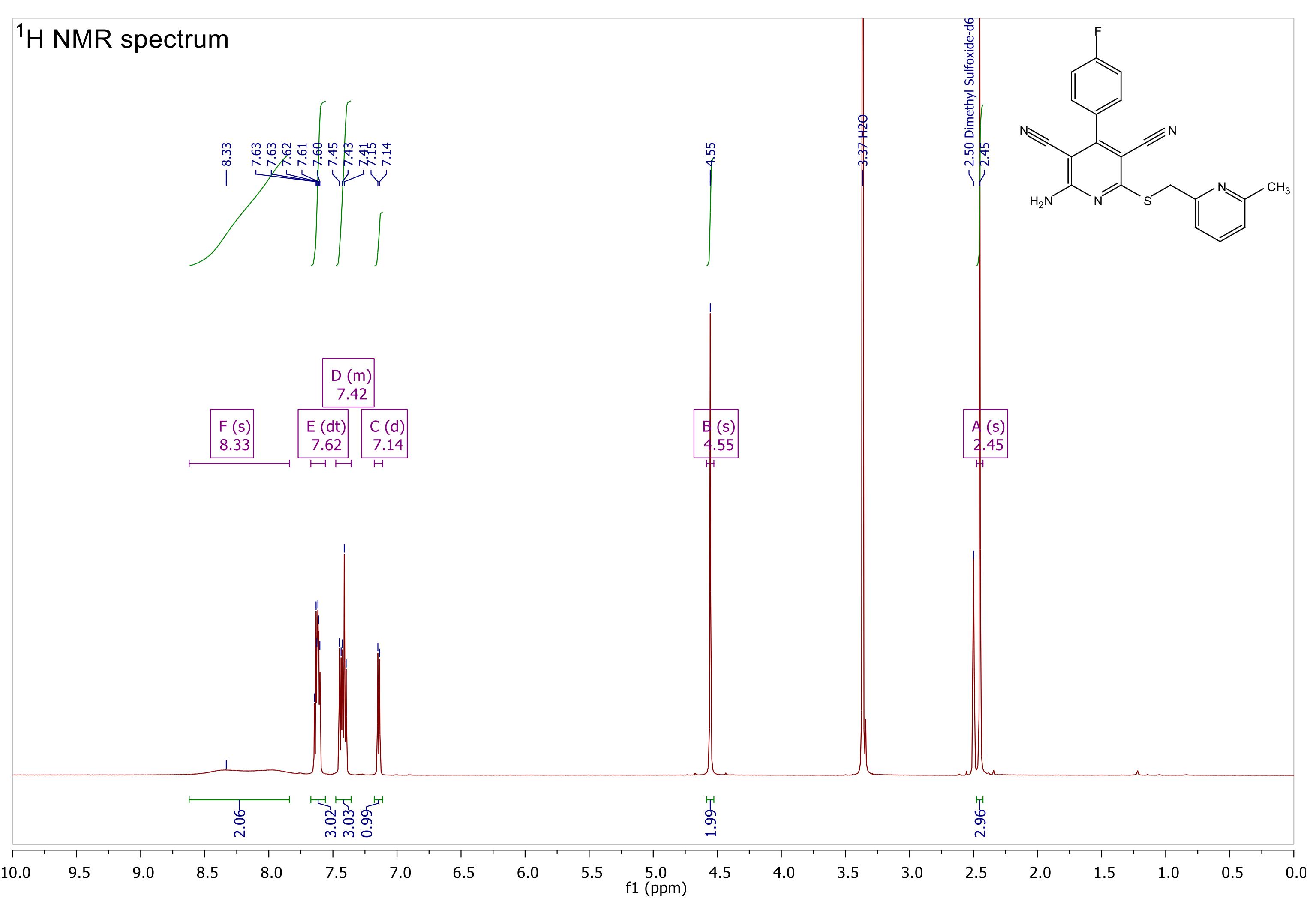
3-(((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (**6o**)

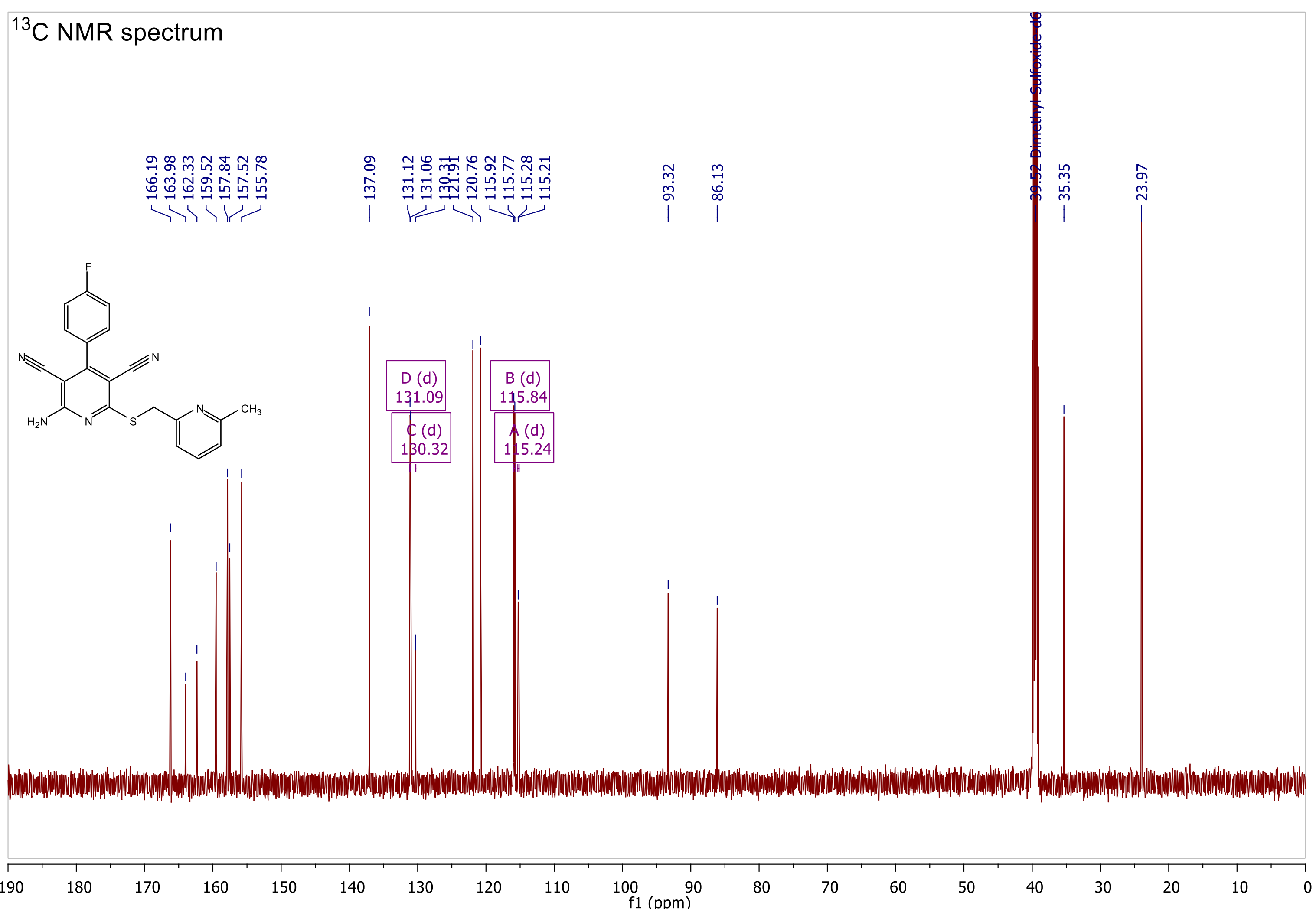




13C NMR spectrum
166.04
159.50
159.04
158.22
137.52
135.18
132.52
130.08
129.95
128.15
120.49
115.87
115.23
115.17
114.03
93.26
86.05
55.34
39.52 Dimethyl Sulfoxide-d6
32.94
f1 (ppm)

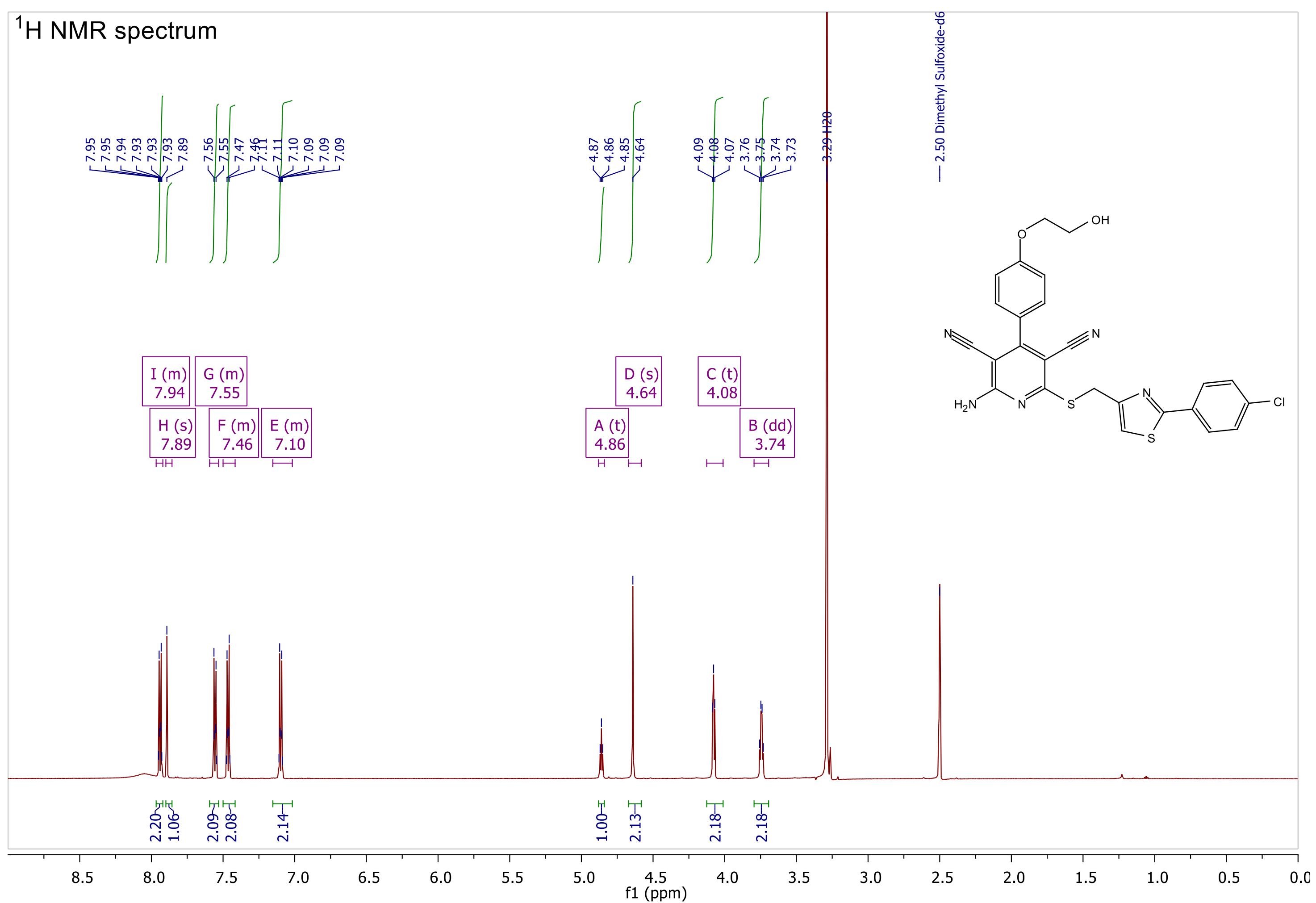
2-amino-4-(4-fluorophenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6p**)

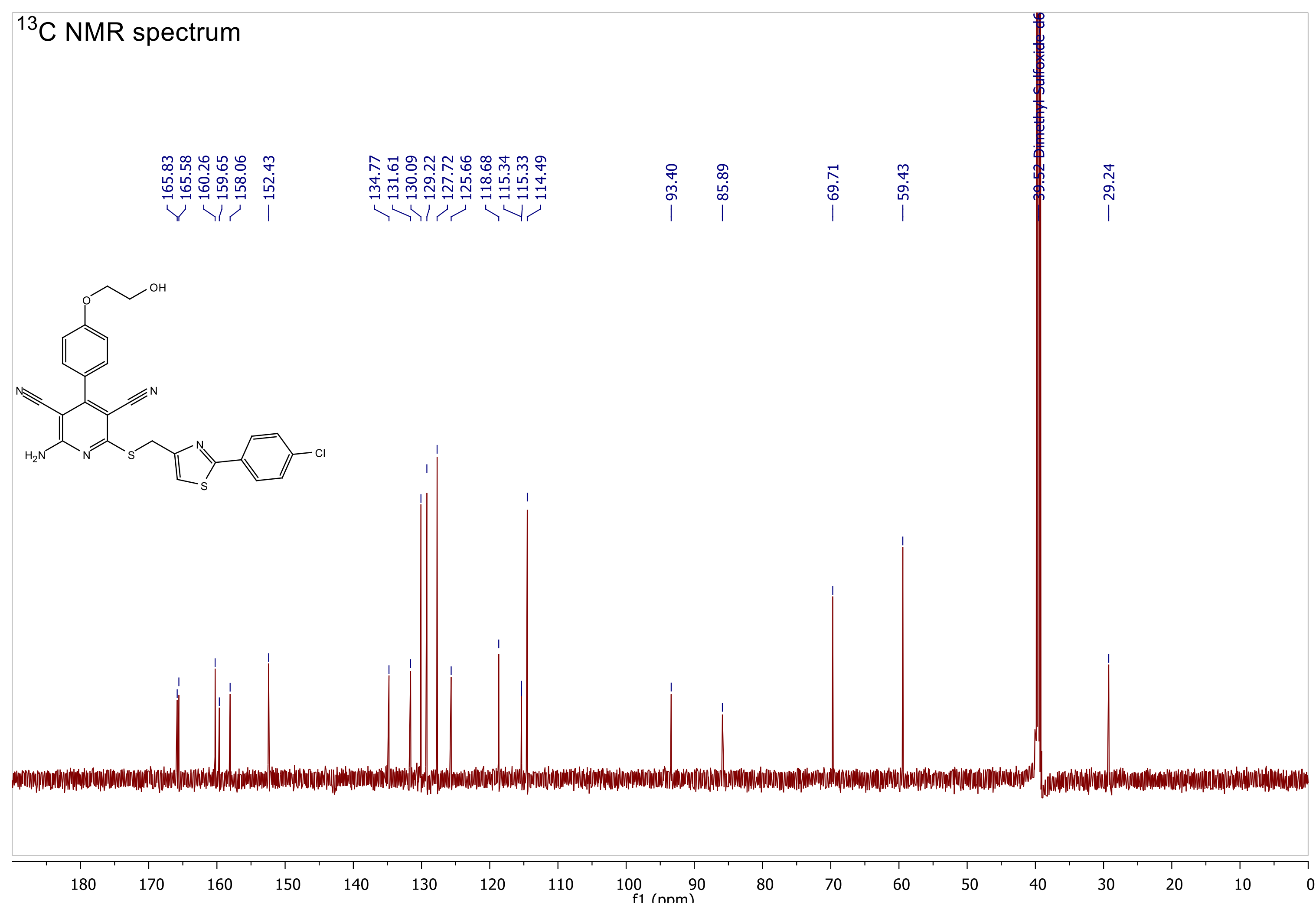




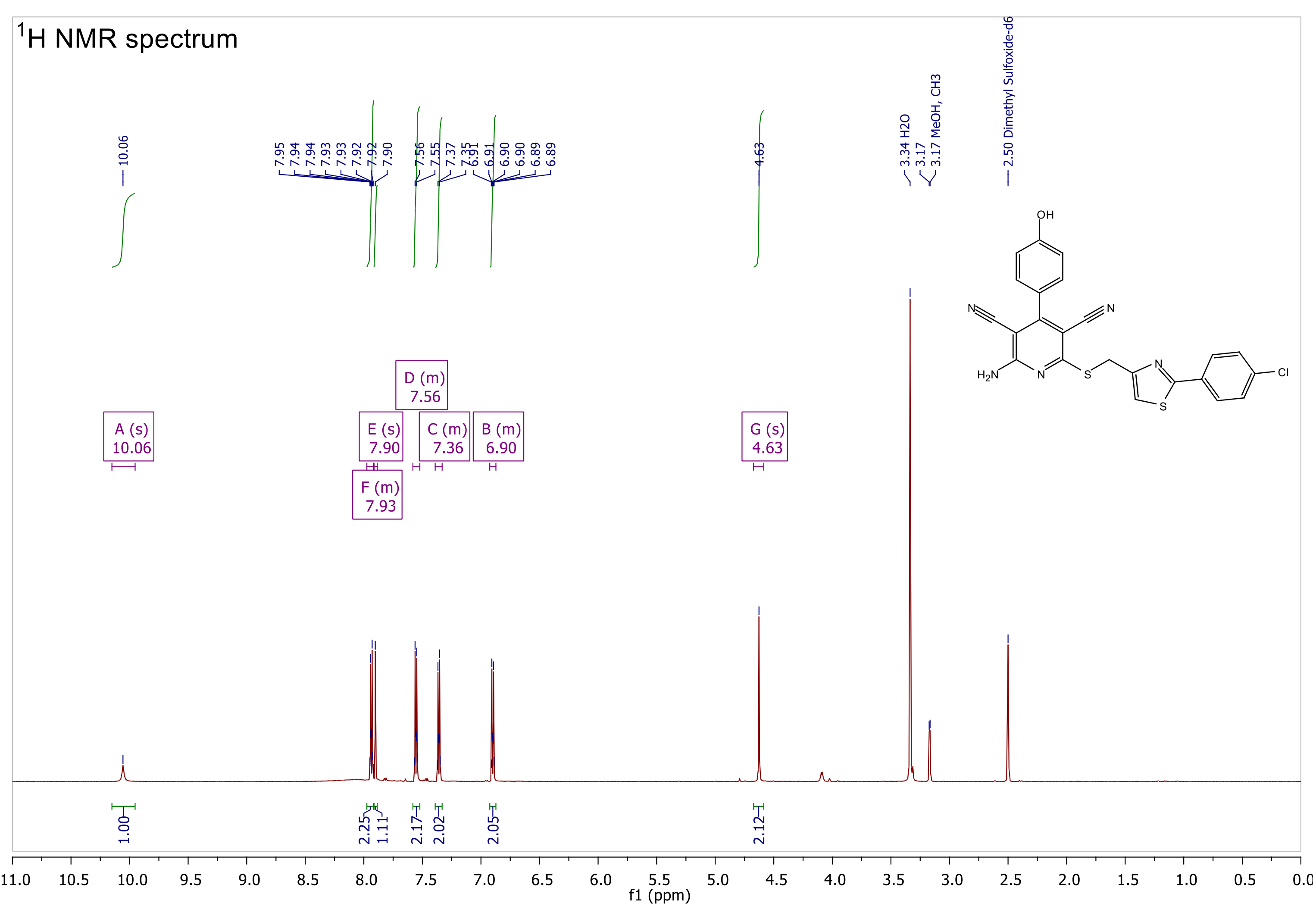
^{13}C NMR spectrum
166.19
163.98
162.33
159.52
157.84
157.52
155.78
137.09
131.12
131.06
130.31
121.91
120.76
115.92
115.77
115.28
115.21
93.32
86.13
39.52 Dimethyl Sulfoxide-d6
35.35
23.97
D (d) 131.09
C (d) 130.32
B (d) 115.84
A (d) 115.24
F
N
N
H2N
N
S
N
CH3
f1 (ppm)

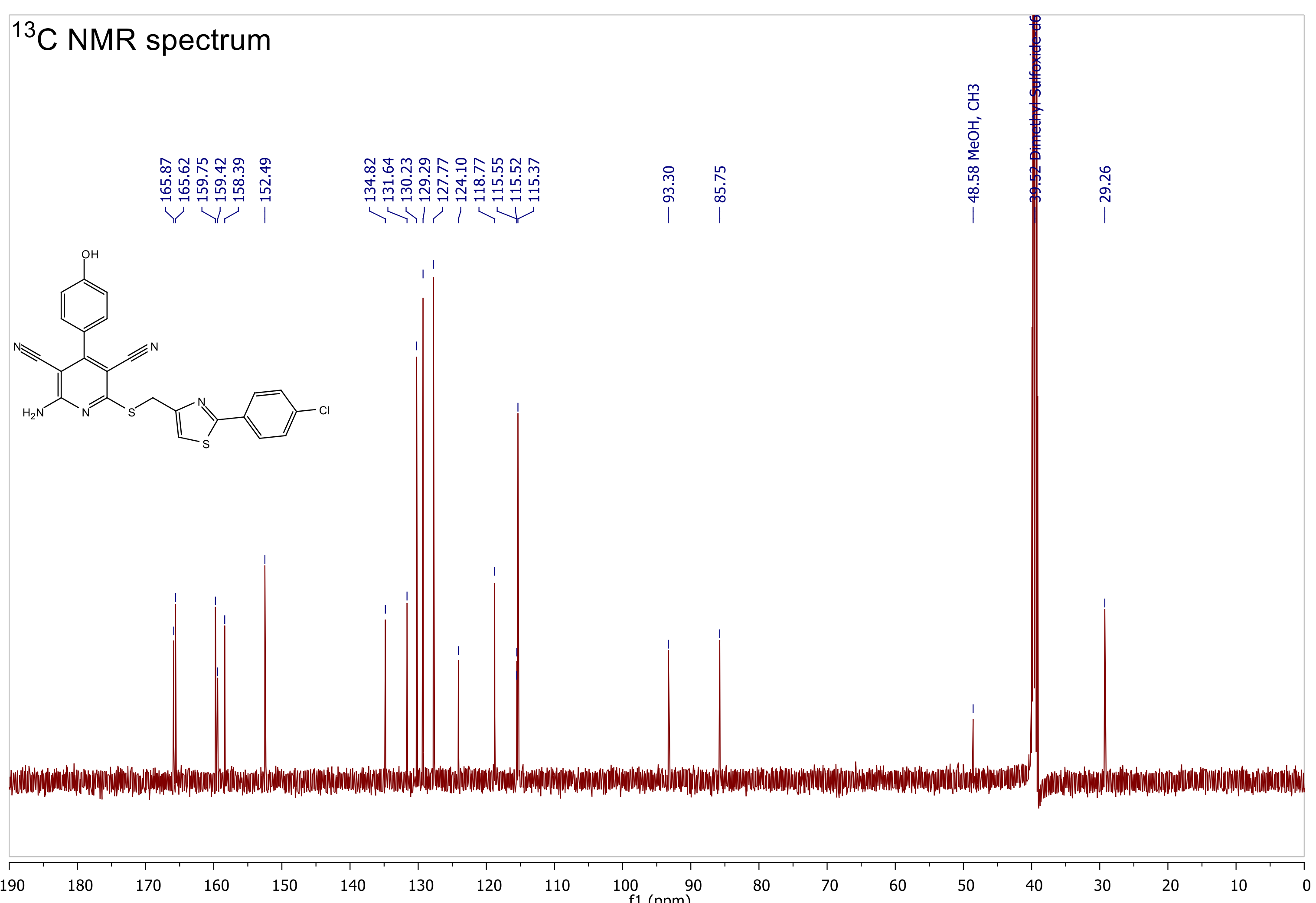
2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-(2-hydroxyethoxy)phenyl)pyridine-3,5-dicarbonitrile (**6q**)




13C NMR spectrum
165.83
165.58
160.26
159.65
158.06
152.43
134.77
131.61
130.09
129.22
127.72
125.66
118.68
115.34
115.33
114.49
93.40
85.89
69.71
59.43
39.52 Dimethyl Sulfoxide-d6
29.24
OH
O
N
N
H2N
N
S
N
S
Cl
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

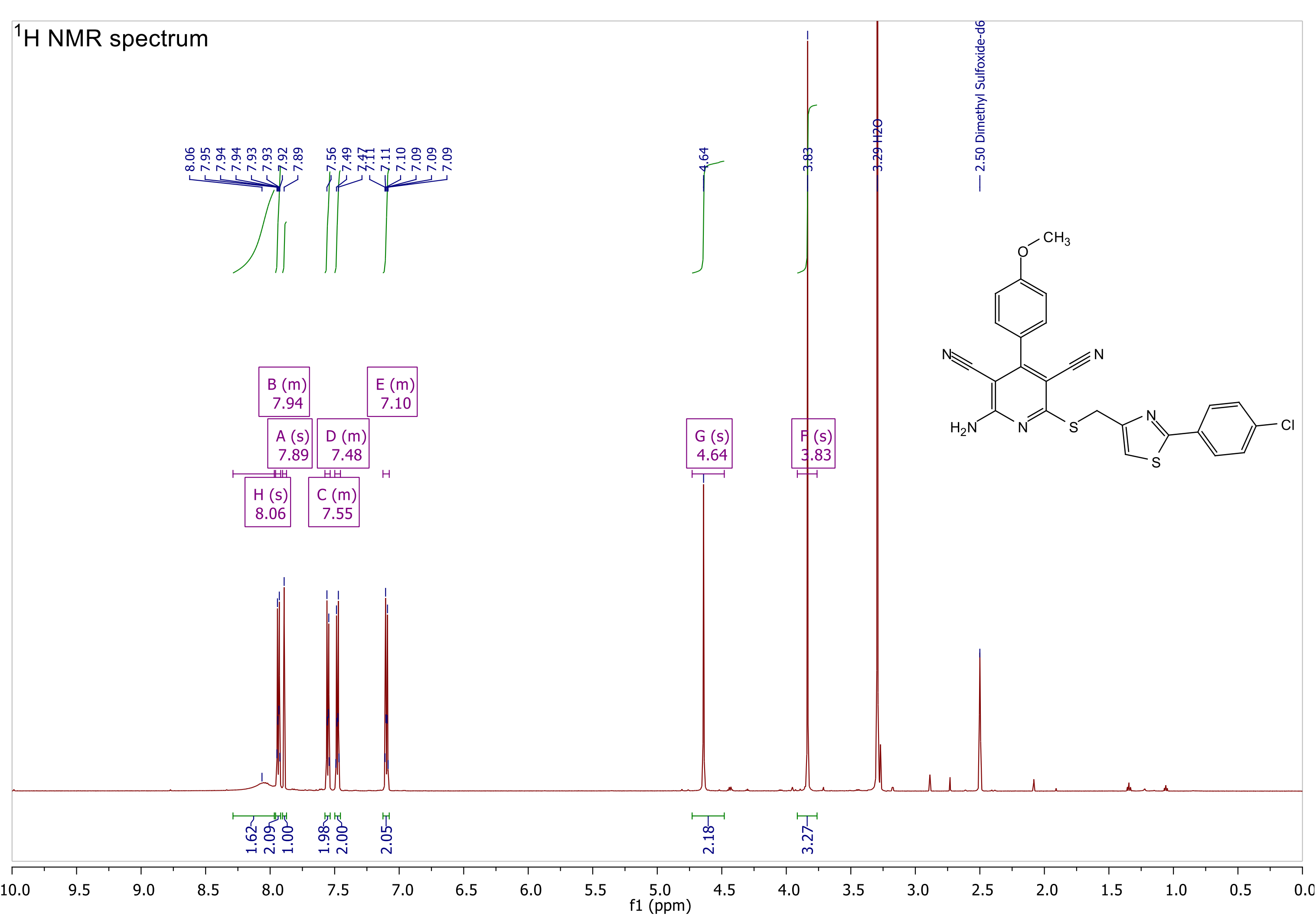
2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-hydroxyphenyl)pyridine-3,5-dicarbonitrile (**6r**)

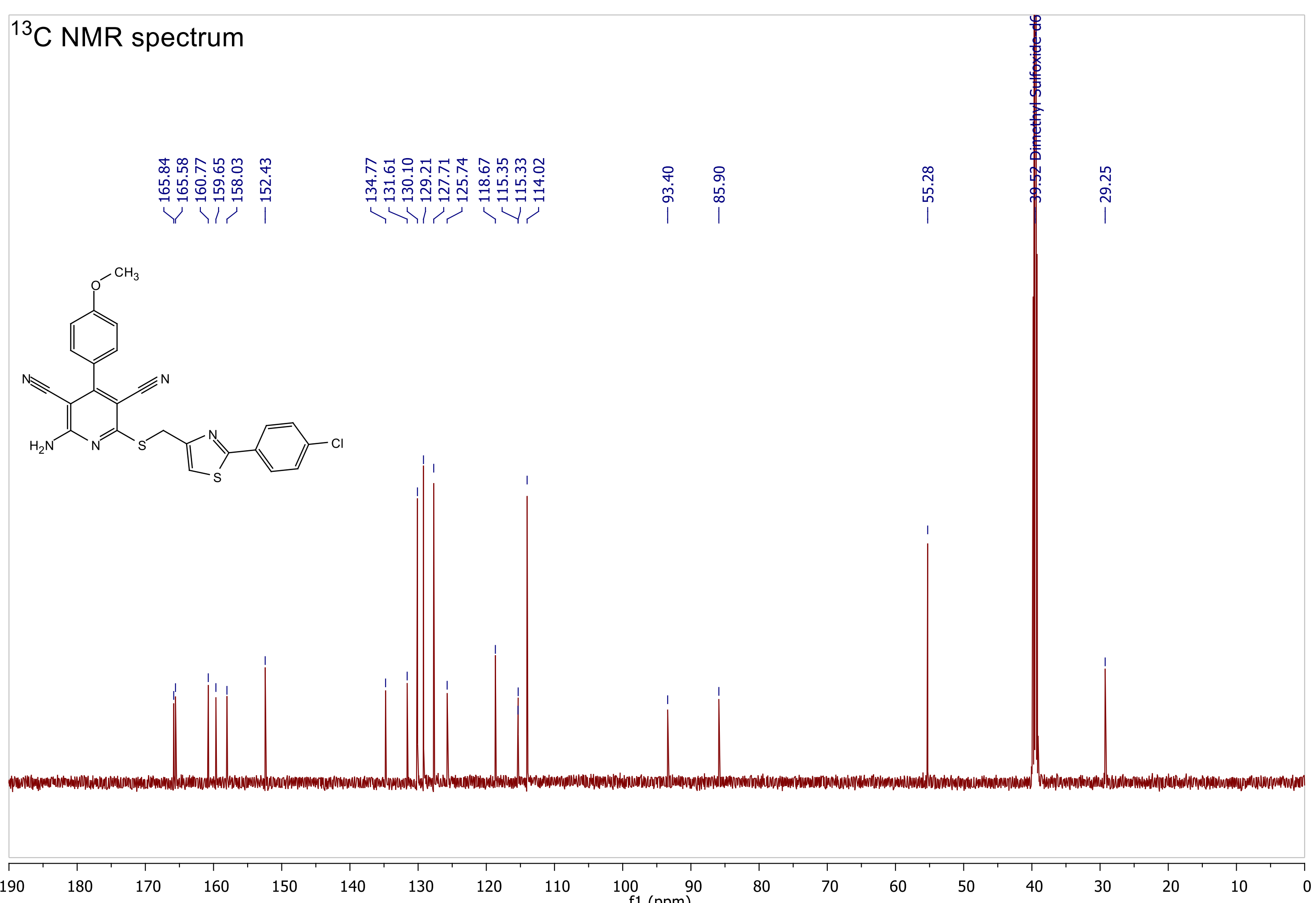




13C NMR spectrum
165.87
165.62
159.75
159.42
158.39
152.49
134.82
131.64
130.23
129.29
127.77
124.10
118.77
115.55
115.52
115.37
93.30
85.75
48.58 MeOH, CH3
39.52 Dimethyl Sulfoxide d6
29.26
OH
N
N
H2N
N
S
N
S
Cl
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

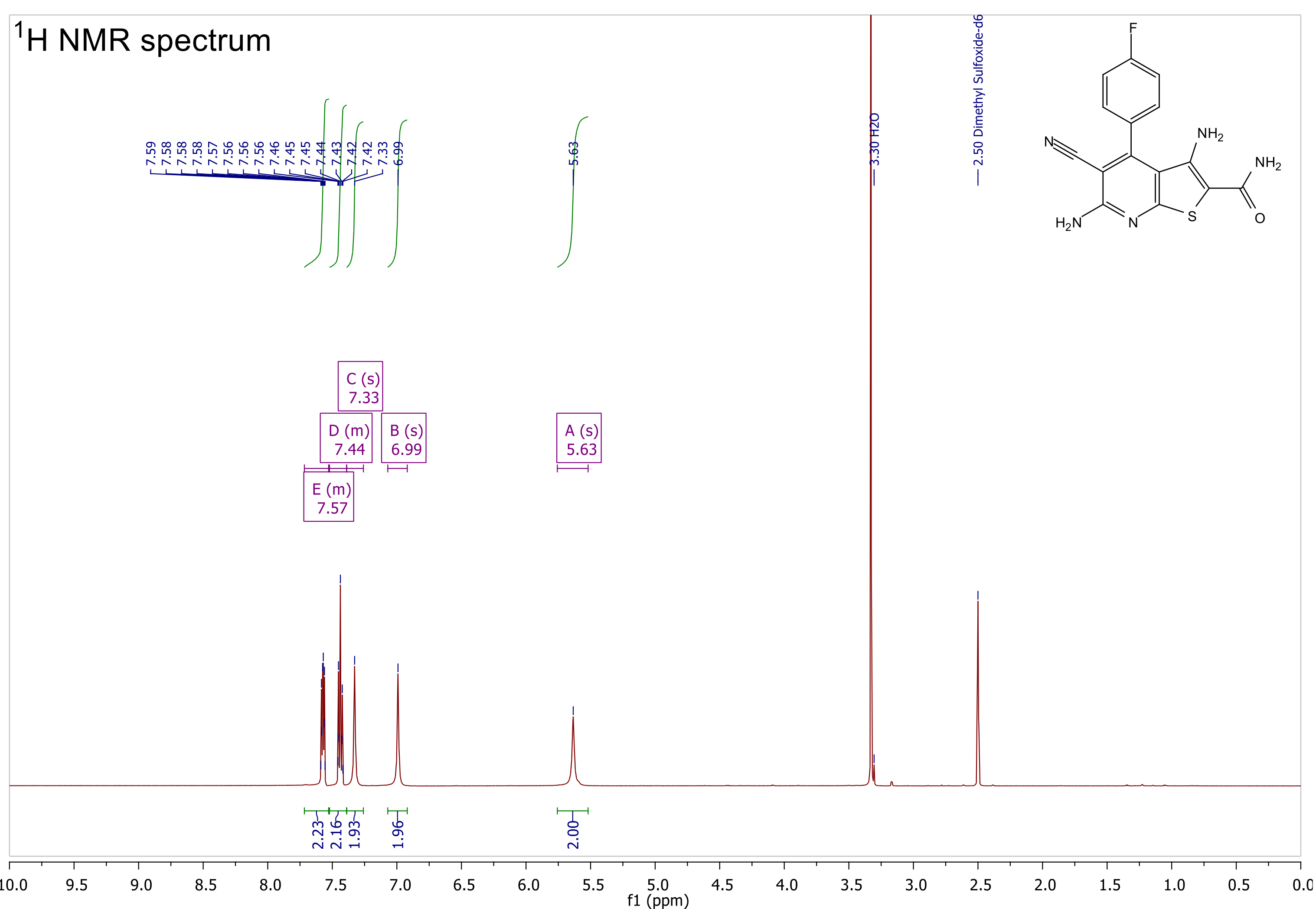
2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (**6s**)

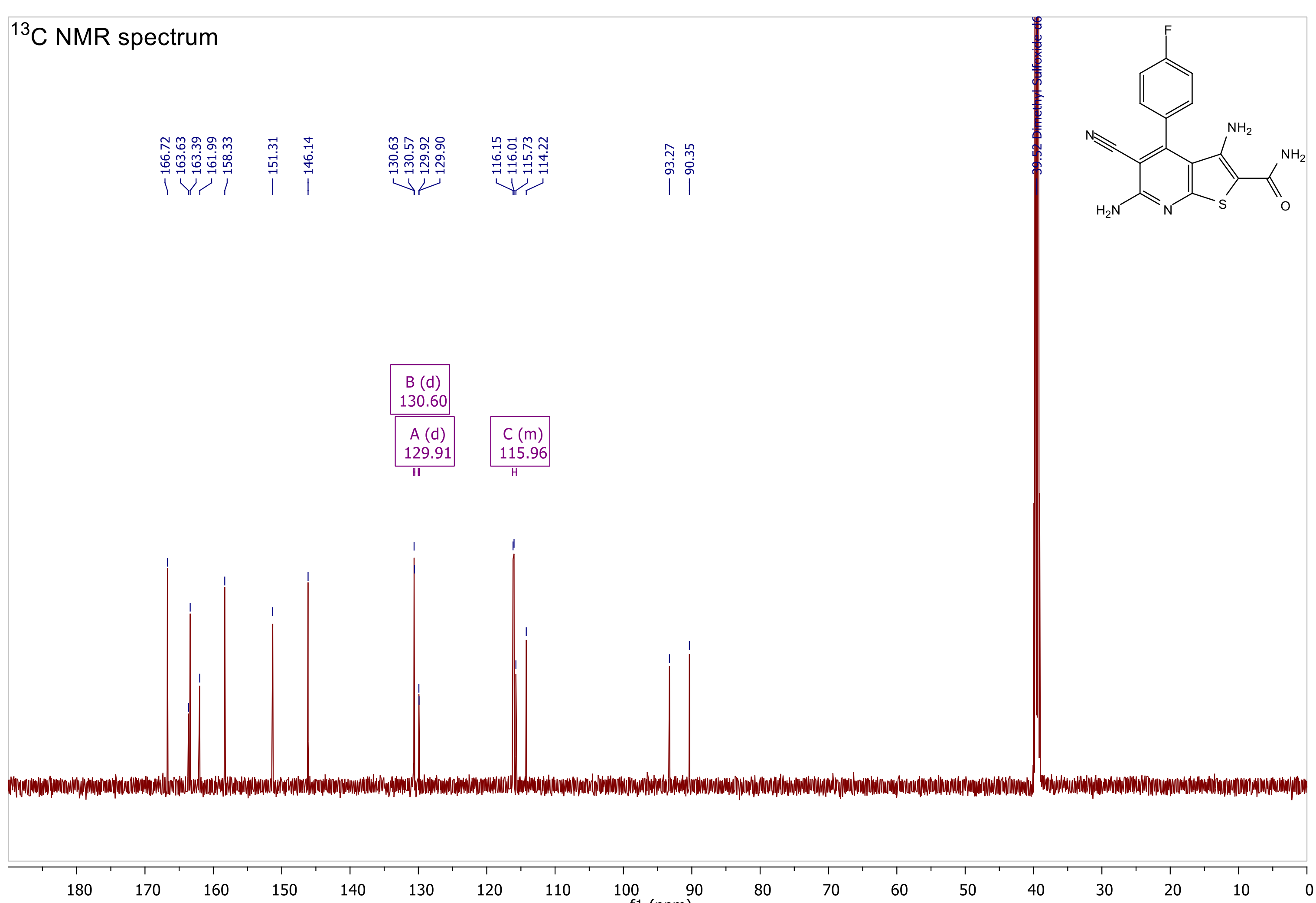




13C NMR spectrum
165.84
165.58
160.77
159.65
158.03
152.43
134.77
131.61
130.10
129.21
127.71
125.74
118.67
115.35
115.33
114.02
93.40
85.90
55.28
39.52 Dimethyl Sulfoxide d6
29.25
O
CH3
N
N
H2N
N
S
N
S
Cl
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

3,6-diamino-5-cyano-4-(4-fluorophenyl)thieno[2,3-b]pyridine-2-carboxamide (**7a**)

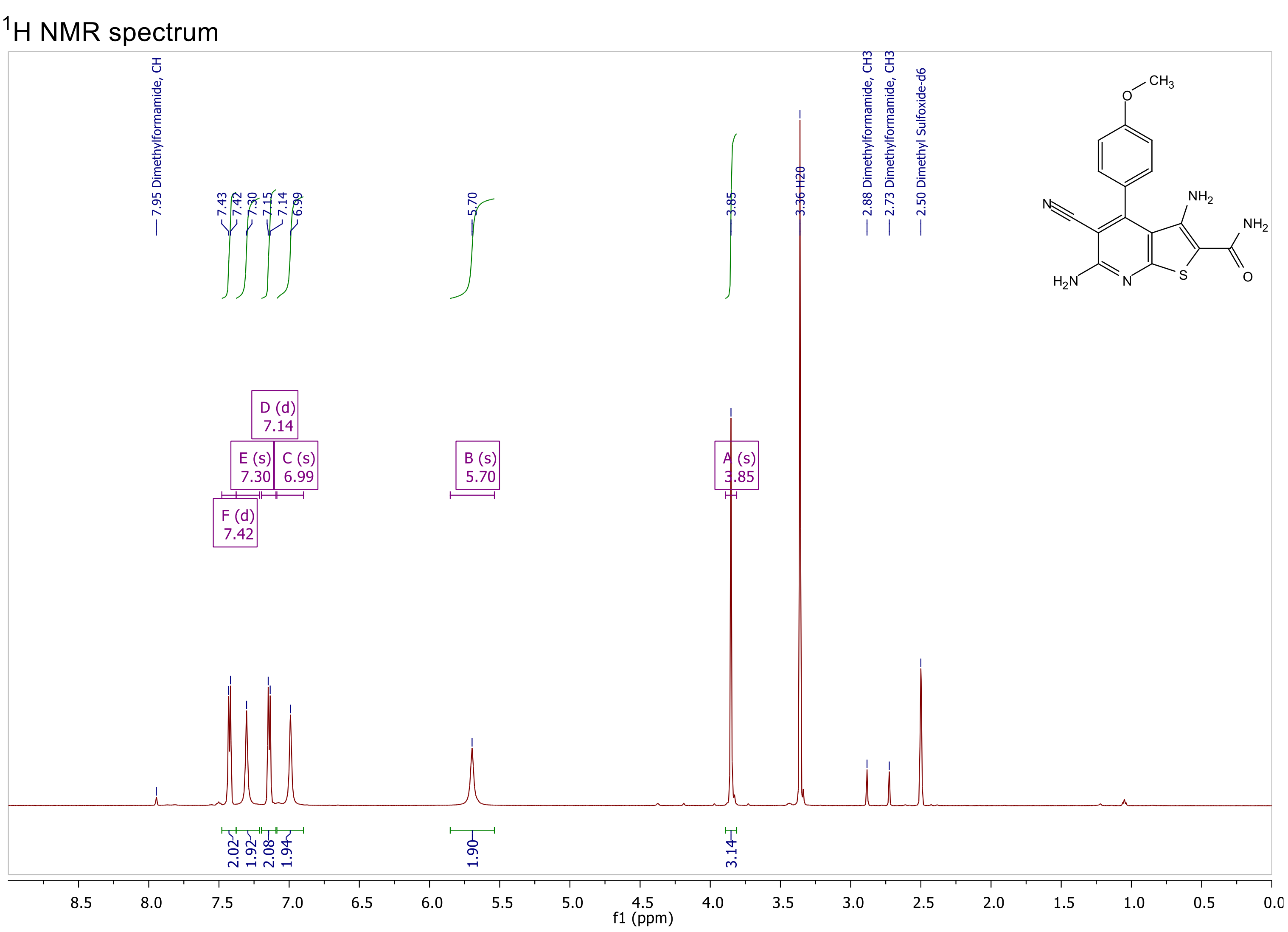




13C NMR spectrum
166.72
163.63
163.39
161.99
158.33
151.31
146.14
130.63
130.57
129.92
129.90
116.15
116.01
115.73
114.22
93.27
90.35
39.52 Dimethyl Sulfoxide-d6
B (d)
130.60
A (d)
129.91
C (m)
115.96
f1 (ppm)
F
NH2
NH2
N
H2N
N
S
O

3,6-diamino-5-cyano-4-(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (**7b**)

^{1}H NMR spectrum

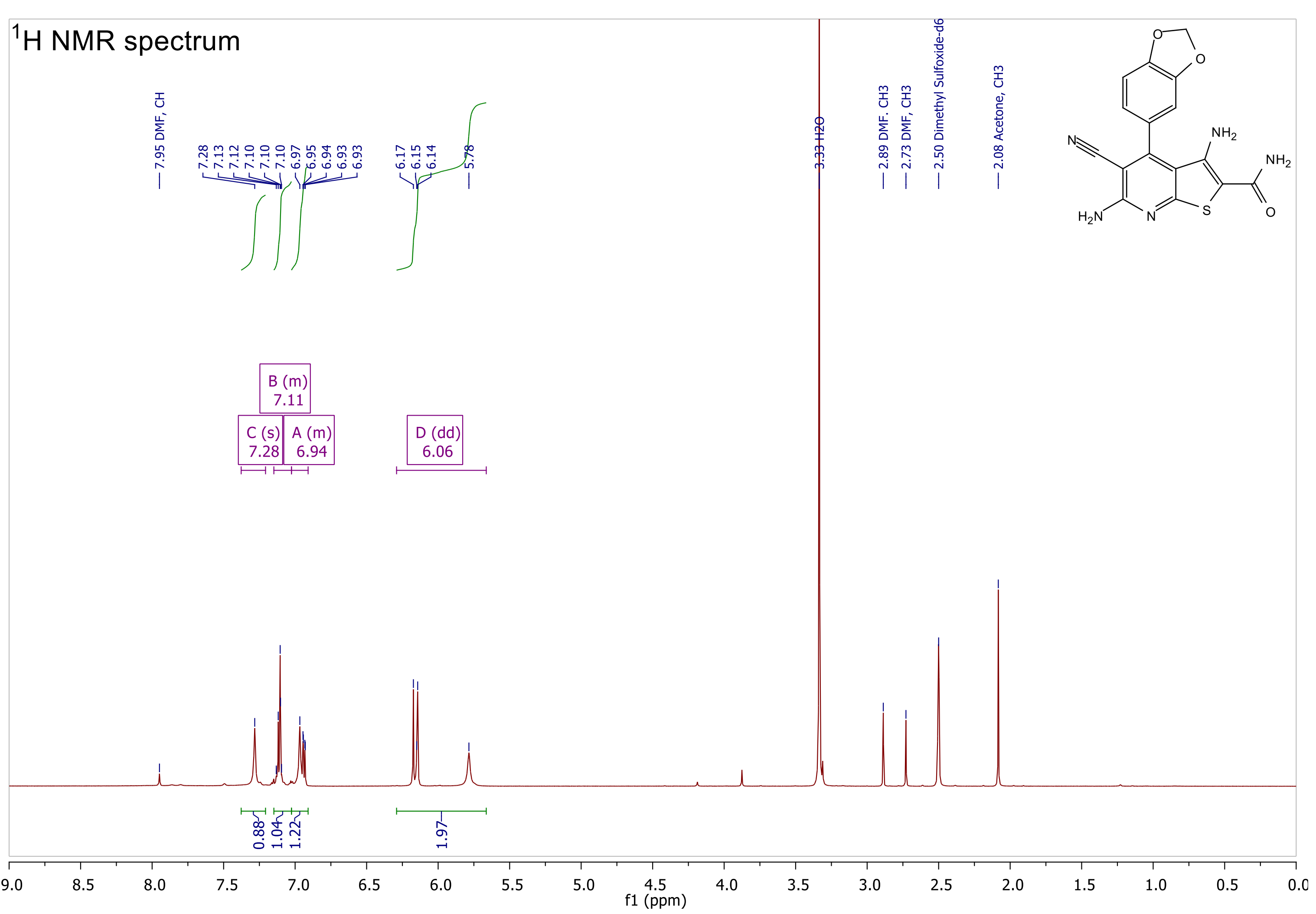


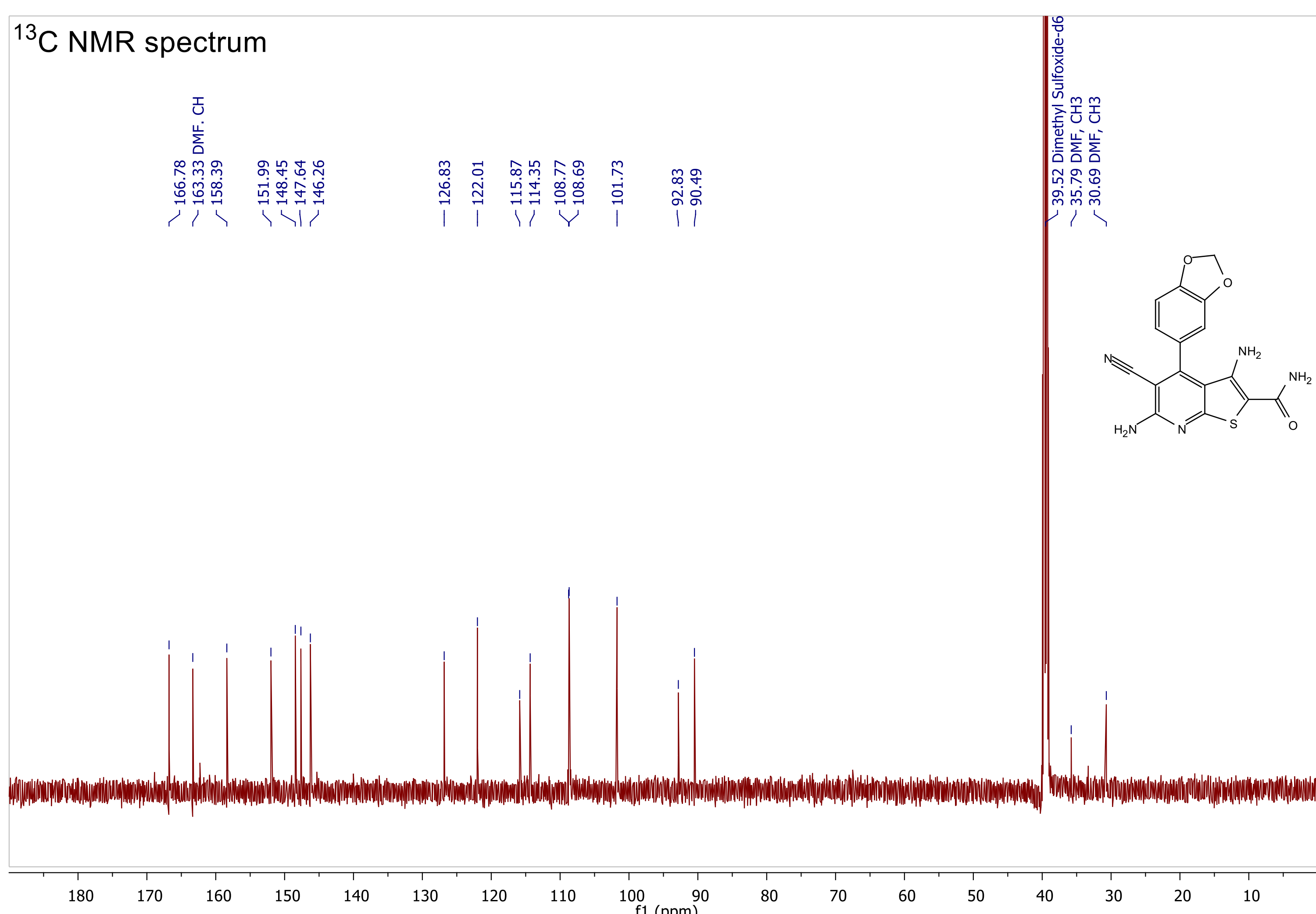
166.79
163.39
162.33 Dimethylformamide, CH
160.22
158.48
152.29
146.31
129.65
125.46
116.00
114.42
114.33
92.91
90.47
55.33
39.52 Dimethyl Sulfoxide-d6
35.82 Dimethylformamide, CH3
30.80 Dimethylformamide, CH3

CH3
O
NH2
N
NH2
H2N
N
S
O

190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

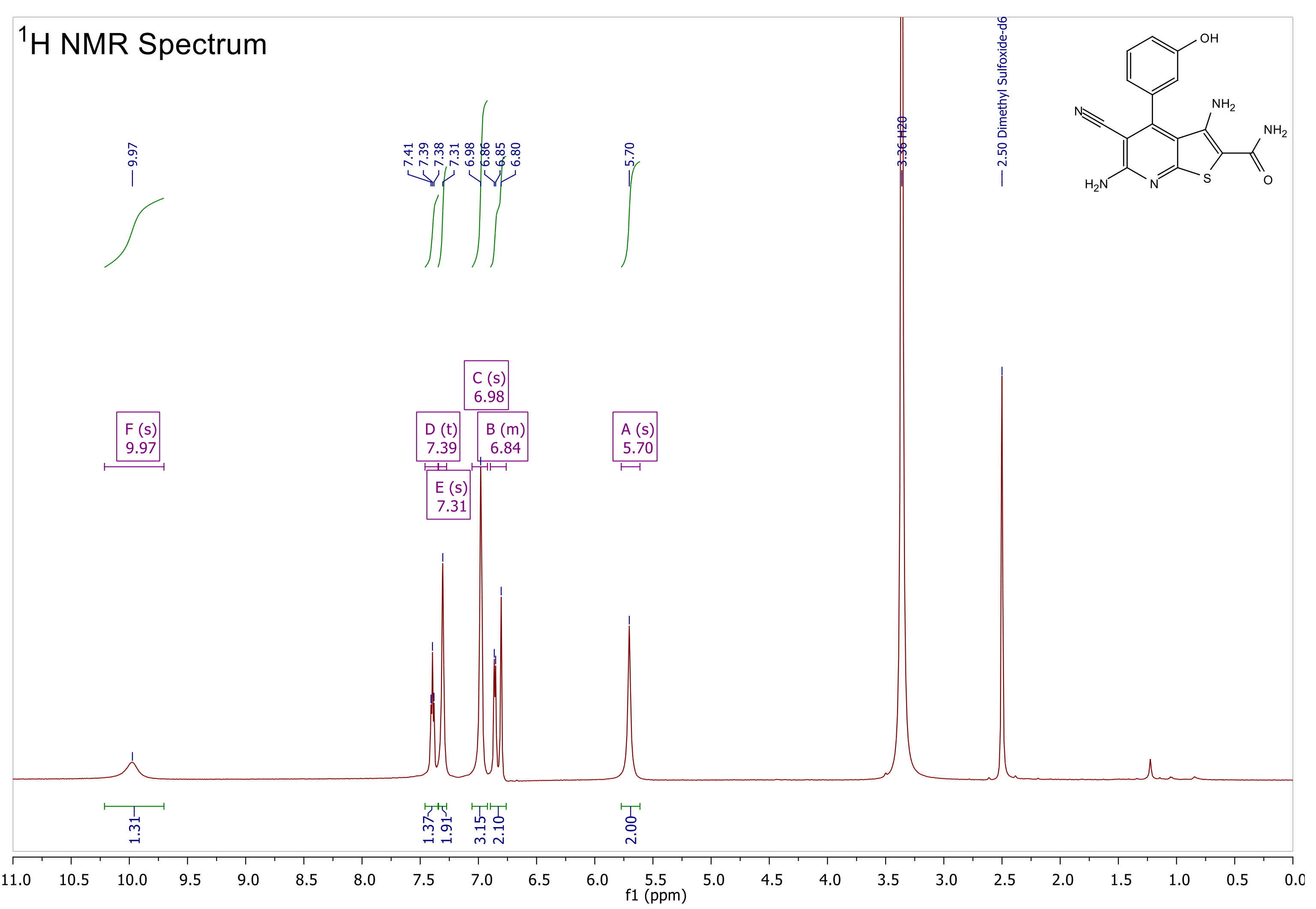
3,6-diamino-4-(benzo[d][1,3]dioxol-5-yl)-5-cyanothieno[2,3-b]pyridine-2-carboxamide (**7c**)

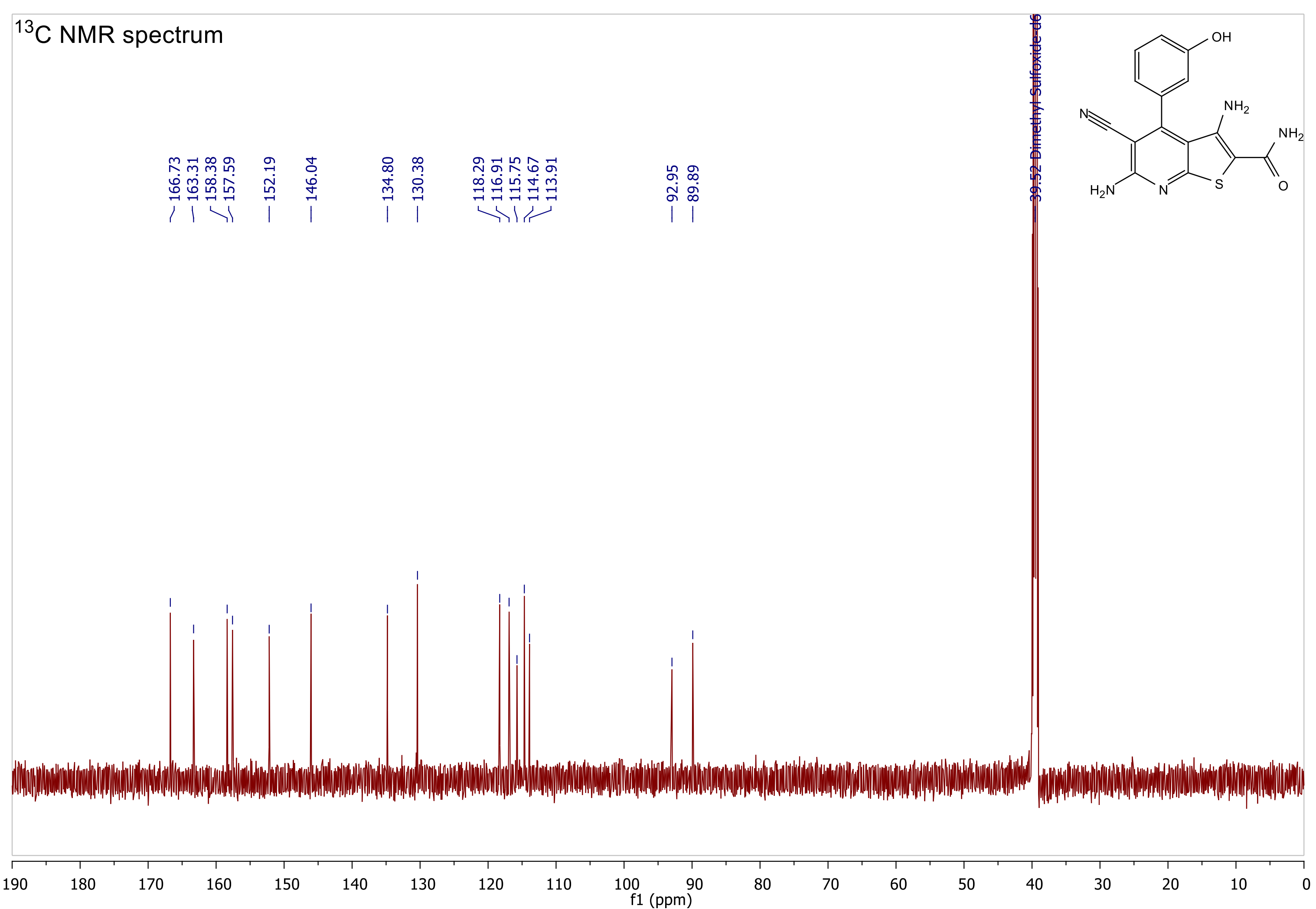




13C NMR spectrum
166.78
163.33 DMF. CH
158.39
151.99
148.45
147.64
146.26
126.83
122.01
115.87
114.35
108.77
108.69
101.73
92.83
90.49
39.52 Dimethyl Sulfoxide-d6
35.79 DMF, CH3
30.69 DMF, CH3
O
O
NH2
NH2
N
H2N
N
S
O
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

3,6-diamino-5-cyano-4-(3-hydroxyphenyl)thieno[2,3-b]pyridine-2-carboxamide(**7d**)



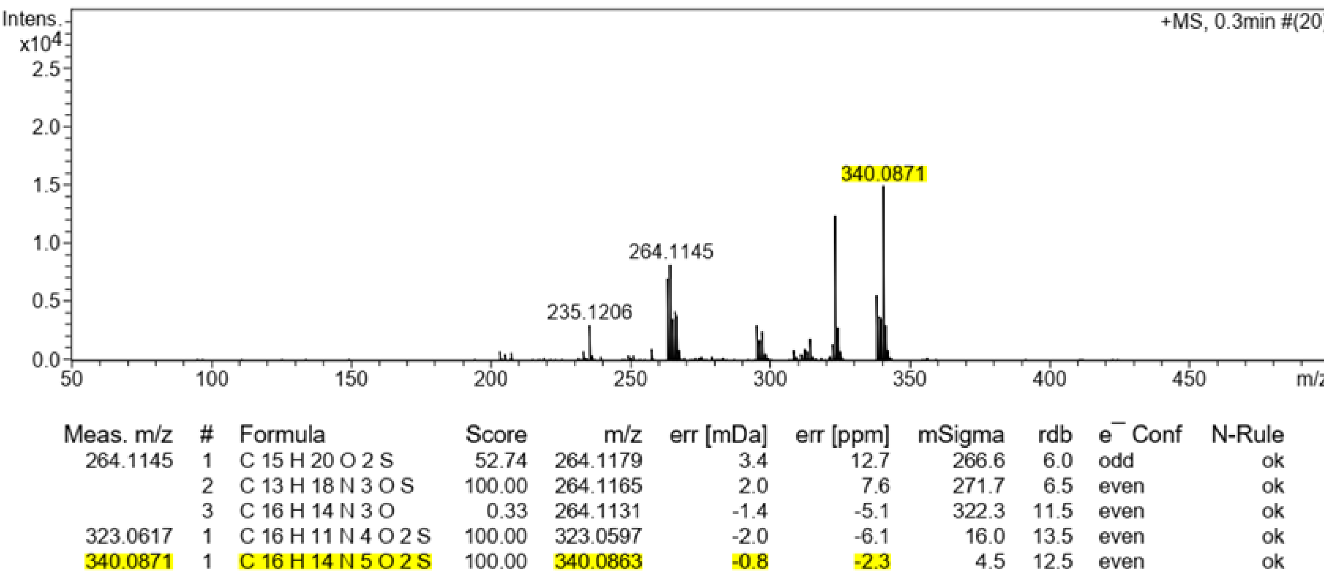


^{13}C NMR spectrum
166.73
163.31
158.38
157.59
152.19
146.04
134.80
130.38
118.29
116.91
115.75
114.67
113.91
92.95
89.89
39.52 Dimethyl Sulfoxide-d6
OH
NH2
NH2
N
H2N
N
S
O
f1 (ppm)

Reference for NMR structure numbering (^{13}C):

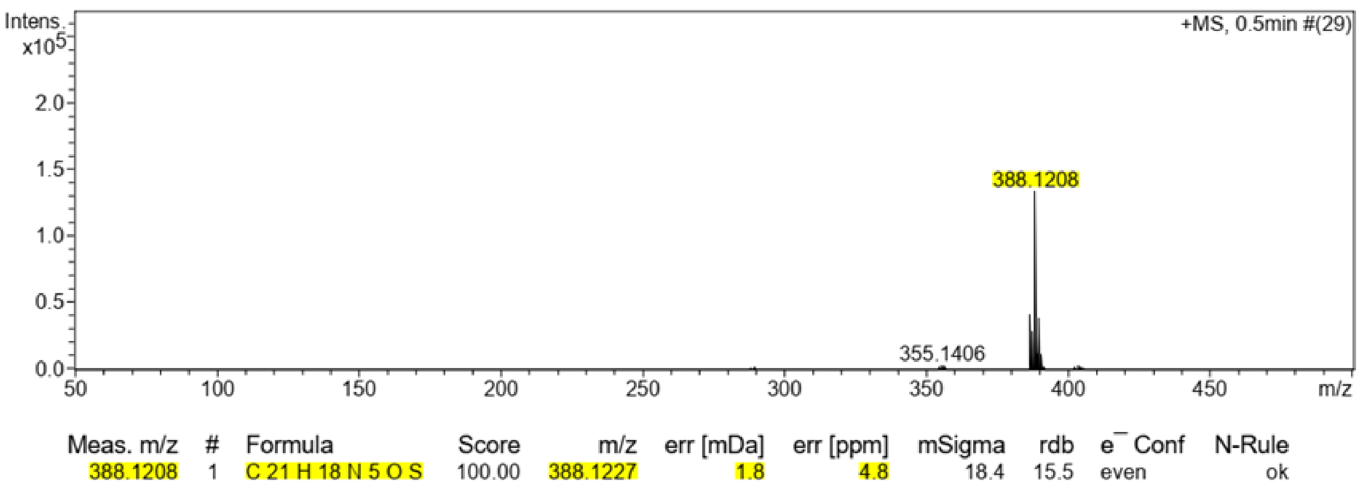
2. Mass Spectrometry (MS) data of test compounds

2-((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)acetamide (**6a**)



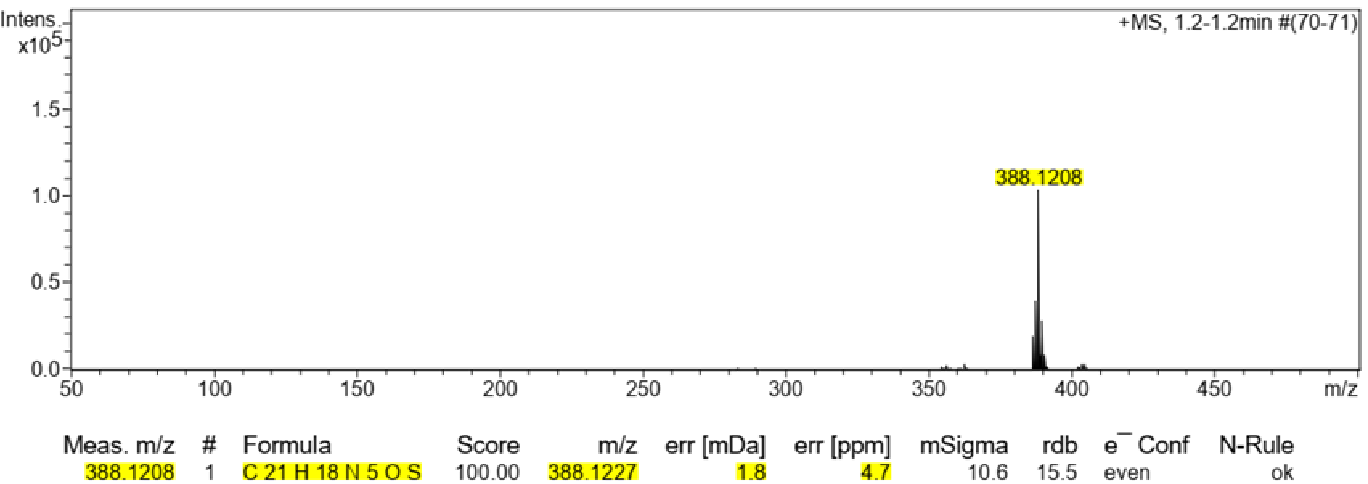
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
264.1145	1	C 15 H 20 O 2 S	52.74	264.1179	3.4	12.7	266.6	6.0	odd	ok
	2	C 13 H 18 N 3 O S	100.00	264.1165	2.0	7.6	271.7	6.5	even	ok
	3	C 16 H 14 N 3 O	0.33	264.1131	-1.4	-5.1	322.3	11.5	even	ok
323.0617	1	C 16 H 11 N 4 O 2 S	100.00	323.0597	-2.0	-6.1	16.0	13.5	even	ok
340.0871	1	C 16 H 14 N 5 O 2 S	100.00	340.0863	-0.8	-2.3	4.5	12.5	even	ok

2-amino-4-(4-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6b**)



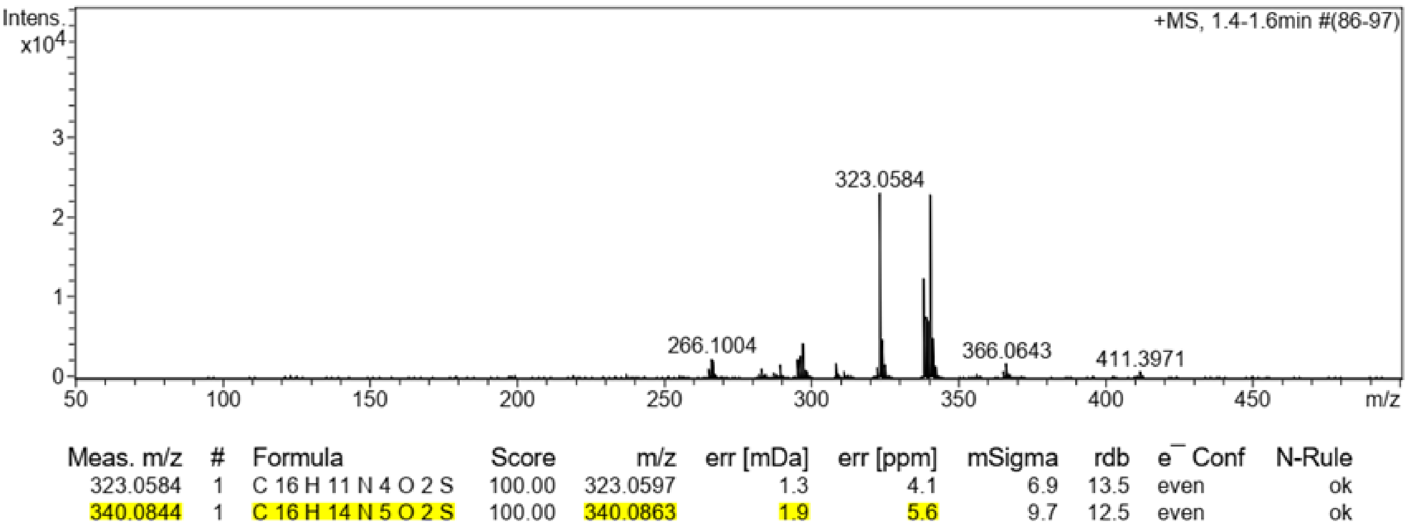
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
388.1208	1	C 21 H 18 N 5 O S	100.00	388.1227	1.8	4.8	18.4	15.5	even	ok

2-amino-4-(3-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6c)**



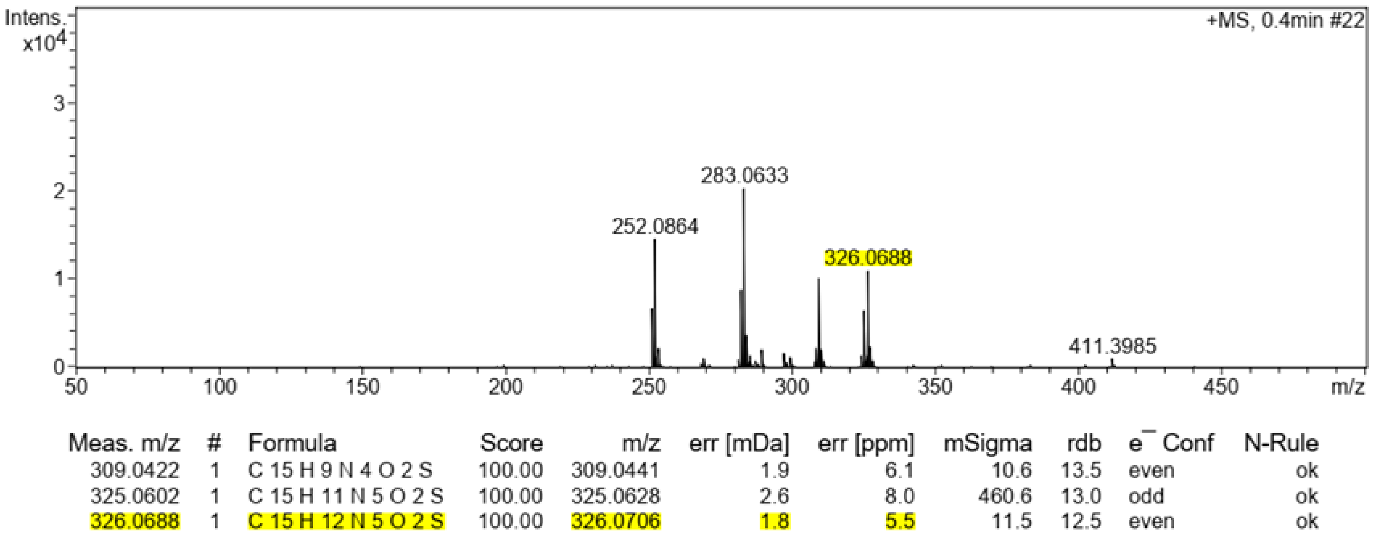
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
388.1208	1	C 21 H 18 N 5 O S	100.00	388.1227	1.8	4.7	10.6	15.5	even	ok

2-((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)acetamide(**6d)**



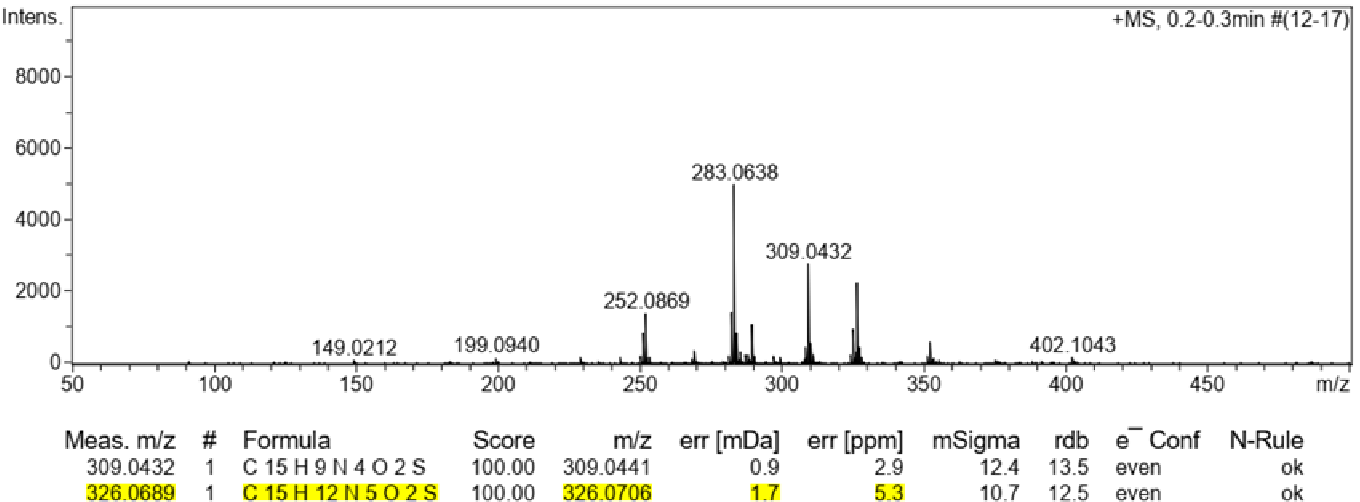
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
323.0584	1	C 16 H 11 N 4 O 2 S	100.00	323.0597	1.3	4.1	6.9	13.5	even	ok
340.0844	1	C 16 H 14 N 5 O 2 S	100.00	340.0863	1.9	5.6	9.7	12.5	even	ok

2-((6-amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-yl)thio)acetamide (**6e**)



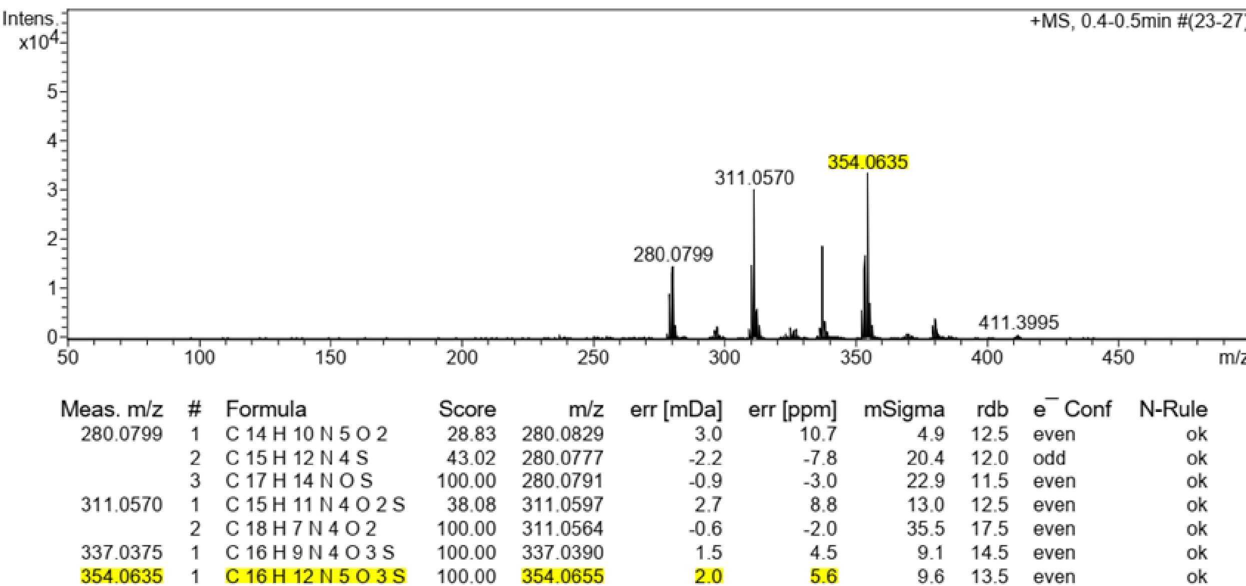
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
309.0422	1	C 15 H 9 N 4 O 2 S	100.00	309.0441	1.9	6.1	10.6	13.5	even	ok
325.0602	1	C 15 H 11 N 5 O 2 S	100.00	325.0628	2.6	8.0	460.6	13.0	odd	ok
326.0688	1	C 15 H 12 N 5 O 2 S	100.00	326.0706	1.8	5.5	11.5	12.5	even	ok

2-((6-amino-3,5-dicyano-4-(3-hydroxyphenyl)pyridin-2-yl)thio)acetamide (**6f**)



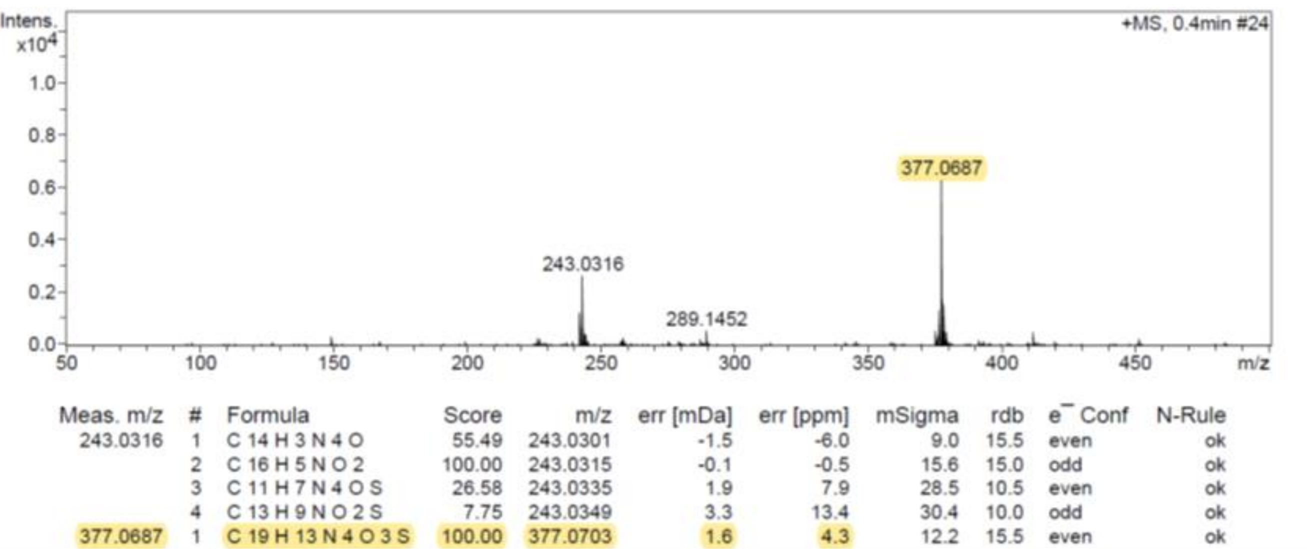
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
309.0432	1	C 15 H 9 N 4 O 2 S	100.00	309.0441	0.9	2.9	12.4	13.5	even	ok
326.0689	1	C 15 H 12 N 5 O 2 S	100.00	326.0706	1.7	5.3	10.7	12.5	even	ok

2-((6-amino-4-(benzo[d][1,3]dioxol-5-yl)-3,5-dicyanopyridin-2-yl)thio)acetamide (**6g**)



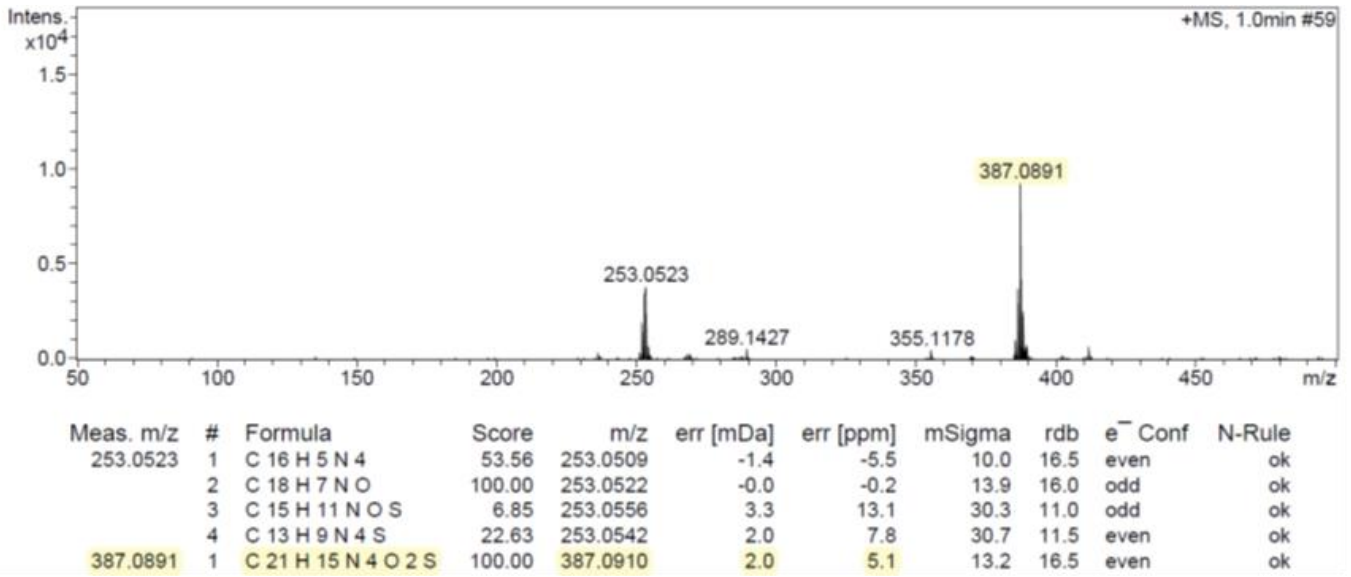
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
280.0799	1	C 14 H 10 N 5 O 2	28.83	280.0829	3.0	10.7	4.9	12.5	even	ok
	2	C 15 H 12 N 4 S	43.02	280.0777	-2.2	-7.8	20.4	12.0	odd	ok
	3	C 17 H 14 N O S	100.00	280.0791	-0.9	-3.0	22.9	11.5	even	ok
311.0570	1	C 15 H 11 N 4 O 2 S	38.08	311.0597	2.7	8.8	13.0	12.5	even	ok
	2	C 18 H 7 N 4 O 2	100.00	311.0564	-0.6	-2.0	35.5	17.5	even	ok
337.0375	1	C 16 H 9 N 4 O 3 S	100.00	337.0390	1.5	4.5	9.1	14.5	even	ok
354.0635	1	C 16 H 12 N 5 O 3 S	100.00	354.0655	2.0	5.6	9.6	13.5	even	ok

3-(((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzoic acid (**6h**)



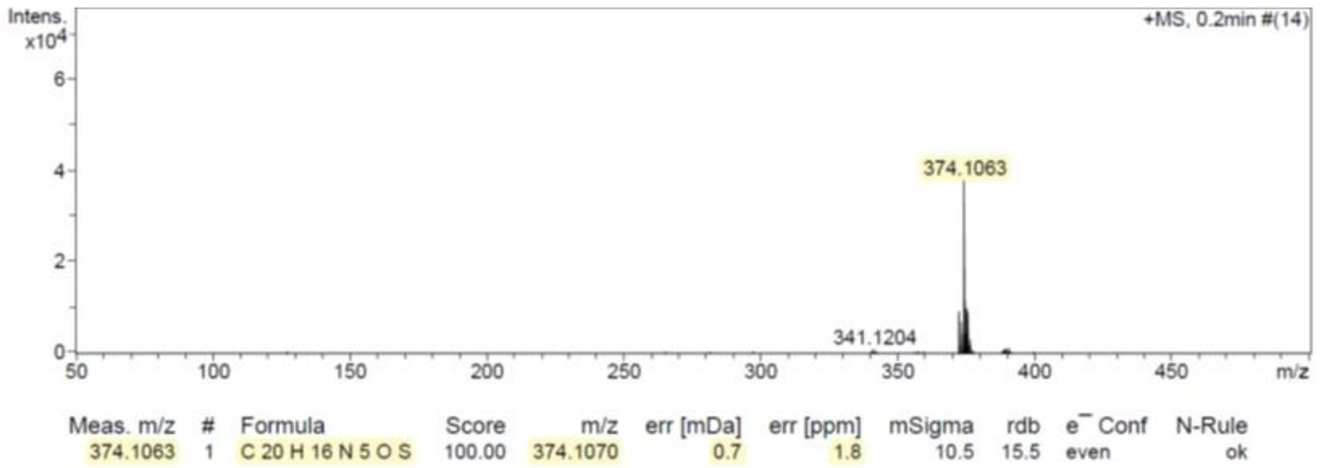
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
243.0316	1	C 14 H 3 N 4 O	55.49	243.0301	-1.5	-6.0	9.0	15.5	even	ok
	2	C 16 H 5 N O 2	100.00	243.0315	-0.1	-0.5	15.6	15.0	odd	ok
	3	C 11 H 7 N 4 O S	26.58	243.0335	1.9	7.9	28.5	10.5	even	ok
	4	C 13 H 9 N O 2 S	7.75	243.0349	3.3	13.4	30.4	10.0	odd	ok
377.0687	1	C 19 H 13 N 4 O 3 S	100.00	377.0703	1.6	4.3	12.2	15.5	even	ok

3-(((6-amino-3,5-dicyano-4-phenylpyridin-2-yl)thio)methyl)benzoic acid (**6i**)



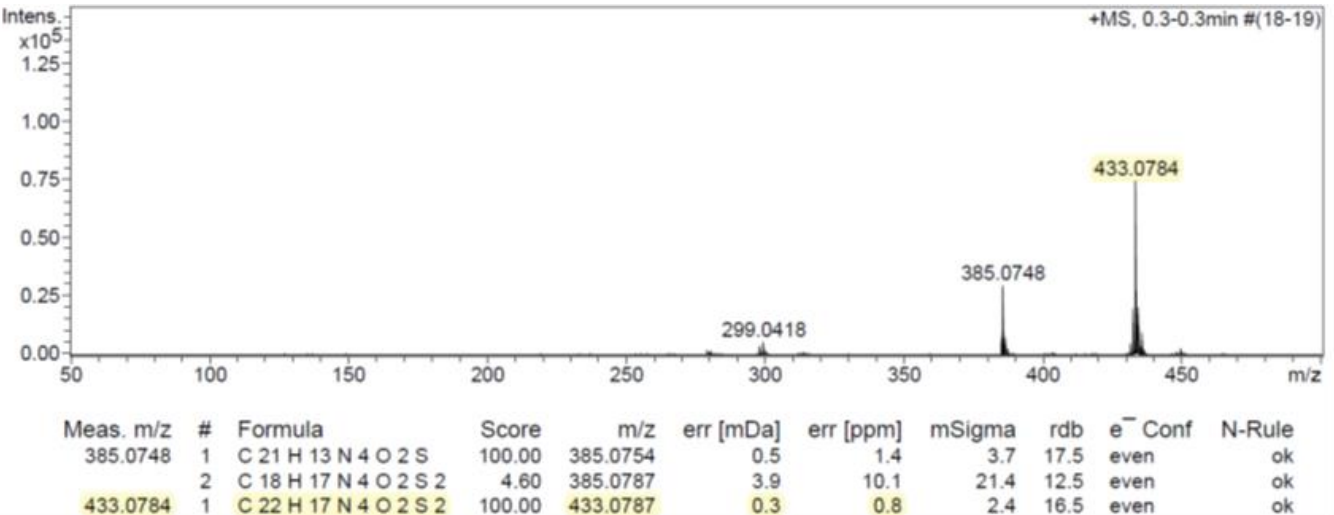
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
253.0523	1	C 16 H 5 N 4	53.56	253.0509	-1.4	-5.5	10.0	16.5	even	ok
	2	C 18 H 7 N O	100.00	253.0522	-0.0	-0.2	13.9	16.0	odd	ok
	3	C 15 H 11 N O S	6.85	253.0556	3.3	13.1	30.3	11.0	odd	ok
	4	C 13 H 9 N 4 S	22.63	253.0542	2.0	7.8	30.7	11.5	even	ok
387.0891	1	C 21 H 15 N 4 O 2 S	100.00	387.0910	2.0	5.1	13.2	16.5	even	ok

2-amino-4-(3-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6j**)



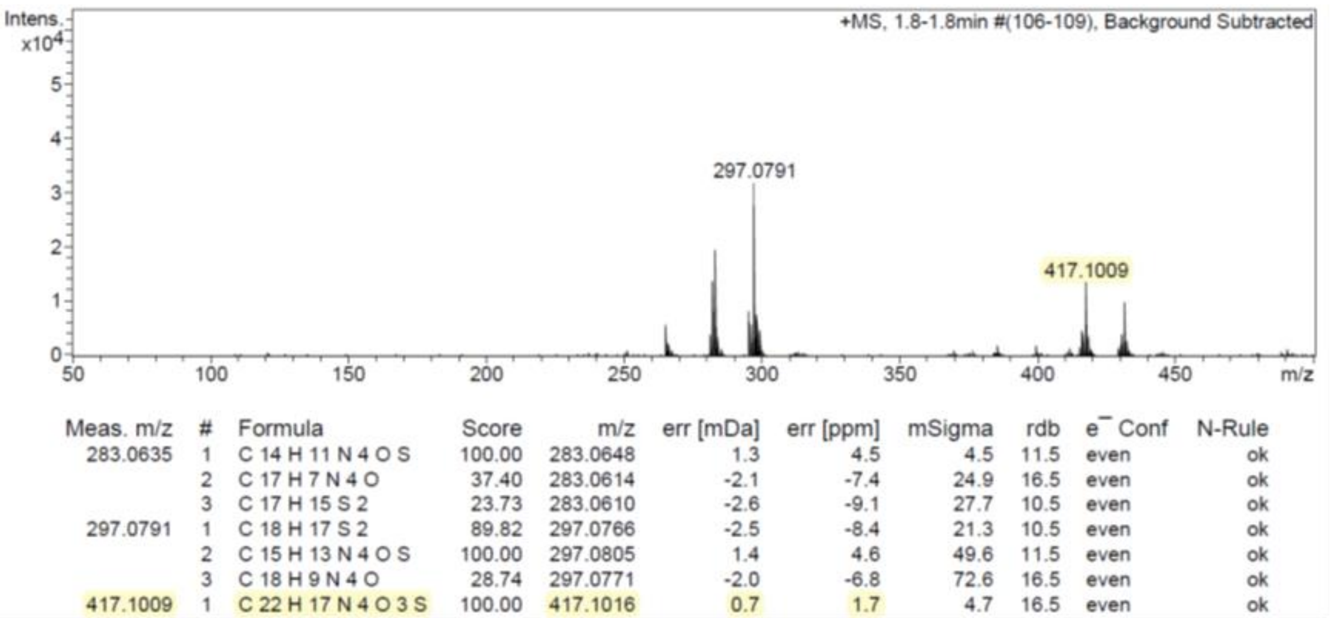
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
374.1063	1	C 20 H 16 N 5 O S	100.00	374.1070	0.7	1.8	10.5	15.5	even	ok

3-(((6-amino-3,5-dicyano-4-(4-(methylthio)phenyl)pyridin-2-yl)thio)methyl)benzoic acid (**6k**)



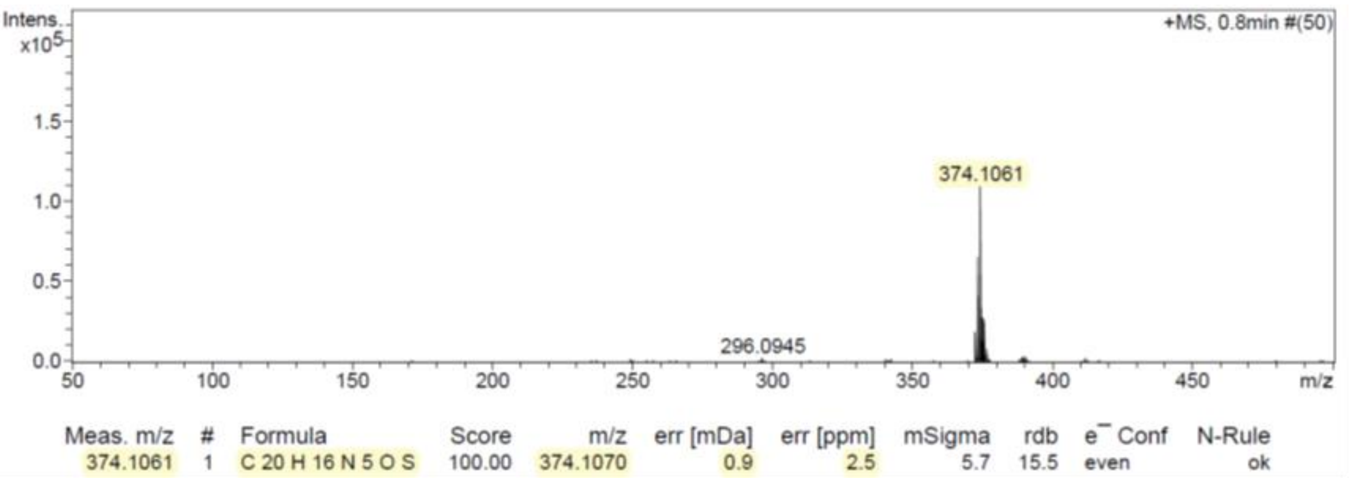
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
385.0748	1	C 21 H 13 N 4 O 2 S	100.00	385.0754	0.5	1.4	3.7	17.5	even	ok
	2	C 18 H 17 N 4 O 2 S 2	4.60	385.0787	3.9	10.1	21.4	12.5	even	ok
433.0784	1	C 22 H 17 N 4 O 2 S 2	100.00	433.0787	0.3	0.8	2.4	16.5	even	ok

3-(((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (**6l**)



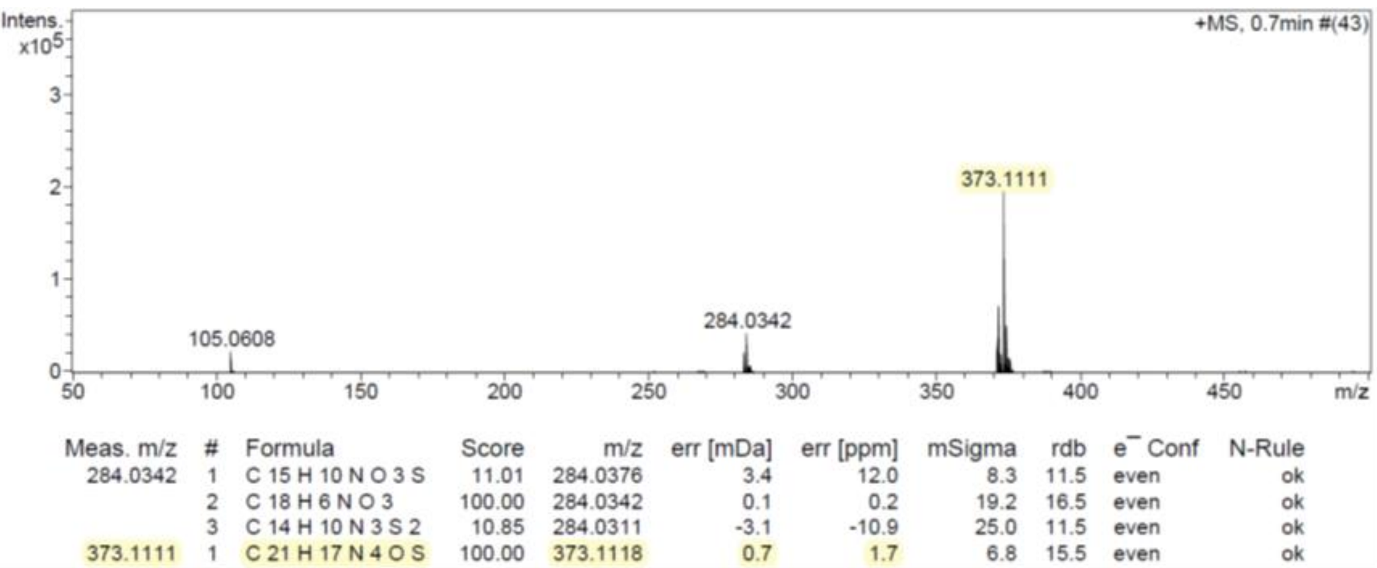
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
283.0635	1	C 14 H 11 N 4 O S	100.00	283.0648	1.3	4.5	4.5	11.5	even	ok
	2	C 17 H 7 N 4 O	37.40	283.0614	-2.1	-7.4	24.9	16.5	even	ok
	3	C 17 H 15 S 2	23.73	283.0610	-2.6	-9.1	27.7	10.5	even	ok
297.0791	1	C 18 H 17 S 2	89.82	297.0766	-2.5	-8.4	21.3	10.5	even	ok
	2	C 15 H 13 N 4 O S	100.00	297.0805	1.4	4.6	49.6	11.5	even	ok
	3	C 18 H 9 N 4 O	28.74	297.0771	-2.0	-6.8	72.6	16.5	even	ok
417.1009	1	C 22 H 17 N 4 O 3 S	100.00	417.1016	0.7	1.7	4.7	16.5	even	ok

2-amino-4-(4-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6m)



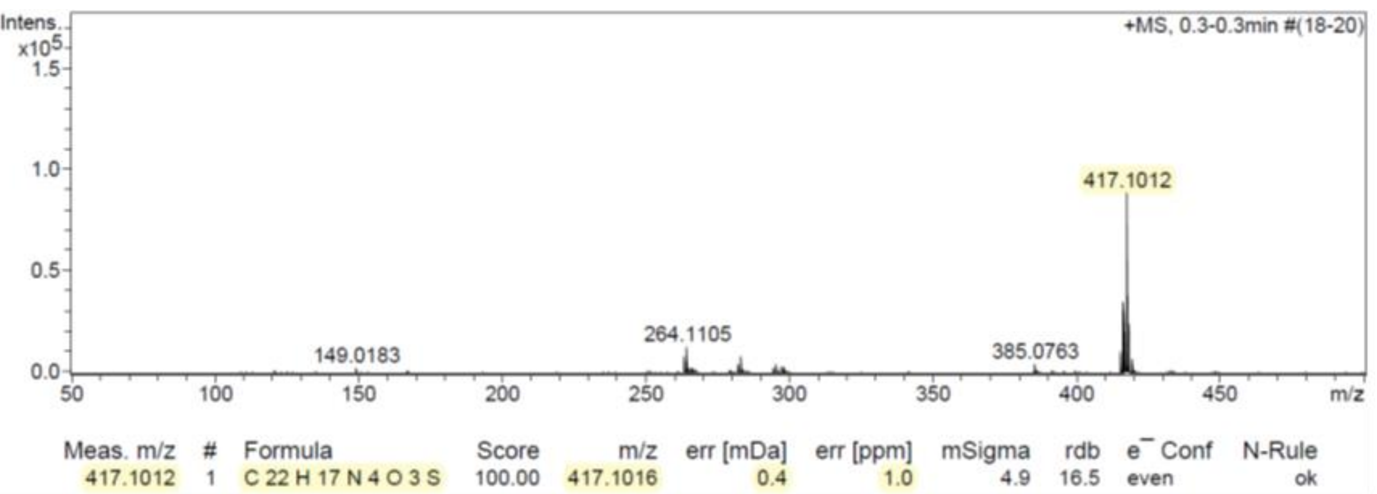
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
374.1061	1	C 20 H 16 N 5 O S	100.00	374.1070	0.9	2.5	5.7	15.5	even	ok

2-amino-4-(4-hydroxyphenyl)-6-((3-methylbenzyl)thio)pyridine-3,5-dicarbonitrile (**6n**)



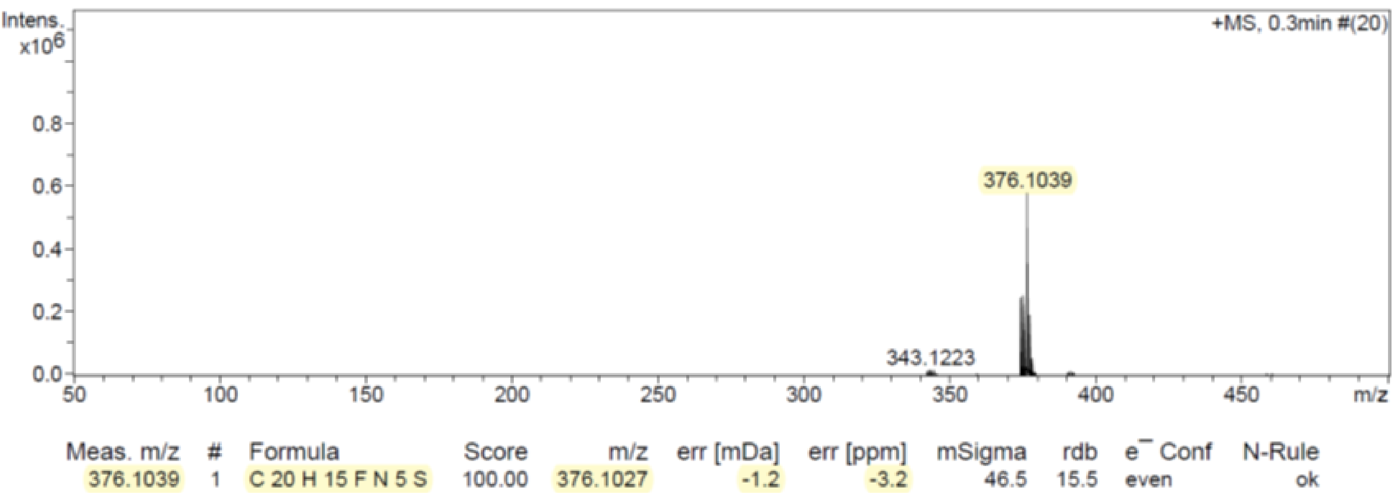
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
284.0342	1	C 15 H 10 N O 3 S	11.01	284.0376	3.4	12.0	8.3	11.5	even	ok
	2	C 18 H 6 N O 3	100.00	284.0342	0.1	0.2	19.2	16.5	even	ok
	3	C 14 H 10 N 3 S 2	10.85	284.0311	-3.1	-10.9	25.0	11.5	even	ok
373.1111	1	C 21 H 17 N 4 O S	100.00	373.1118	0.7	1.7	6.8	15.5	even	ok

3-(((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (**6o**)



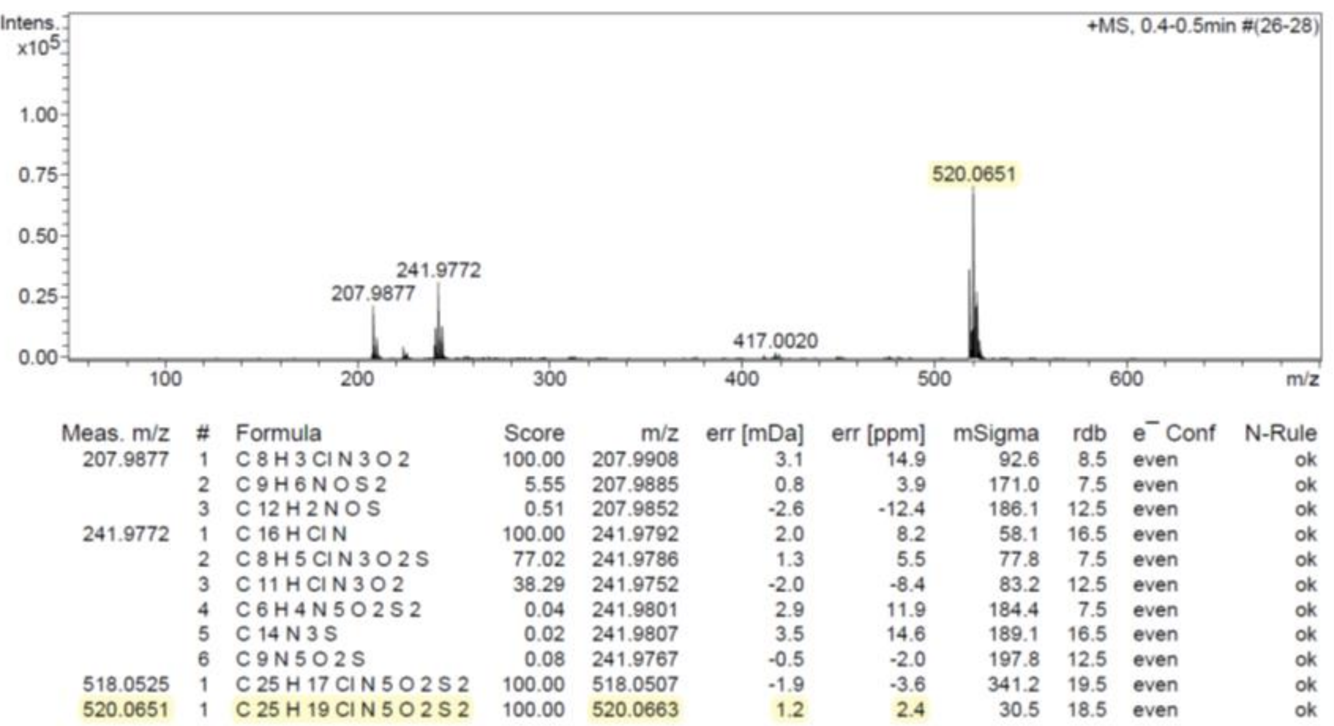
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
417.1012	1	C 22 H 17 N 4 O 3 S	100.00	417.1016	0.4	1.0	4.9	16.5	even	ok

2-amino-4-(4-fluorophenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (**6p**)



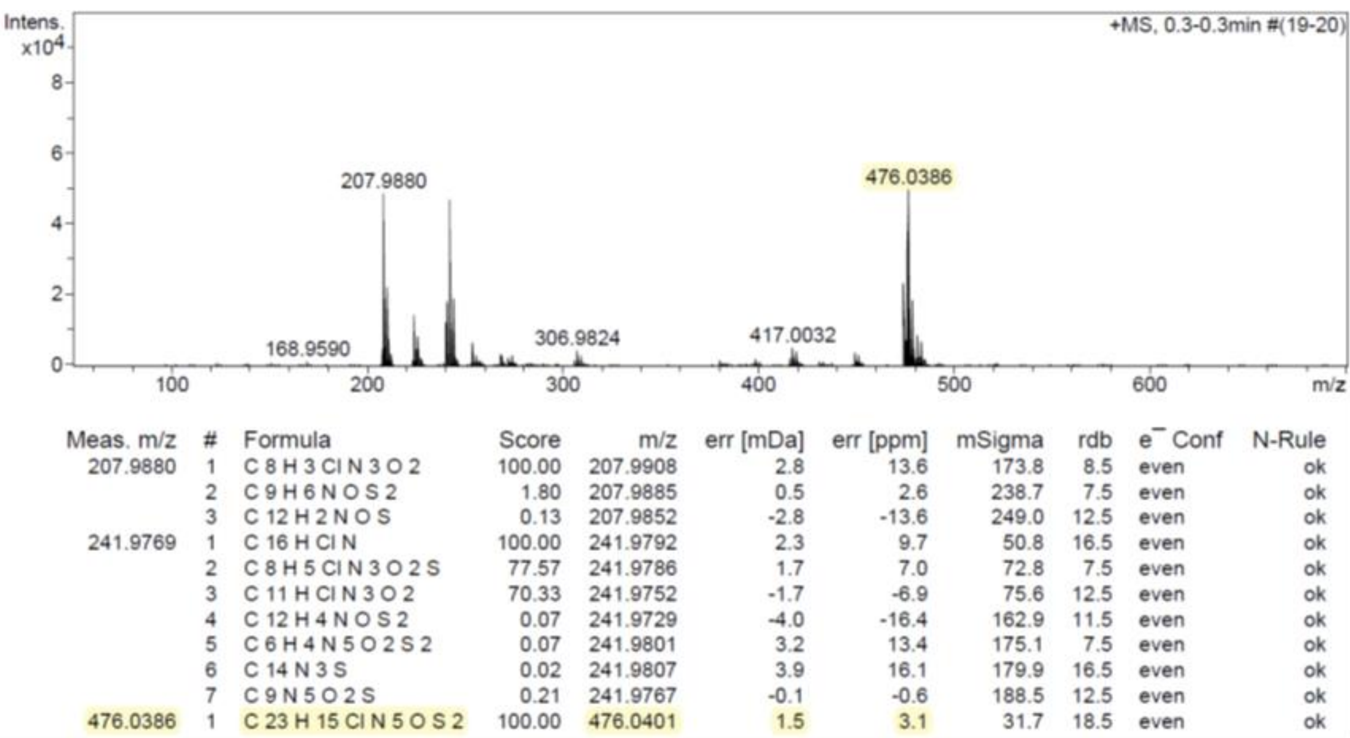
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
376.1039	1	C 20 H 15 F N 5 S	100.00	376.1027	-1.2	-3.2	46.5	15.5	even	ok

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-(2-hydroxyethoxy)phenyl)pyridine-3,5-dicarbonitrile (**6q**)



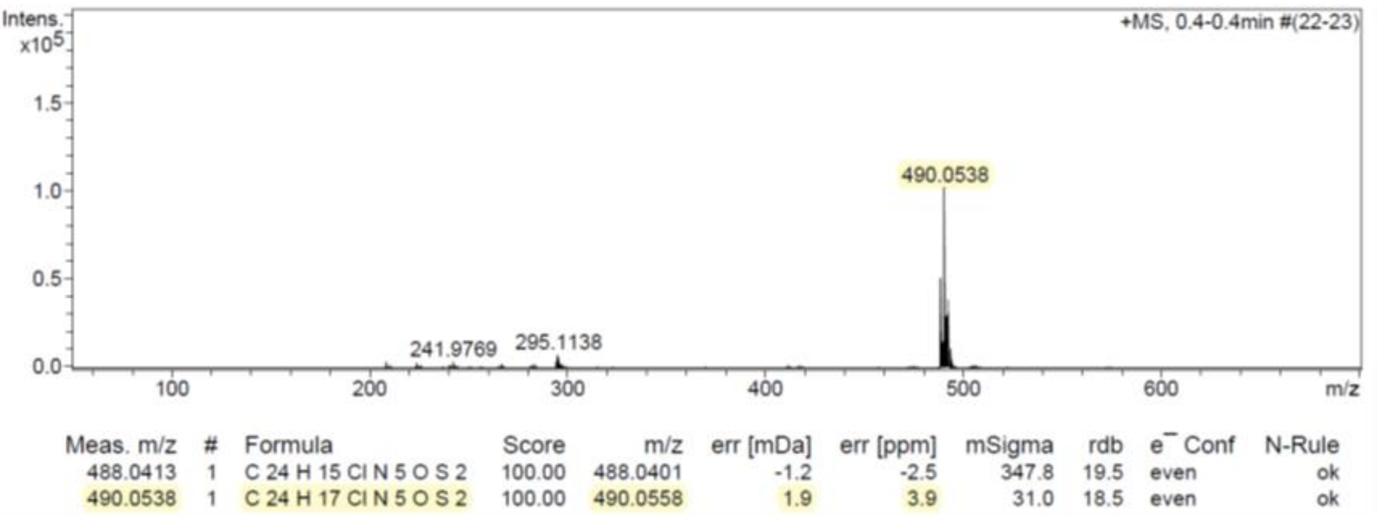
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
207.9877	1	C 8 H 3 Cl N 3 O 2	100.00	207.9908	3.1	14.9	92.6	8.5	even	ok
	2	C 9 H 6 N O S 2	5.55	207.9885	0.8	3.9	171.0	7.5	even	ok
	3	C 12 H 2 N O S	0.51	207.9852	-2.6	-12.4	186.1	12.5	even	ok
241.9772	1	C 16 H Cl N	100.00	241.9792	2.0	8.2	58.1	16.5	even	ok
	2	C 8 H 5 Cl N 3 O 2 S	77.02	241.9786	1.3	5.5	77.8	7.5	even	ok
	3	C 11 H Cl N 3 O 2	38.29	241.9752	-2.0	-8.4	83.2	12.5	even	ok
	4	C 6 H 4 N 5 O 2 S 2	0.04	241.9801	2.9	11.9	184.4	7.5	even	ok
	5	C 14 N 3 S	0.02	241.9807	3.5	14.6	189.1	16.5	even	ok
	6	C 9 N 5 O 2 S	0.08	241.9767	-0.5	-2.0	197.8	12.5	even	ok
518.0525	1	C 25 H 17 Cl N 5 O 2 S 2	100.00	518.0507	-1.9	-3.6	341.2	19.5	even	ok
520.0651	1	C 25 H 19 Cl N 5 O 2 S 2	100.00	520.0663	1.2	2.4	30.5	18.5	even	ok

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-hydroxyphenyl)pyridine-3,5-dicarbonitrile (**6r**)



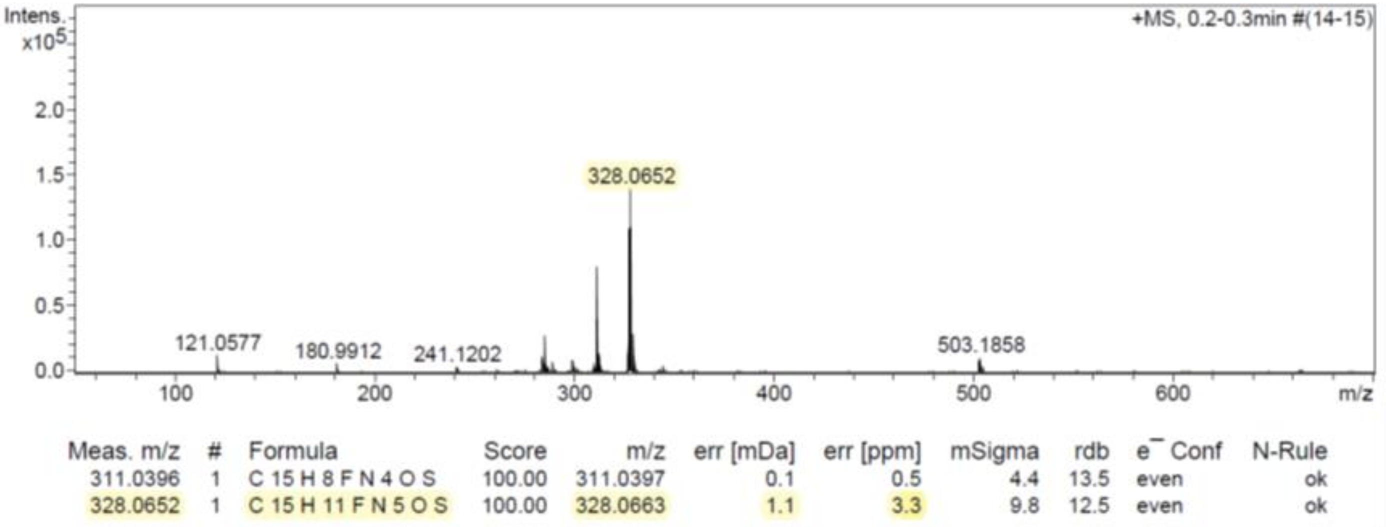
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
207.9880	1	C 8 H 3 Cl N 3 O 2	100.00	207.9908	2.8	13.6	173.8	8.5	even	ok
	2	C 9 H 6 N O S 2	1.80	207.9885	0.5	2.6	238.7	7.5	even	ok
	3	C 12 H 2 N O S	0.13	207.9852	-2.8	-13.6	249.0	12.5	even	ok
241.9769	1	C 16 H Cl N	100.00	241.9792	2.3	9.7	50.8	16.5	even	ok
	2	C 8 H 5 Cl N 3 O 2 S	77.57	241.9786	1.7	7.0	72.8	7.5	even	ok
	3	C 11 H Cl N 3 O 2	70.33	241.9752	-1.7	-6.9	75.6	12.5	even	ok
	4	C 12 H 4 N O S 2	0.07	241.9729	-4.0	-16.4	162.9	11.5	even	ok
	5	C 6 H 4 N 5 O 2 S 2	0.07	241.9801	3.2	13.4	175.1	7.5	even	ok
	6	C 14 N 3 S	0.02	241.9807	3.9	16.1	179.9	16.5	even	ok
	7	C 9 N 5 O 2 S	0.21	241.9767	-0.1	-0.6	188.5	12.5	even	ok
476.0386	1	C 23 H 15 Cl N 5 O S 2	100.00	476.0401	1.5	3.1	31.7	18.5	even	ok

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (**6s**)



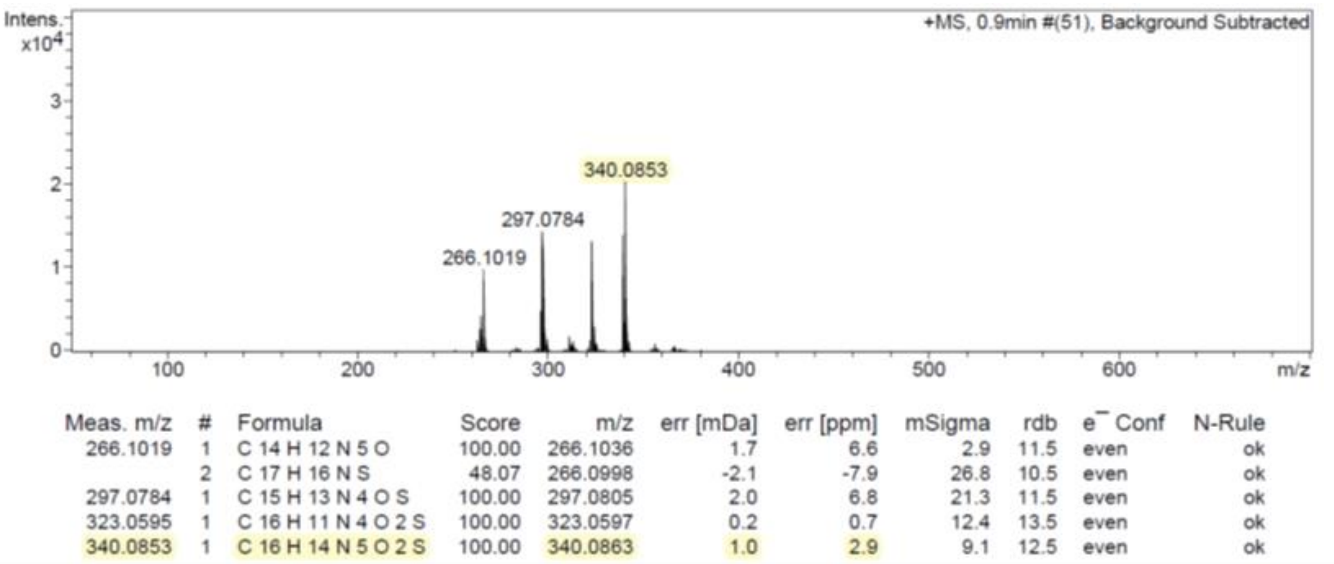
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
488.0413	1	C 24 H 15 Cl N 5 O S 2	100.00	488.0401	-1.2	-2.5	347.8	19.5	even	ok
490.0538	1	C 24 H 17 Cl N 5 O S 2	100.00	490.0558	1.9	3.9	31.0	18.5	even	ok

3,6-diamino-5-cyano-4-(4-fluorophenyl)thieno[2,3-b]pyridine-2-carboxamide (**7a**)



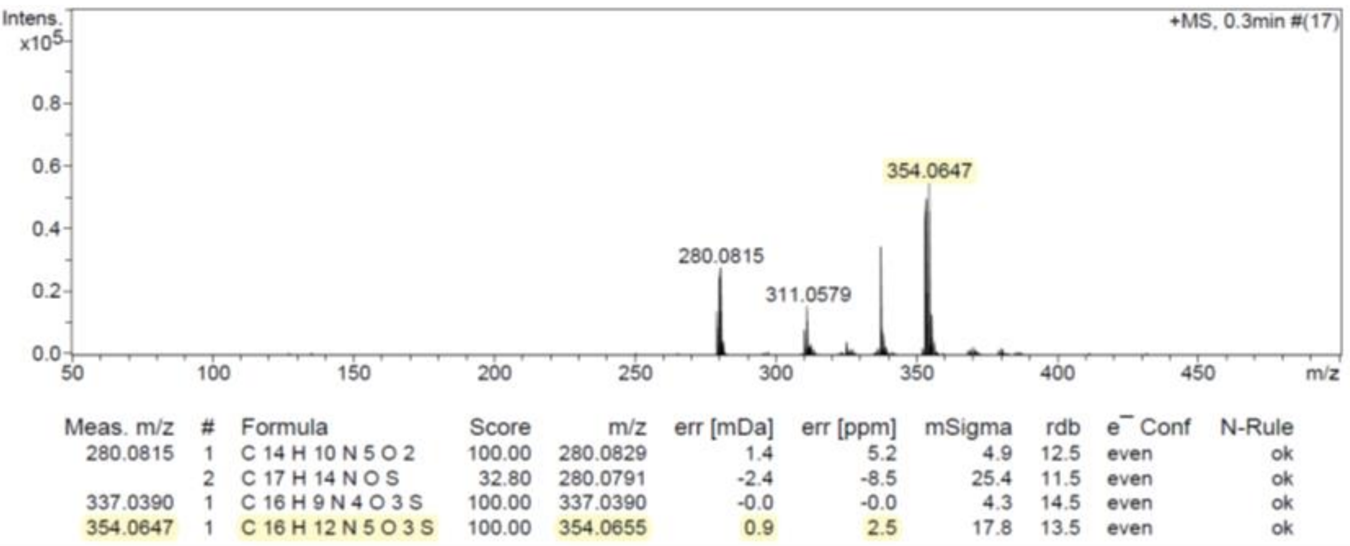
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
311.0396	1	C 15 H 8 F N 4 O S	100.00	311.0397	0.1	0.5	4.4	13.5	even	ok
328.0652	1	C 15 H 11 F N 5 O S	100.00	328.0663	1.1	3.3	9.8	12.5	even	ok

3,6-diamino-5-cyano-4-(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (**7b**)



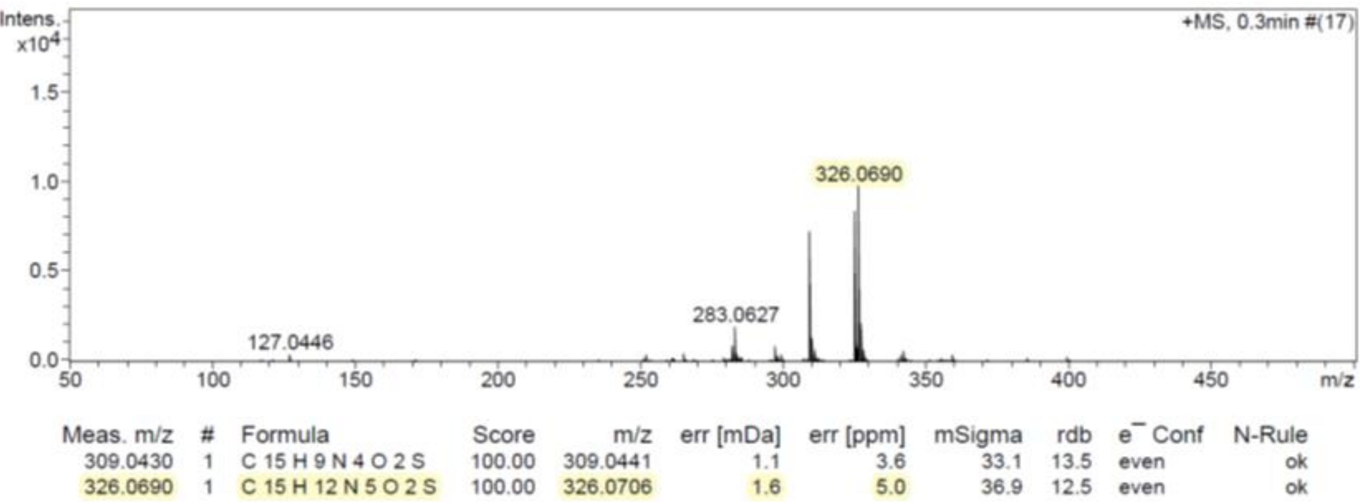
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
266.1019	1	C 14 H 12 N 5 O	100.00	266.1036	1.7	6.6	2.9	11.5	even	ok
	2	C 17 H 16 N S	48.07	266.0998	-2.1	-7.9	26.8	10.5	even	ok
297.0784	1	C 15 H 13 N 4 O S	100.00	297.0805	2.0	6.8	21.3	11.5	even	ok
323.0595	1	C 16 H 11 N 4 O 2 S	100.00	323.0597	0.2	0.7	12.4	13.5	even	ok
340.0853	1	C 16 H 14 N 5 O 2 S	100.00	340.0863	1.0	2.9	9.1	12.5	even	ok

3,6-diamino-4-(benzo[d][1,3]dioxol-5-yl)-5-cyanothieno[2,3-b]pyridine-2-carboxamide (**7c**)



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
280.0815	1	C 14 H 10 N 5 O 2	100.00	280.0829	1.4	5.2	4.9	12.5	even	ok
	2	C 17 H 14 N O S	32.80	280.0791	-2.4	-8.5	25.4	11.5	even	ok
337.0390	1	C 16 H 9 N 4 O 3 S	100.00	337.0390	-0.0	-0.0	4.3	14.5	even	ok
354.0647	1	C 16 H 12 N 5 O 3 S	100.00	354.0655	0.9	2.5	17.8	13.5	even	ok

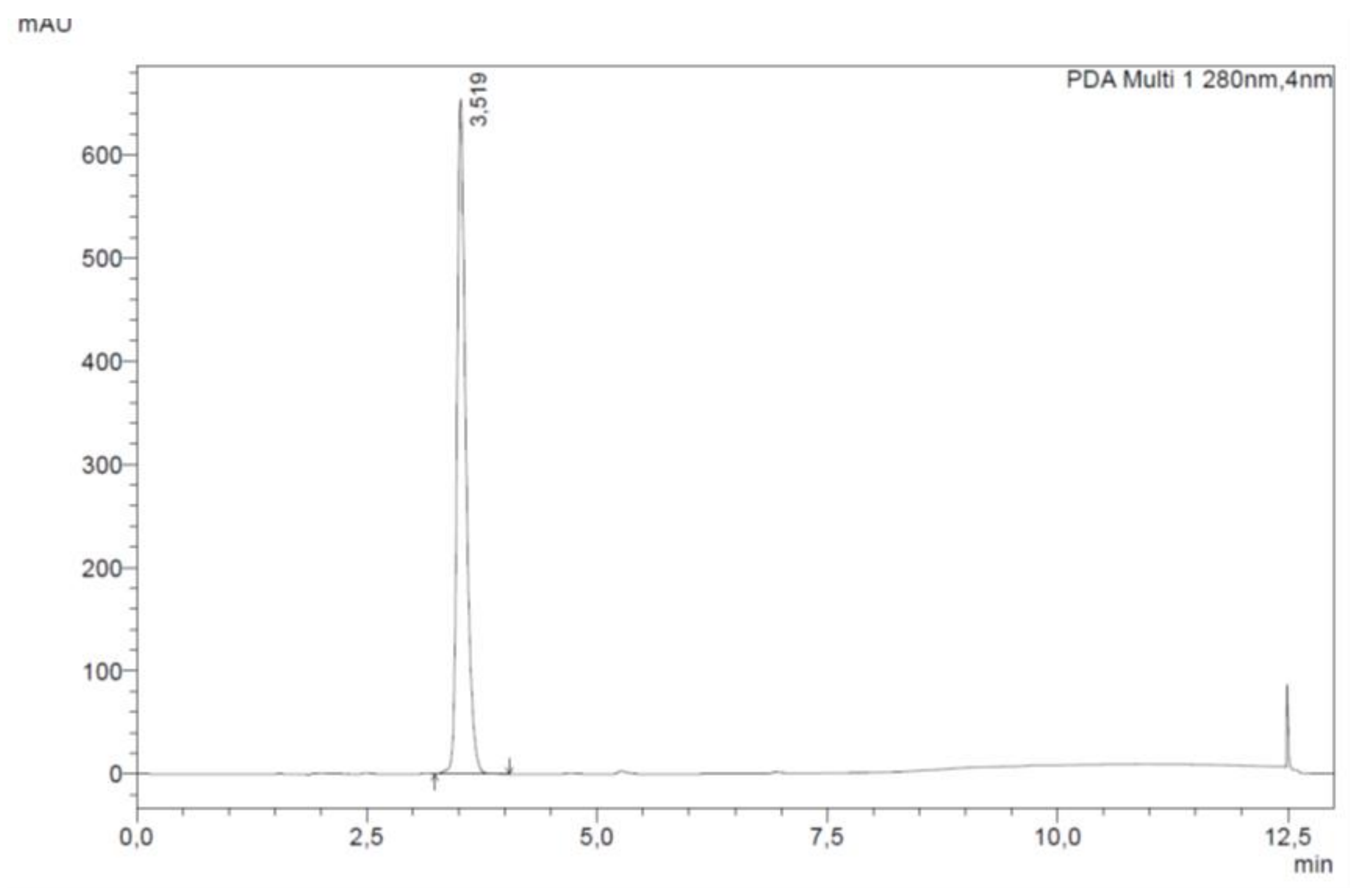
3,6-diamino-5-cyano-4-(3-hydroxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (**7d**)



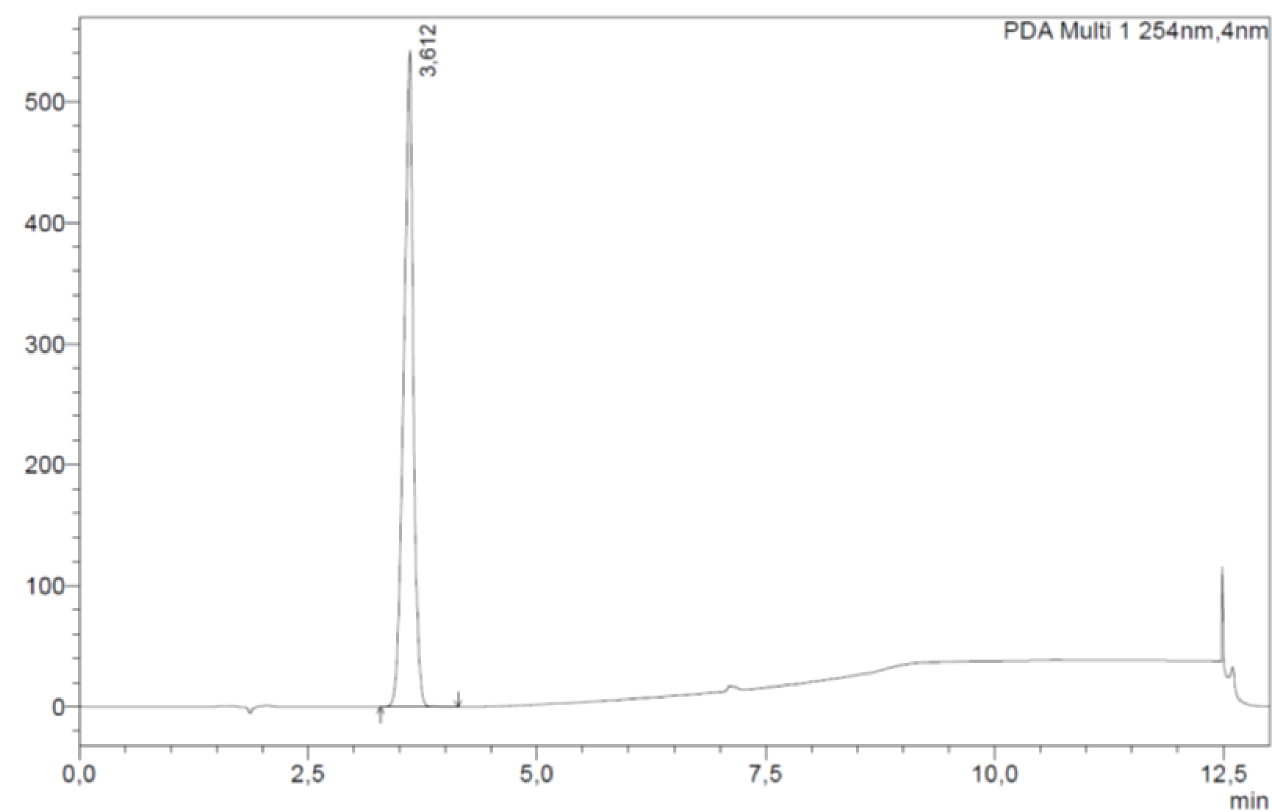
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
309.0430	1	C 15 H 9 N 4 O 2 S	100.00	309.0441	1.1	3.6	33.1	13.5	even	ok
326.0690	1	C 15 H 12 N 5 O 2 S	100.00	326.0706	1.6	5.0	36.9	12.5	even	ok

3. HLPC CHROMATOFGRAMS of test compounds

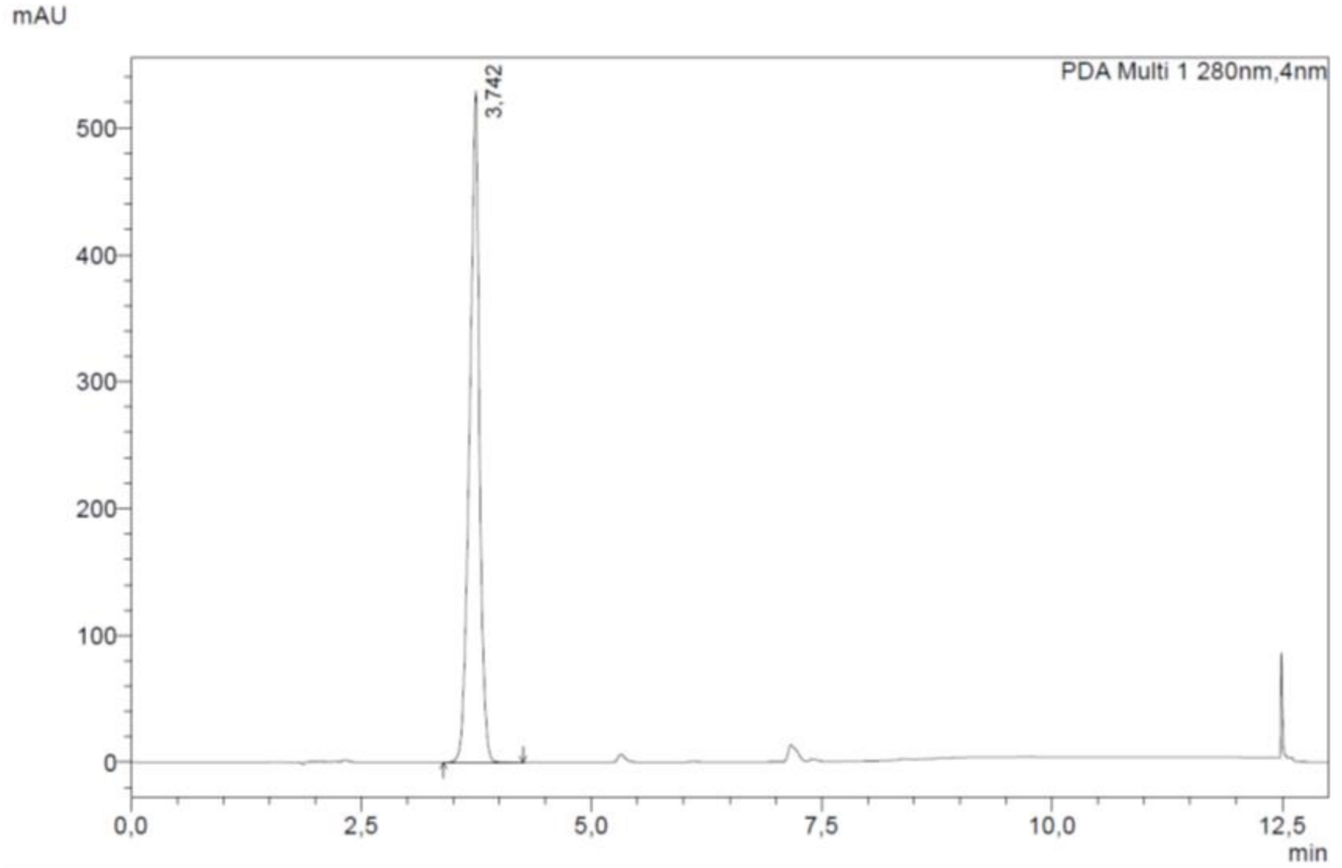
2-((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)acetamide (6a)



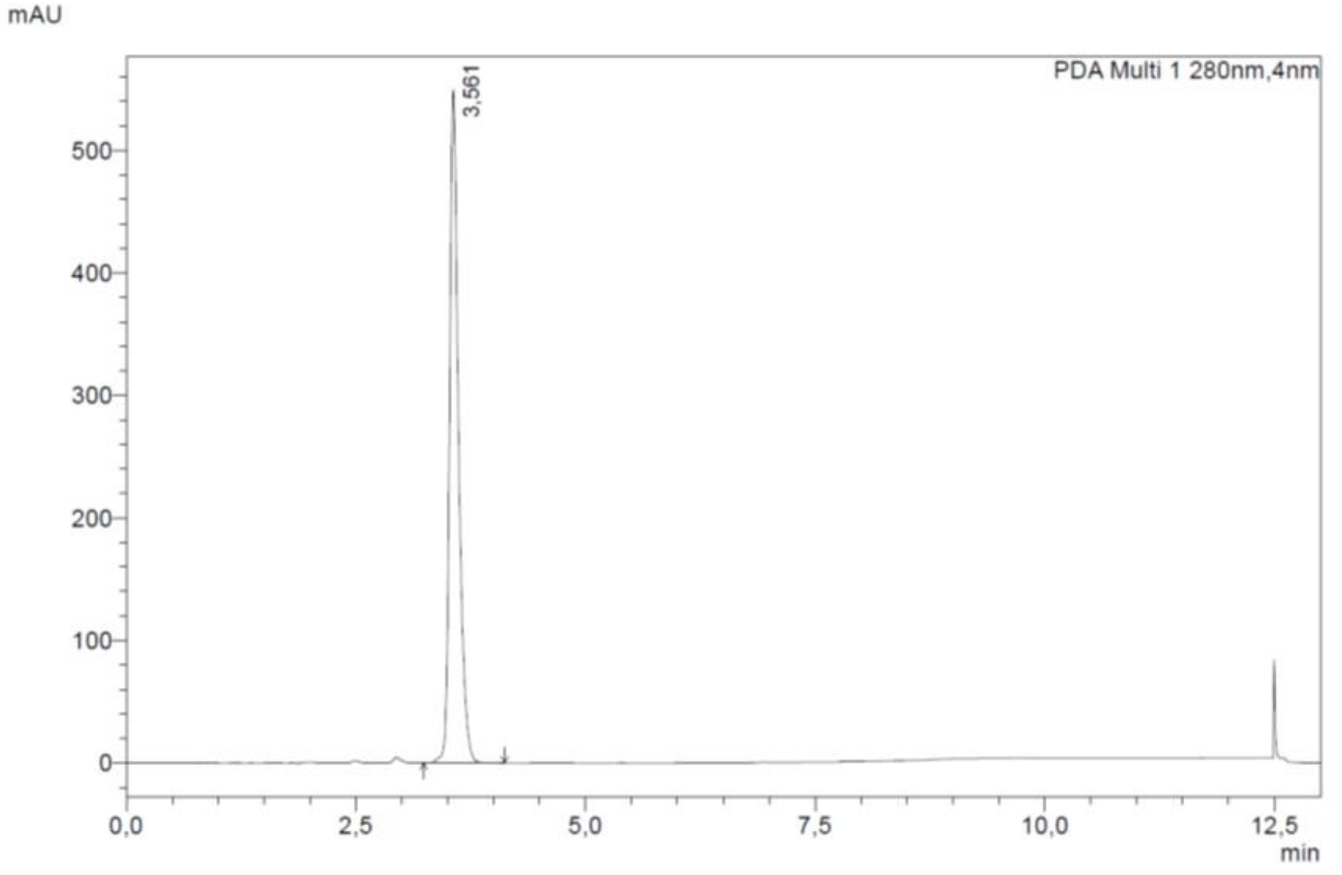
2-amino-4-(4-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6b)



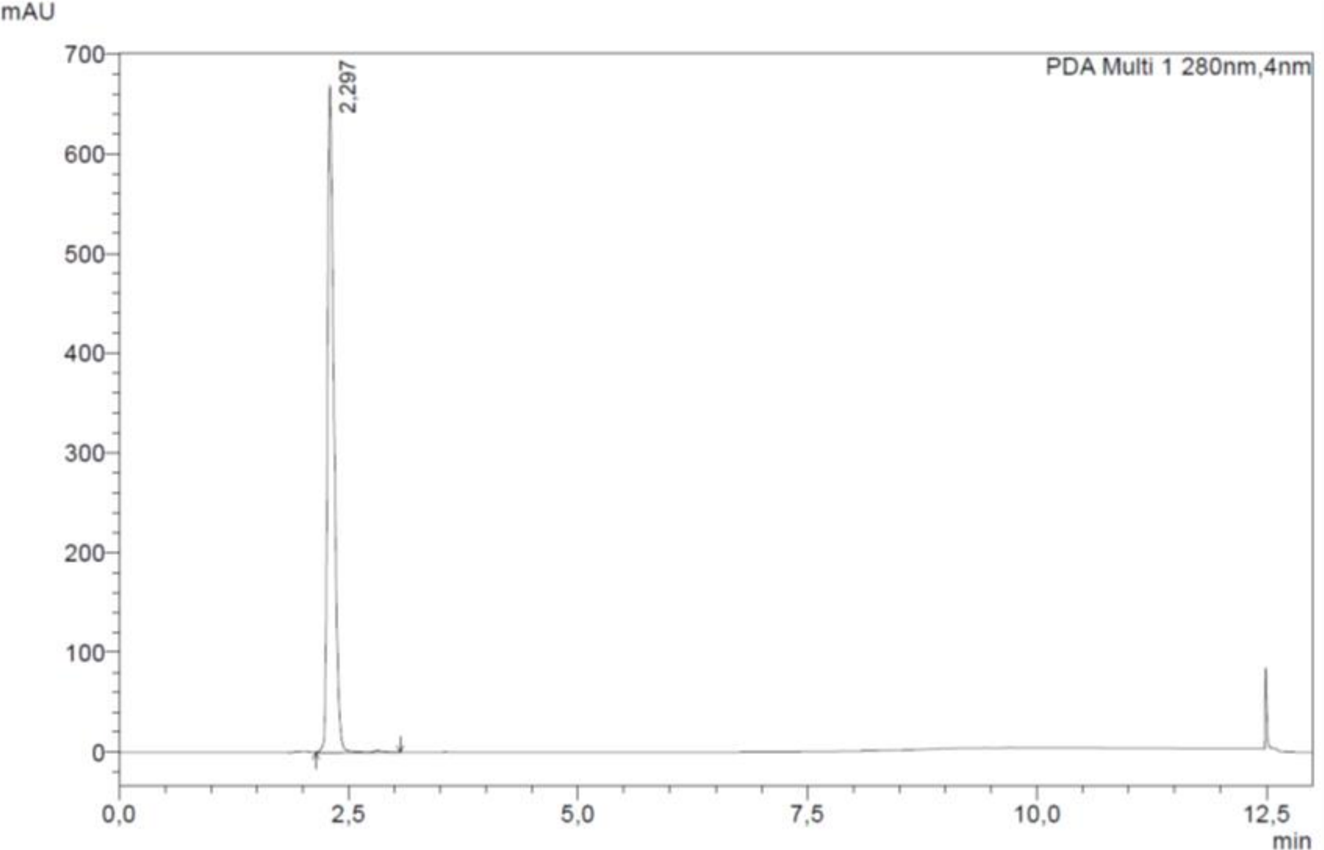
2-amino-4-(3-methoxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6c)



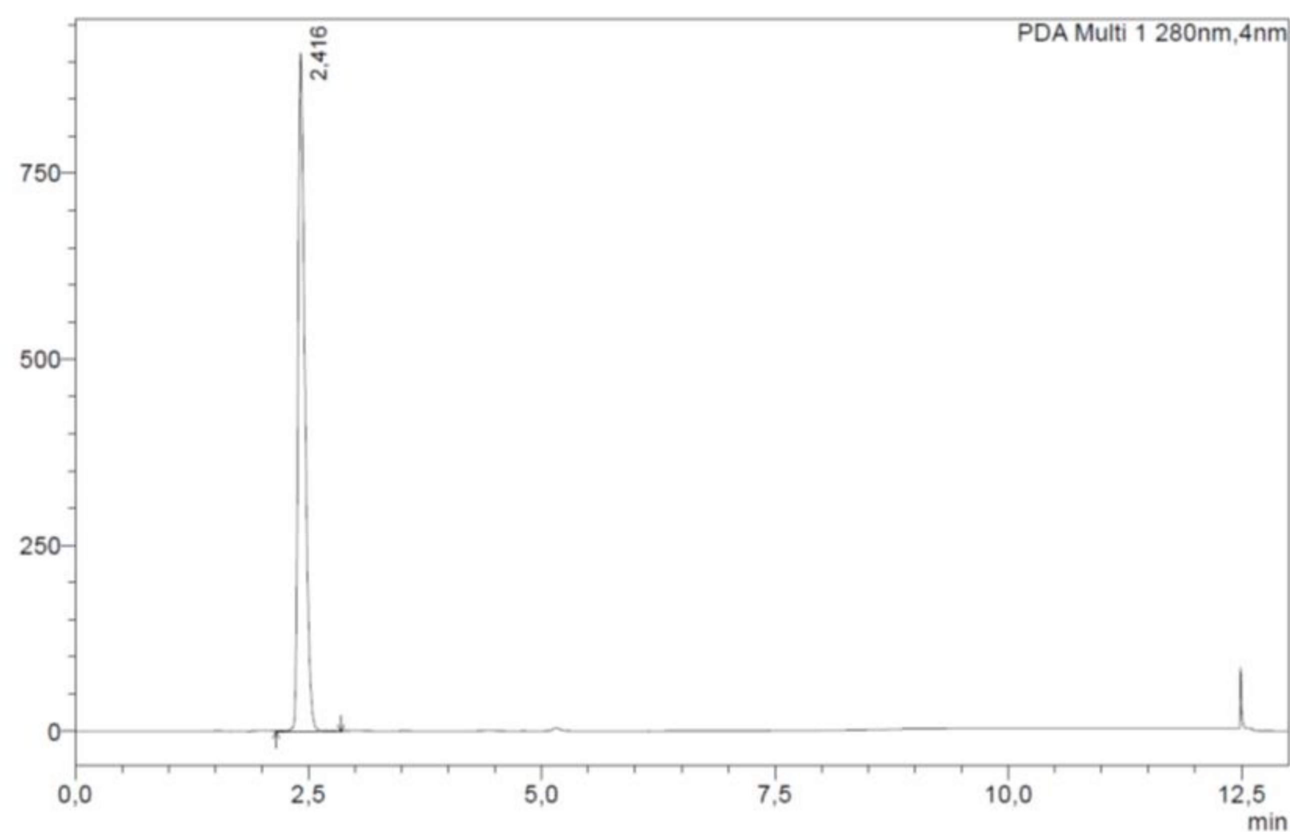
2-((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)acetamide(6d)



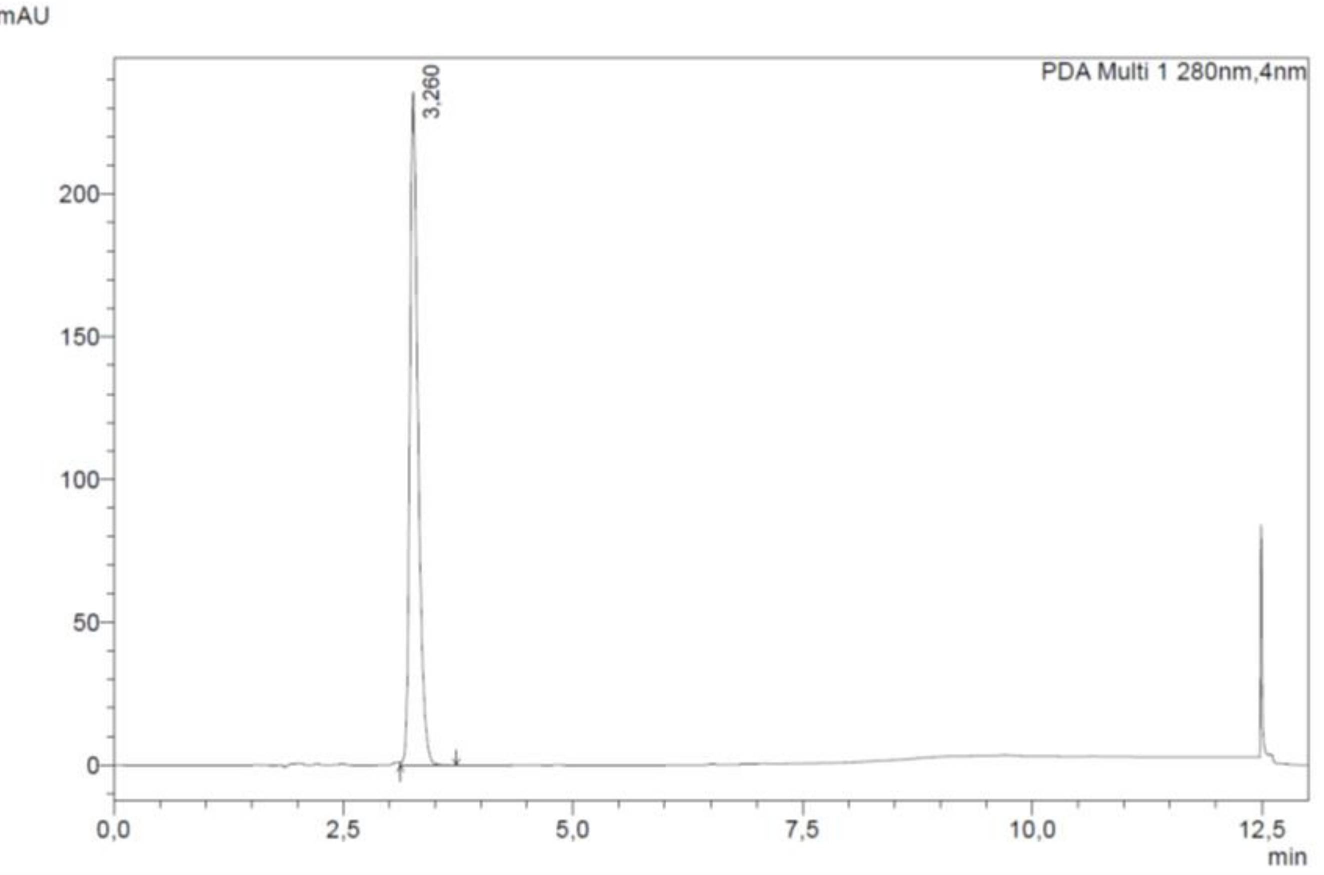
2-((6-amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-yl)thio)acetamide (6e)



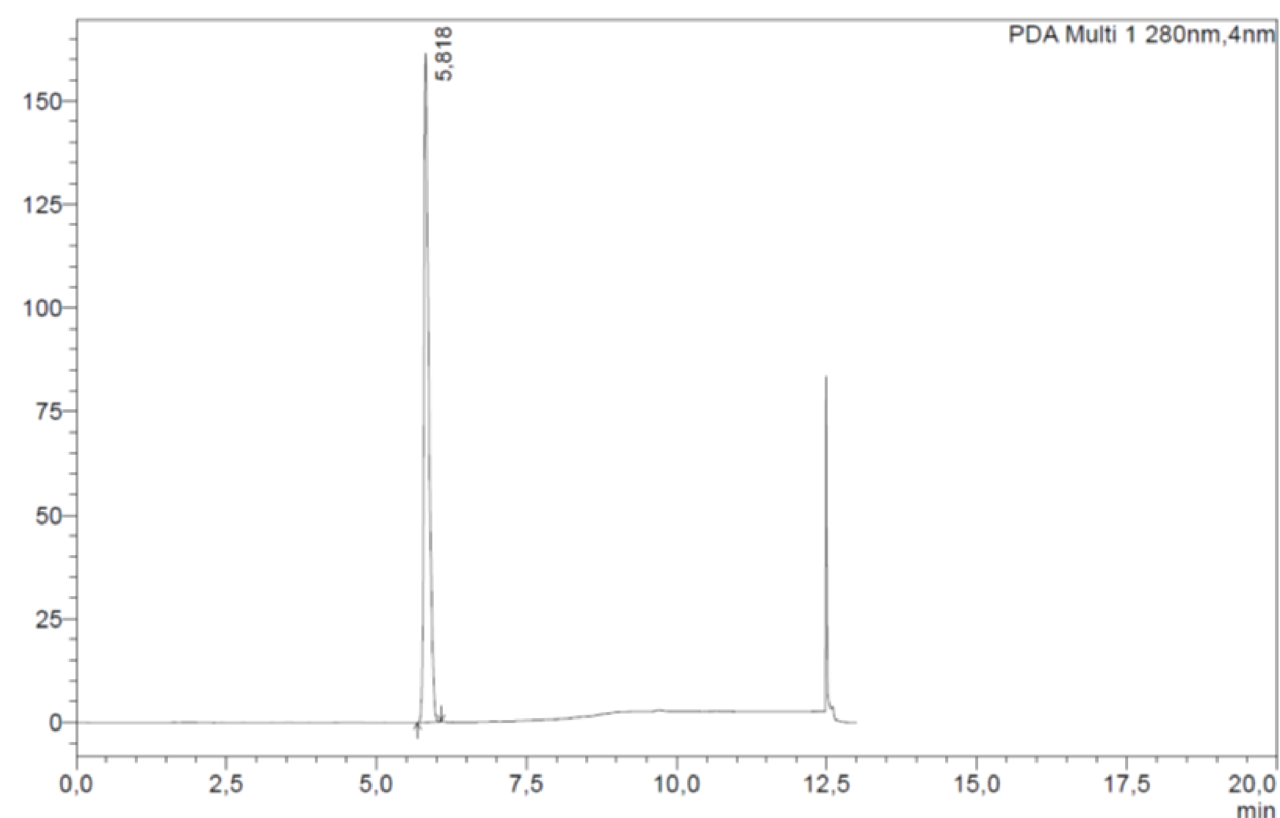
2-((6-amino-3,5-dicyano-4-(3-hydroxyphenyl)pyridin-2-yl)thio)acetamide (6f)



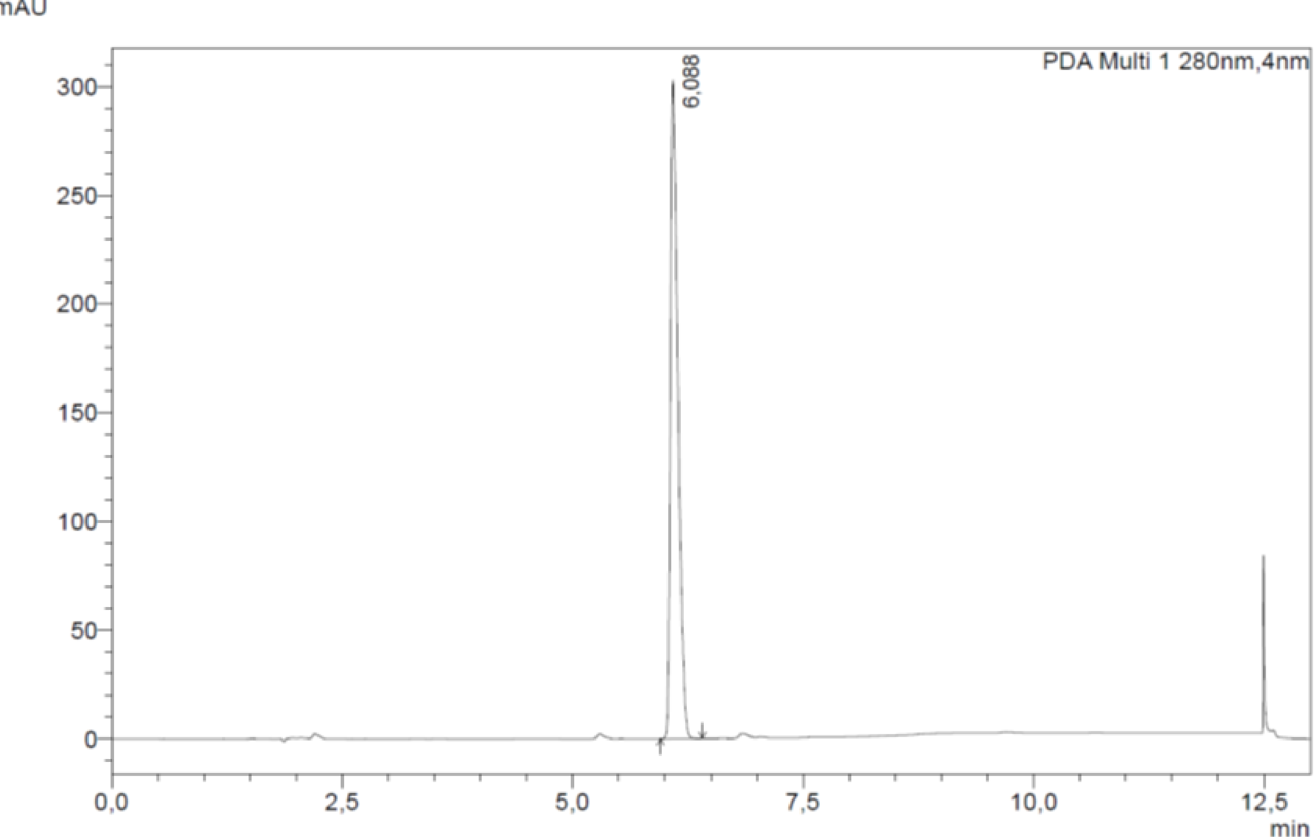
2-((6-amino-4-(benzo[d][1,3]dioxol-5-yl)-3,5-dicyanopyridin-2-yl)thio)acetamide (6g)



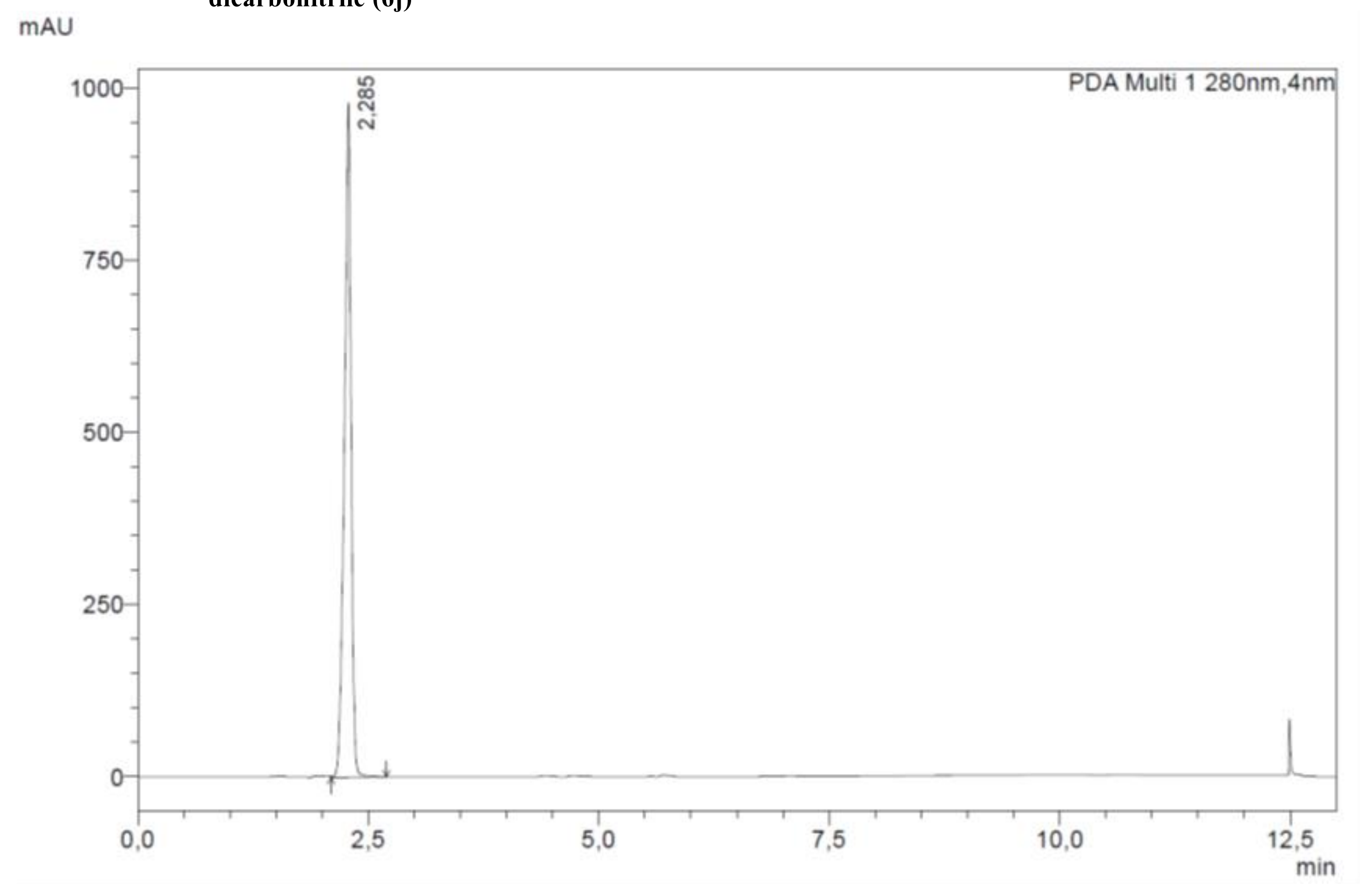
3-(((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzoic acid (6h)



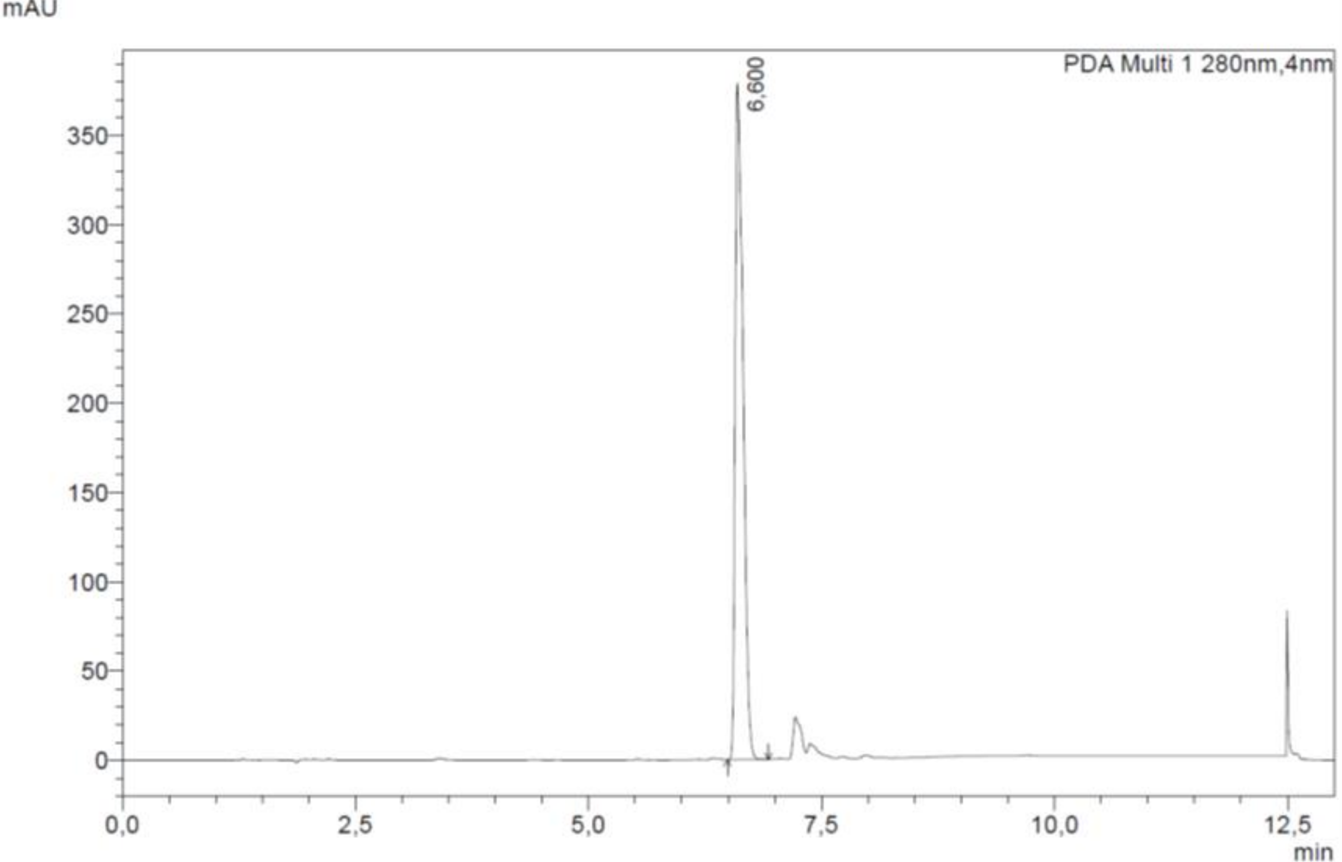
3-(((6-amino-3,5-dicyano-4-phenylpyridin-2-yl)thio)methyl)benzoic acid (6i-GN-010)



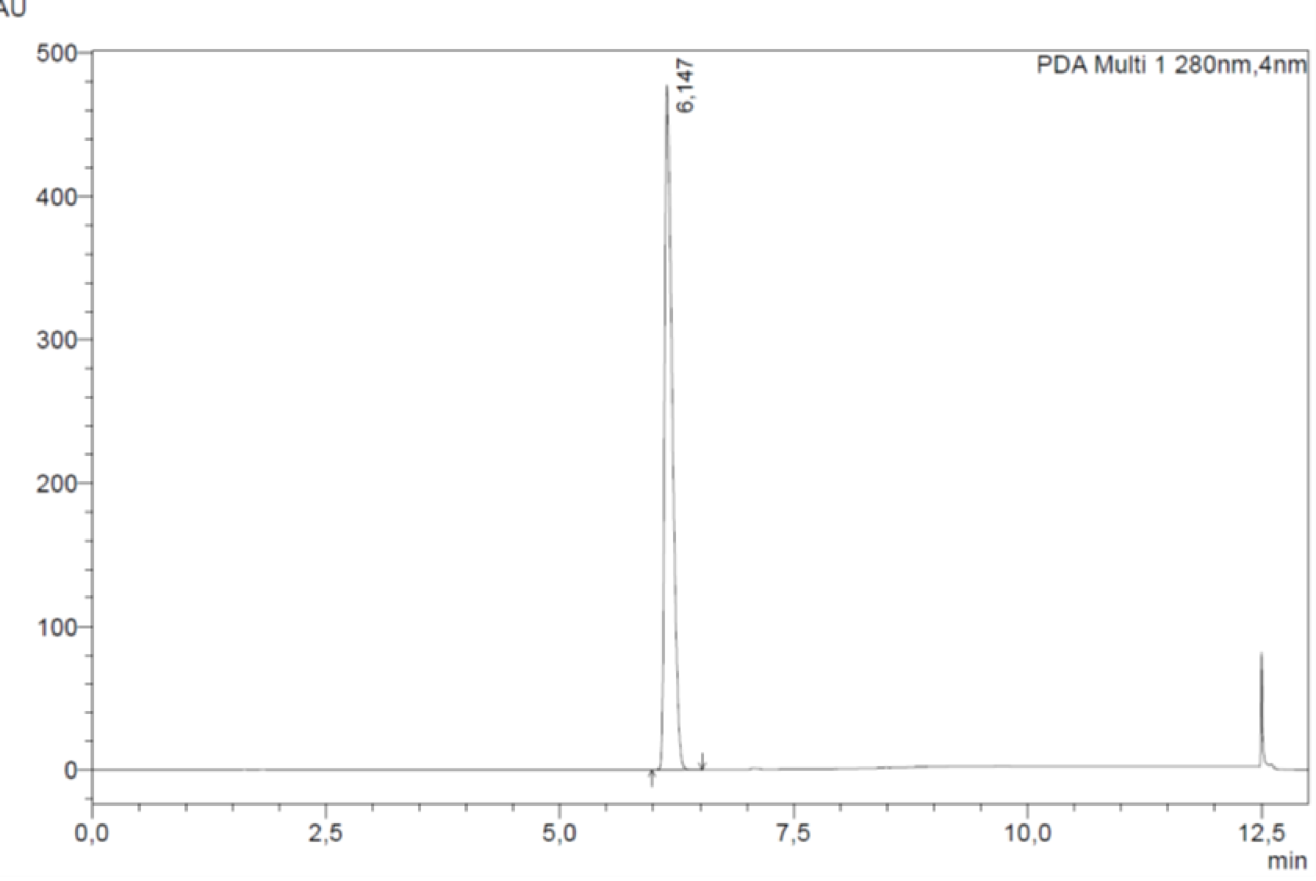
2-amino-4-(3-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6j)



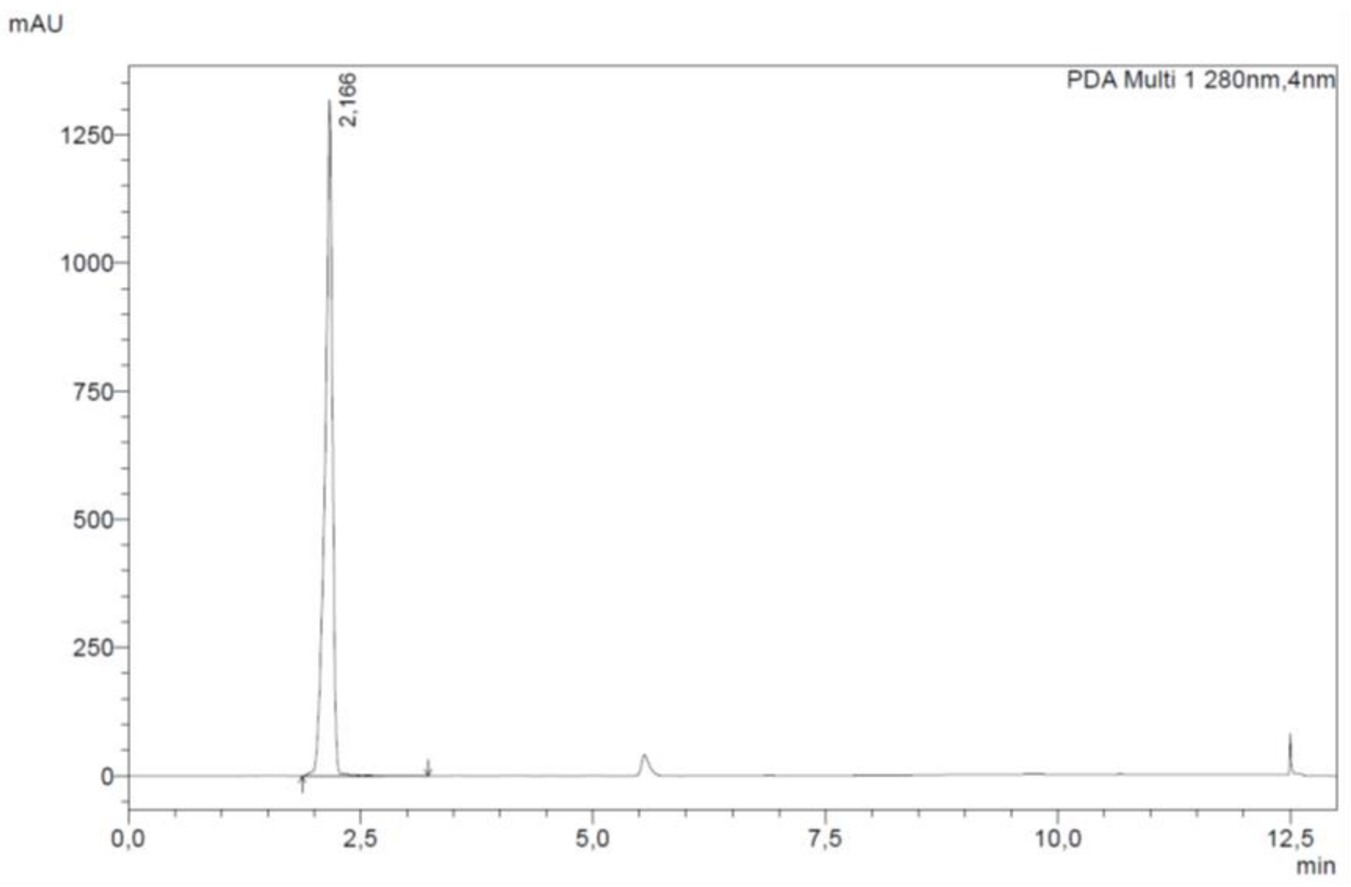
3-(((6-amino-3,5-dicyano-4-(4-(methylthio)phenyl)pyridin-2-yl)thio)methyl)benzoic acid (6k)



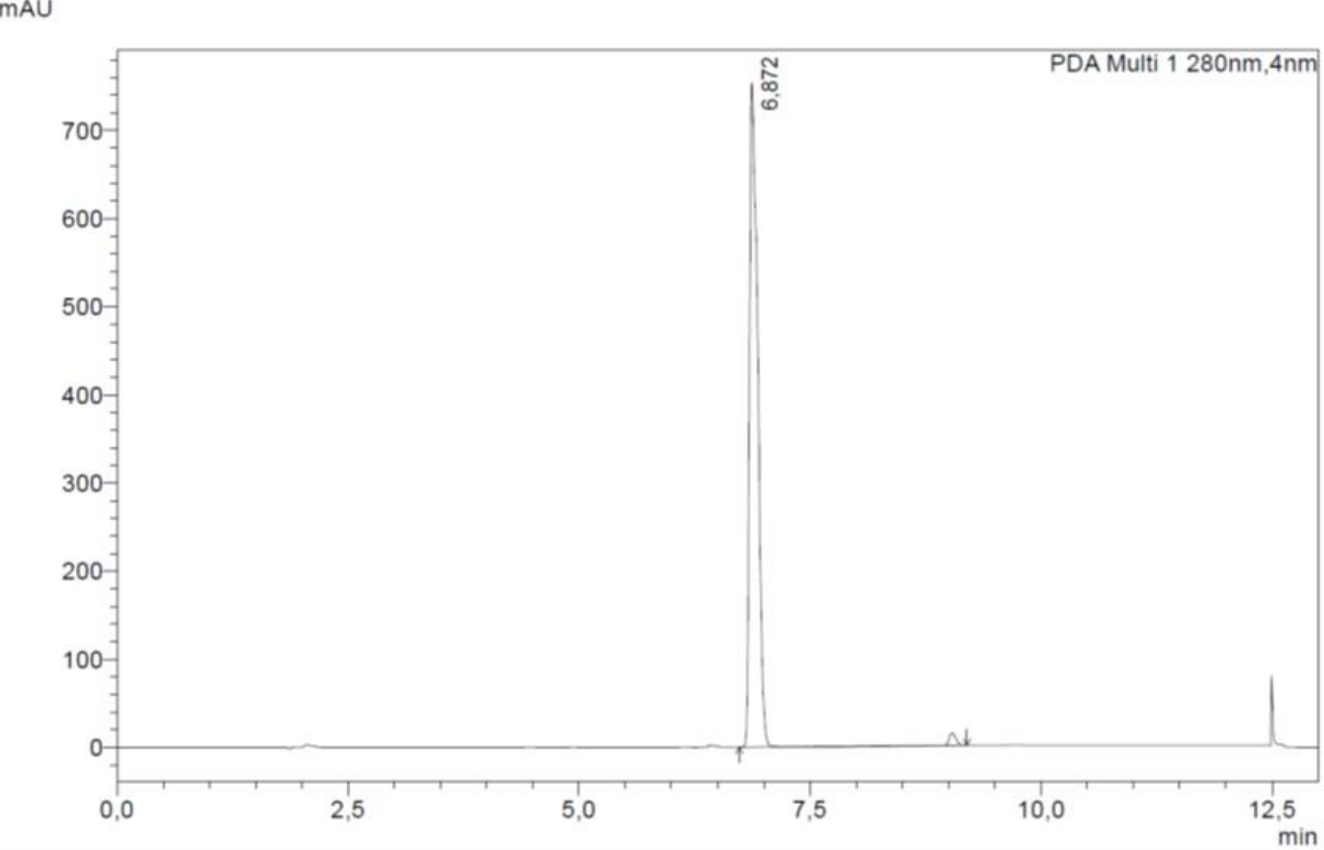
3-(((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (6l)



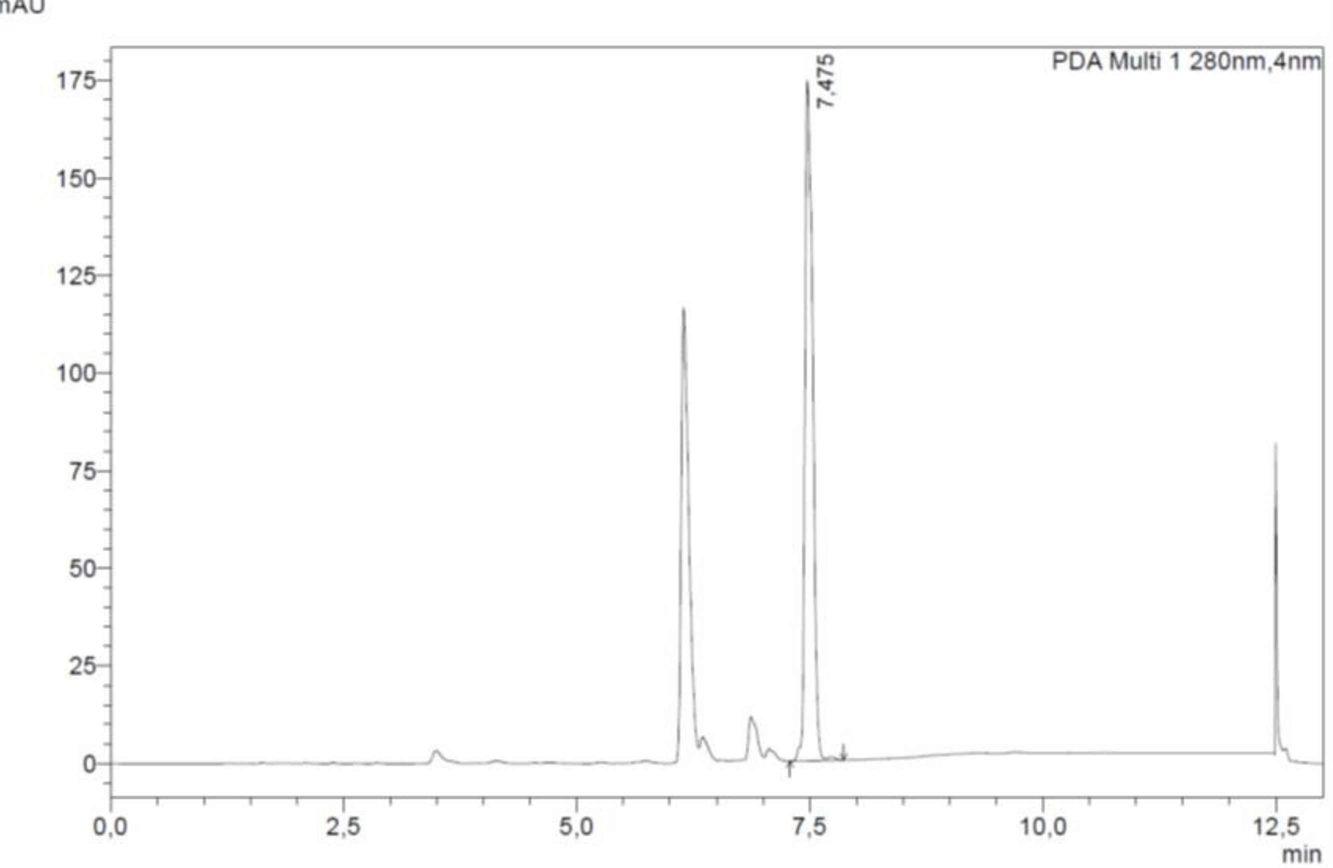
2-amino-4-(4-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6m)



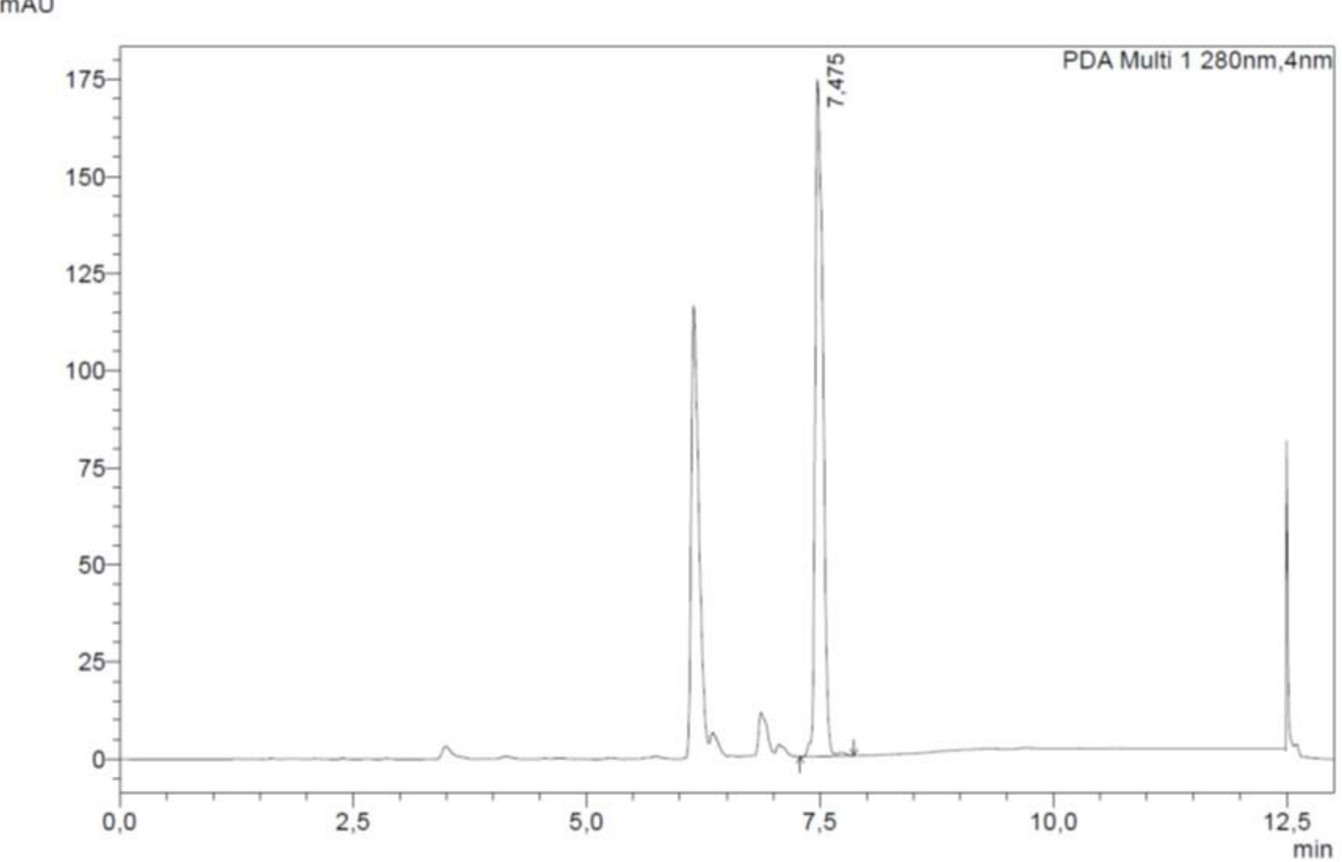
2-amino-4-(4-hydroxyphenyl)-6-((3-methylbenzyl)thio)pyridine-3,5-dicarbonitrile (6n)



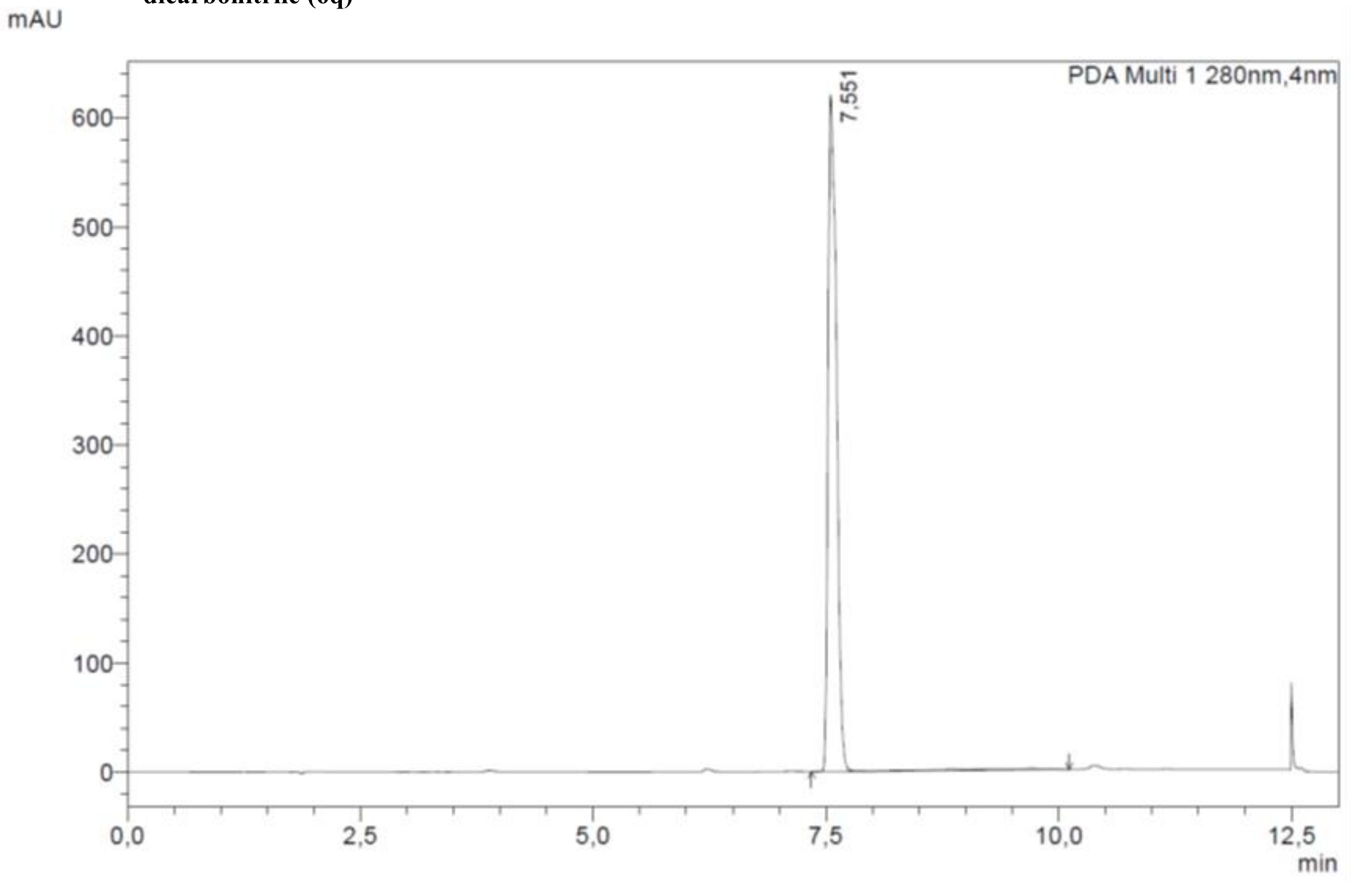
3-(((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (6o)



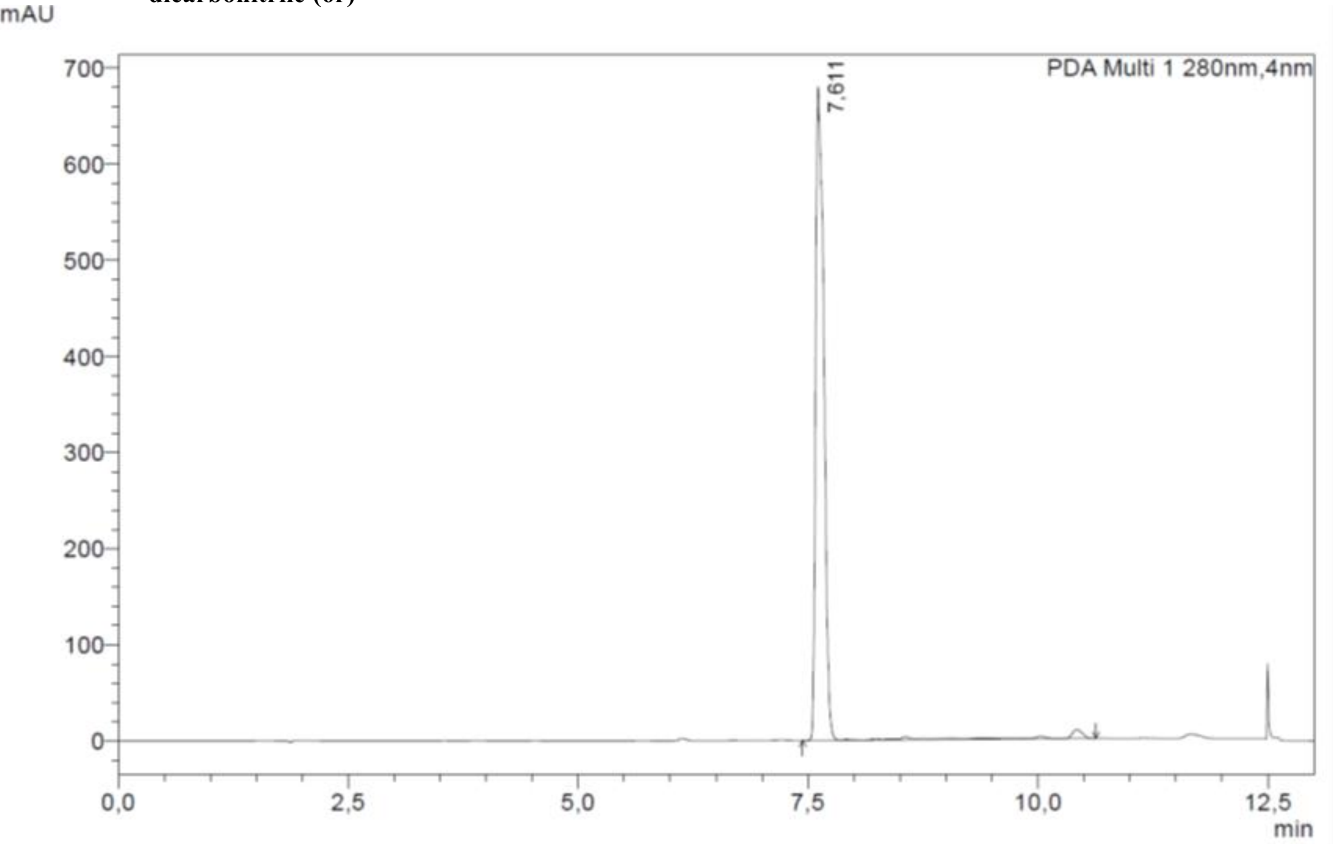
2-amino-4-(4-fluorophenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6p)



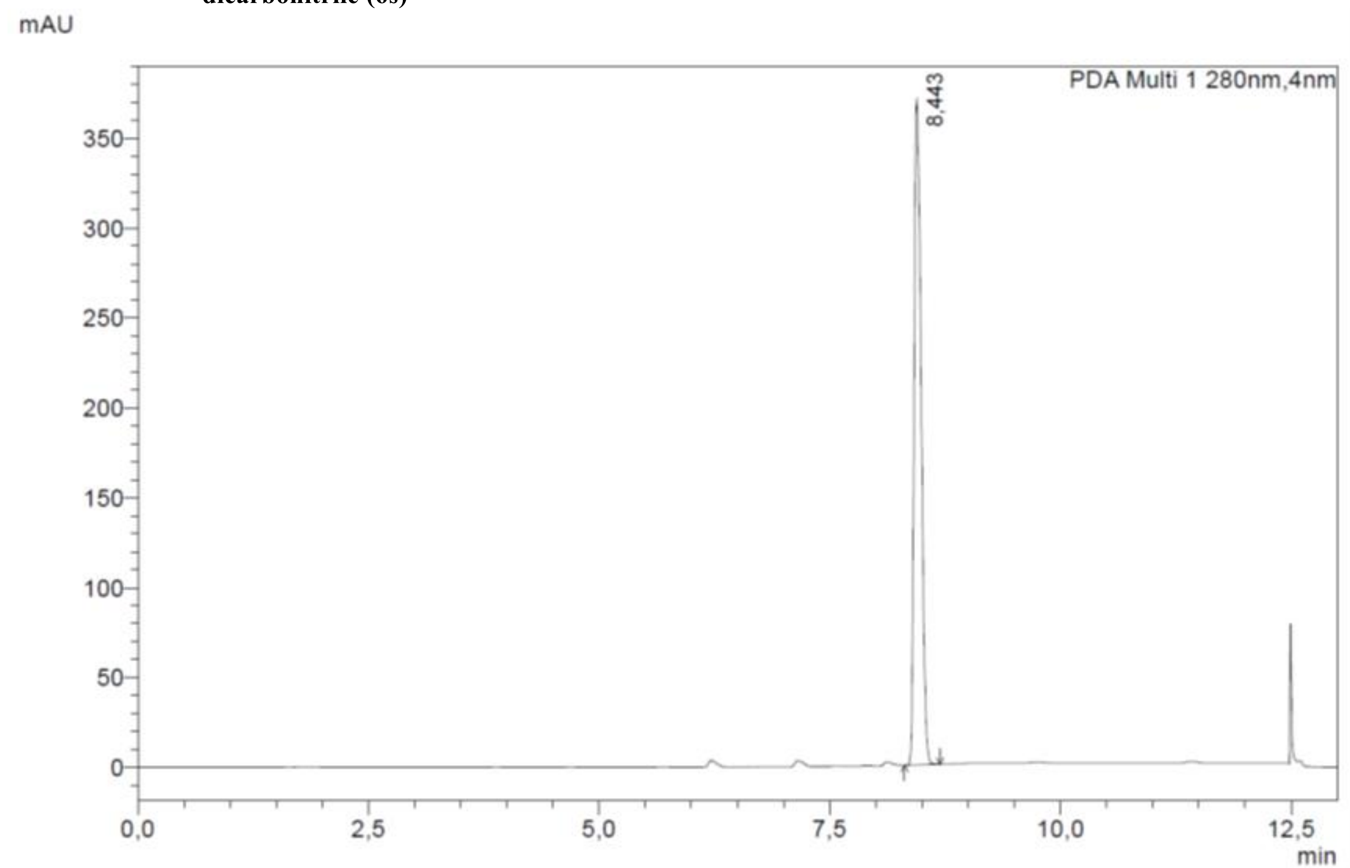
2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-(2-hydroxyethoxy)phenyl)pyridine-3,5-dicarbonitrile (6q)



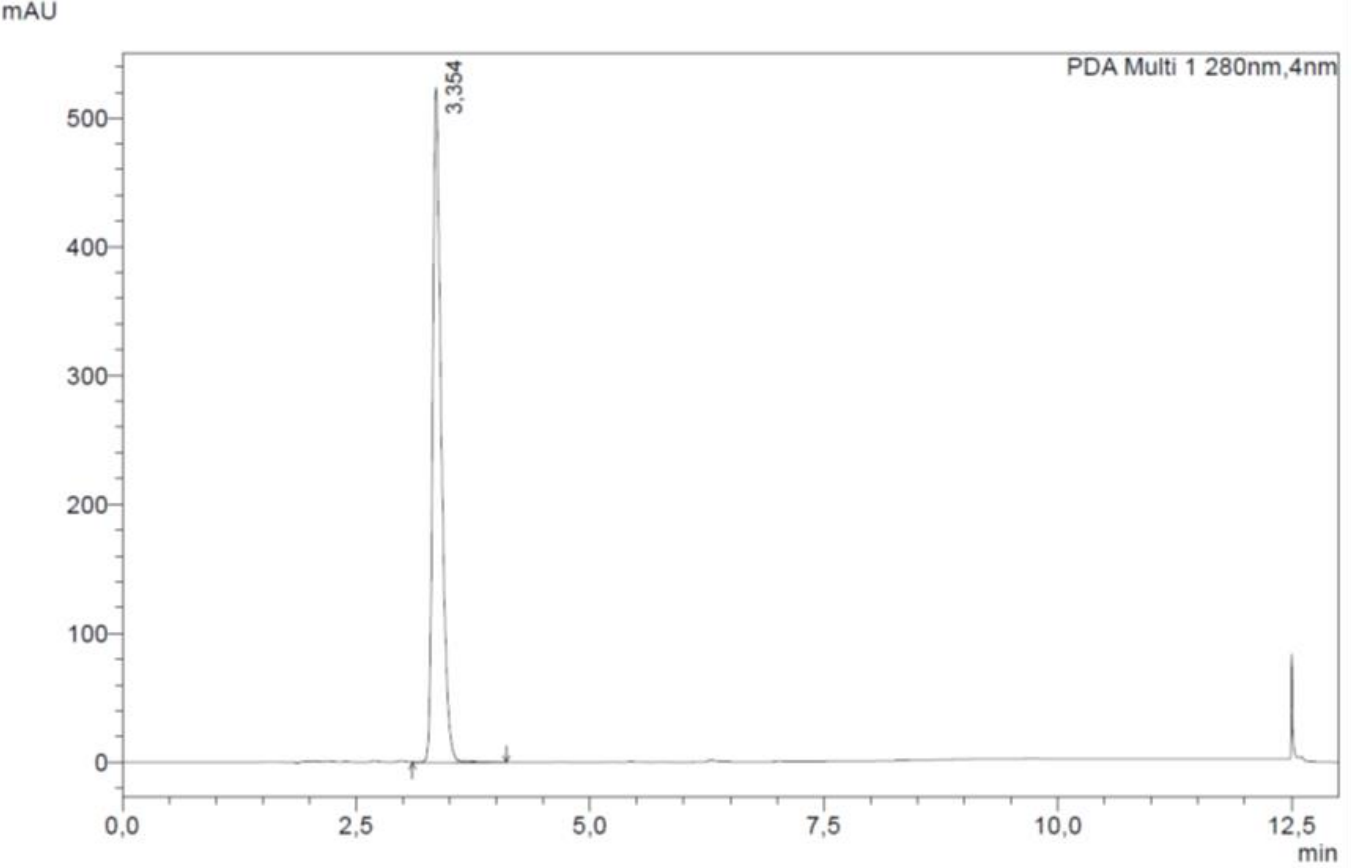
2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-hydroxyphenyl)pyridine-3,5-dicarbonitrile (6r)



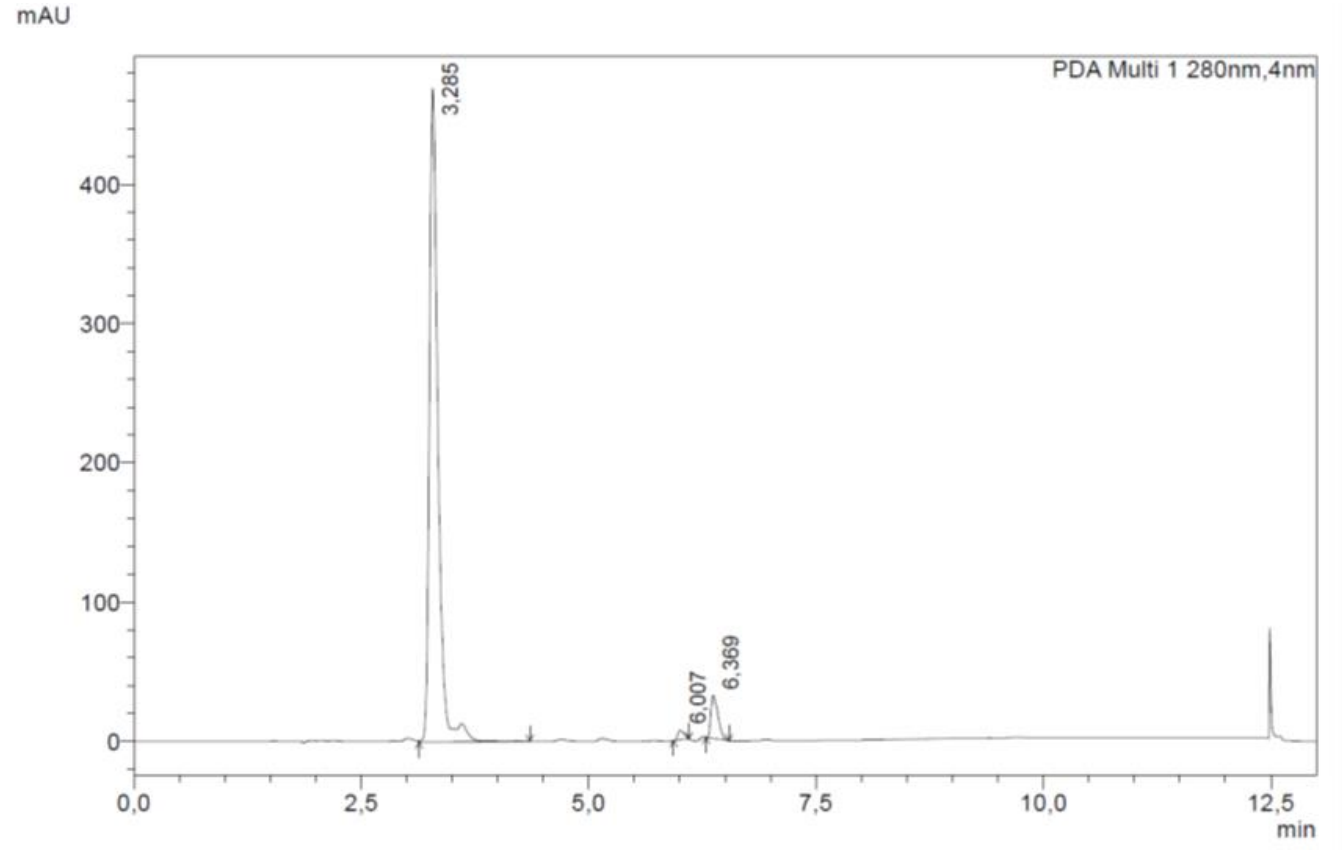
2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (6s)



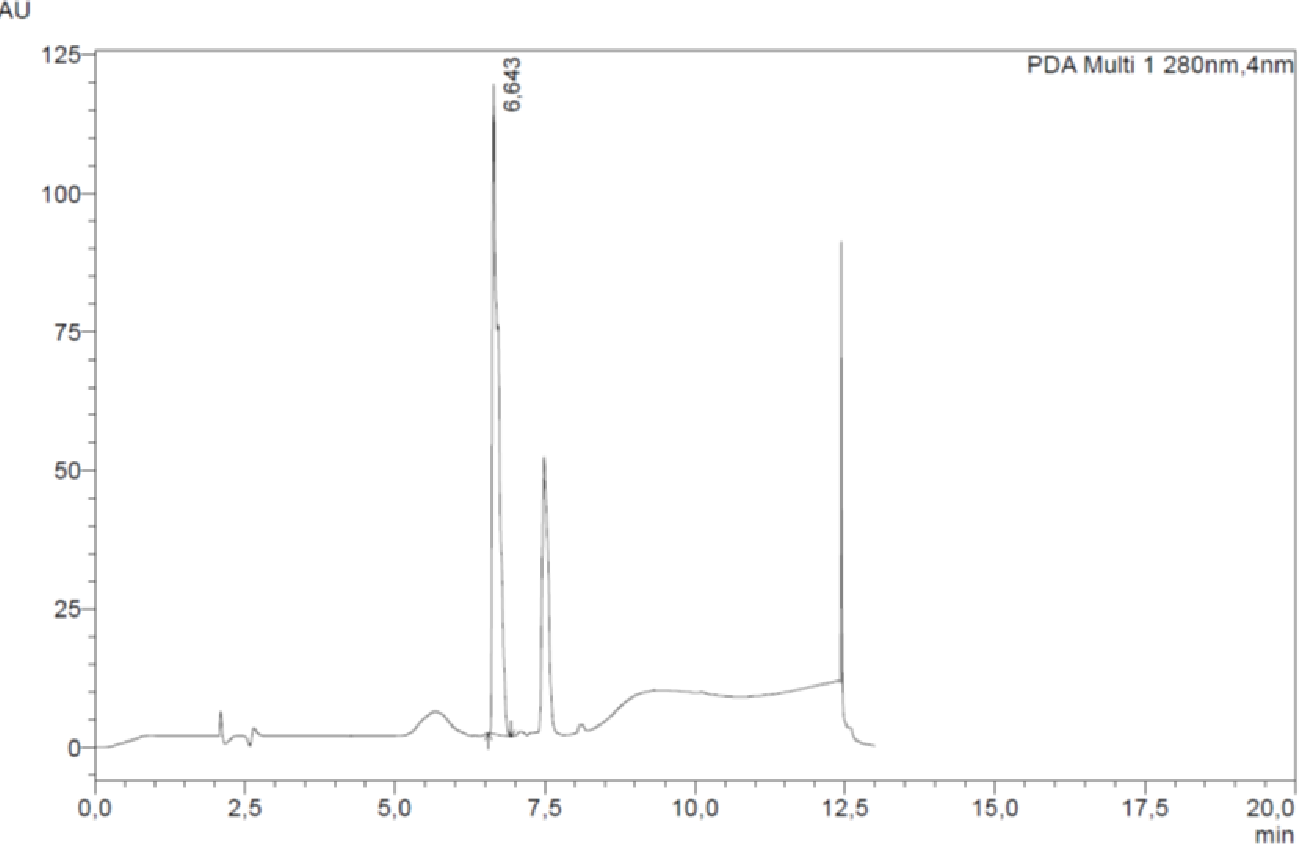
3,6-diamino-5-cyano-4-(4-fluorophenyl)thieno[2,3-b]pyridine-2-carboxamide (7a)



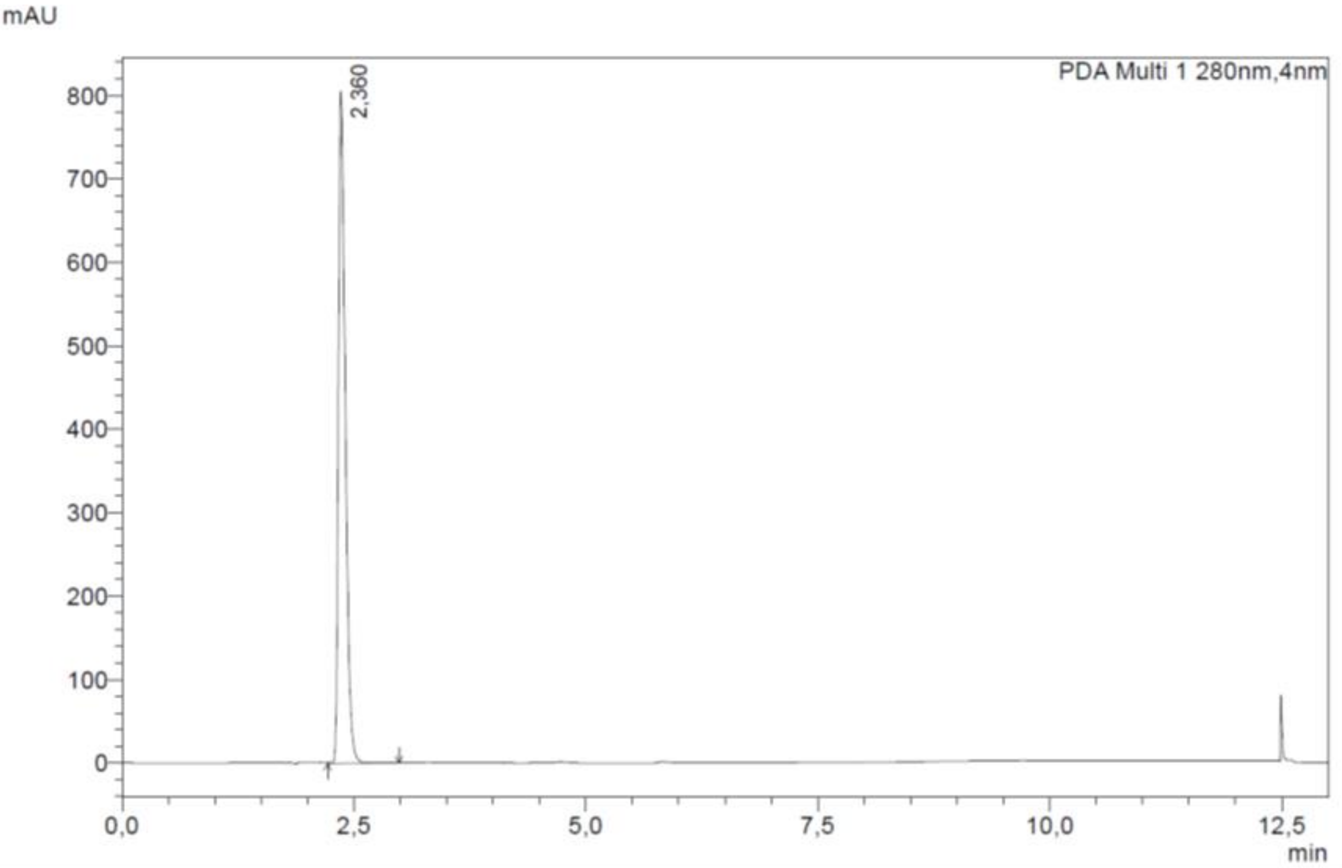
3,6-diamino-5-cyano-4-(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (7b)



3,6-diamino-4-(benzo[d][1,3]dioxol-5-yl)-5-cyanothieno[2,3-b]pyridine-2-carboxamide (7c)

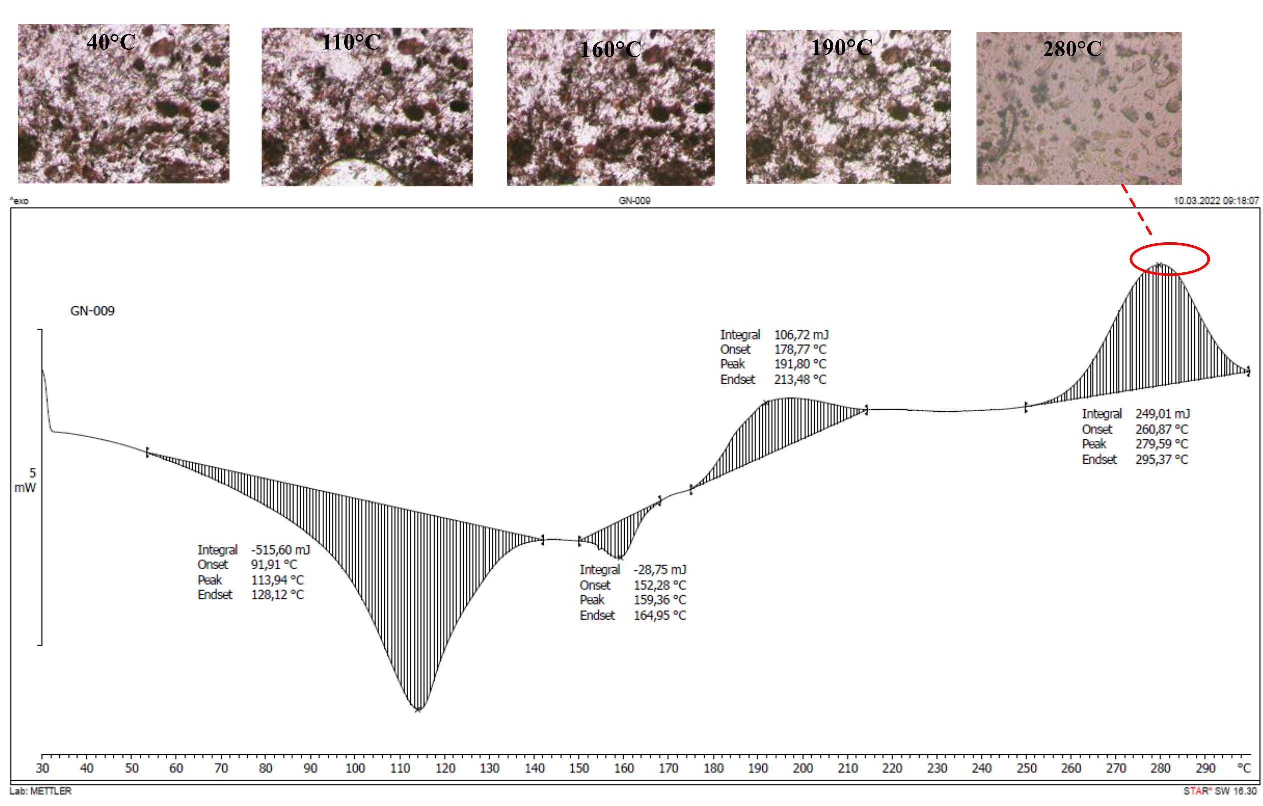


3,6-diamino-5-cyano-4-(3-hydroxyphenyl)thieno[2,3-b]pyridine-2-carboxamide(7d)



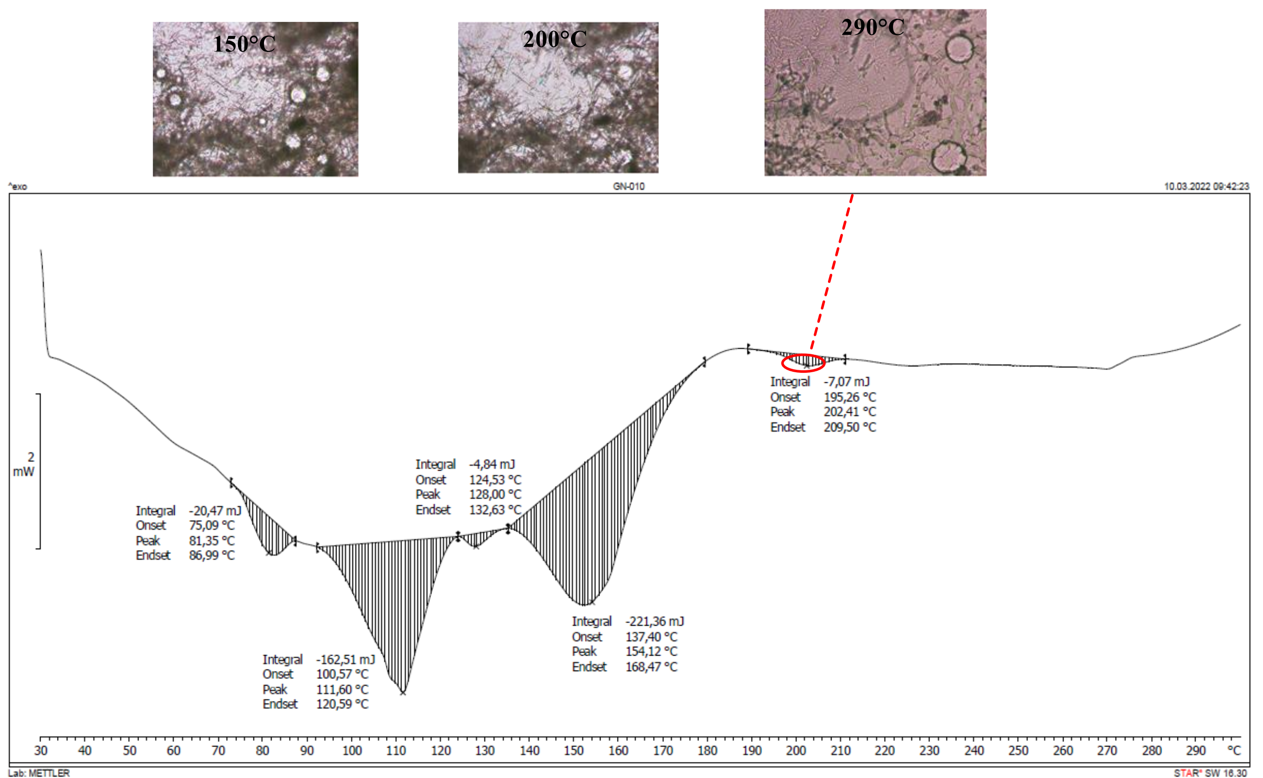
4. DTC/DSC THERMOGRAMS & HSM MICROGRAMS

Compound **6h**



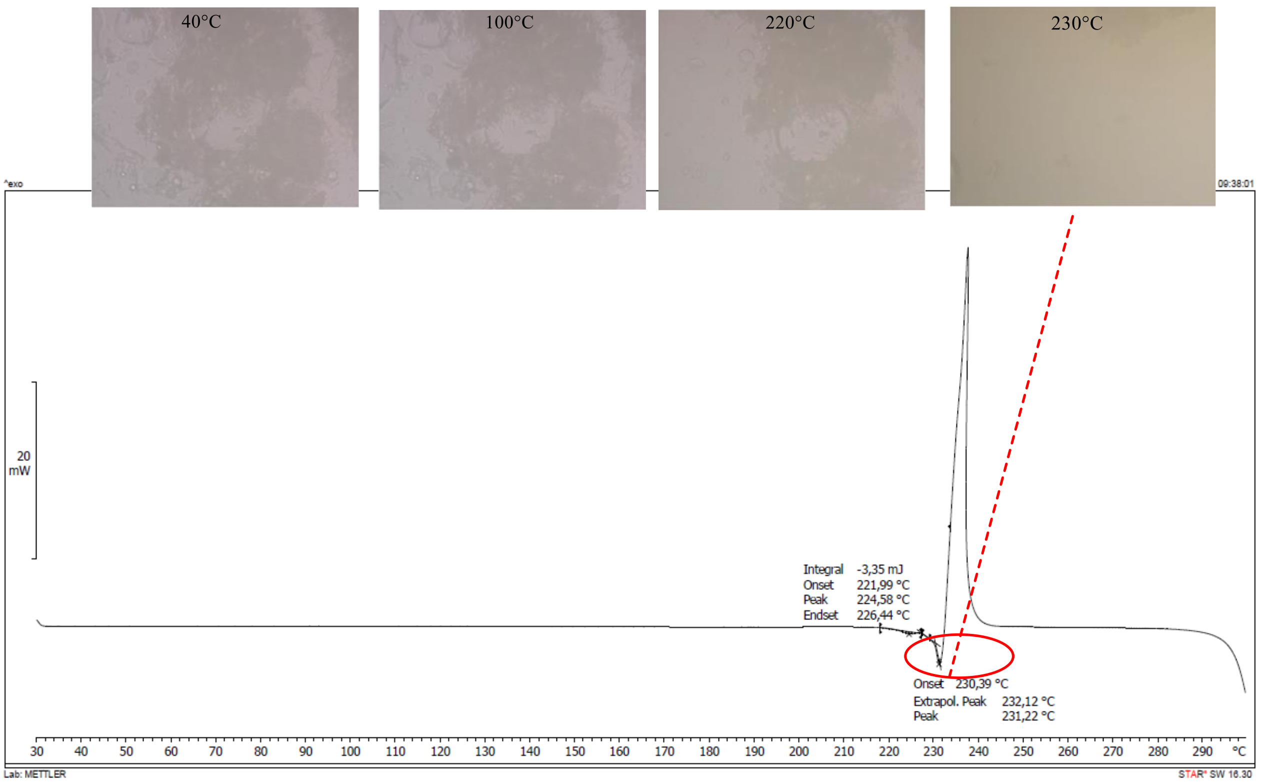
Compound displayed multiple thermal events. Hot-stage microscopy showed that the large endothermal event at 113.94°C is associated with partial melting of the sample. Despite the exothermal peak at 191.80°C, hot-stage microscopy shows further melting of the sample at that temperature with complete melting and recrystallisation, possibly of a decomposition product, starting at 260.87°C and peaking at 279.59°C.

Compound **6i**



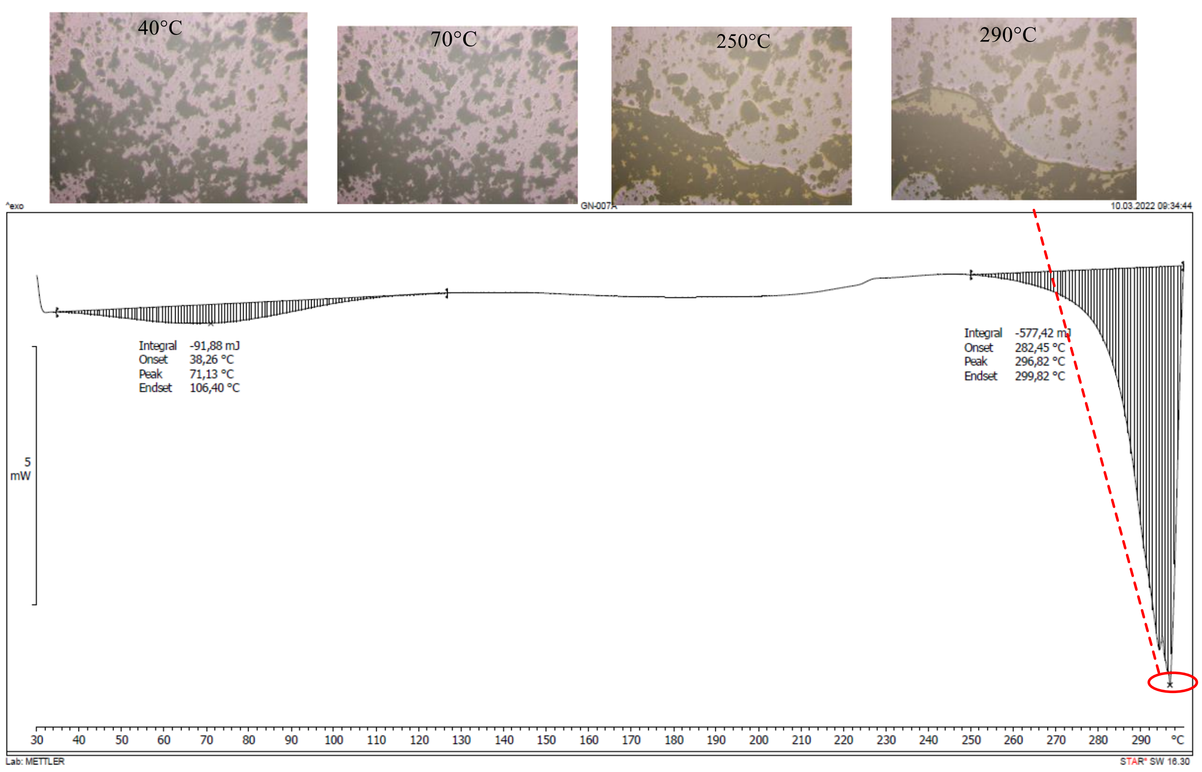
Compound displayed multiple thermal events. However, hot-stage microscopy suggests no visual changes in the sample, with melting first visible at 200°C, from where it gradually progresses until total melting of the sample is seen at around 290°C.

Compound **6j**



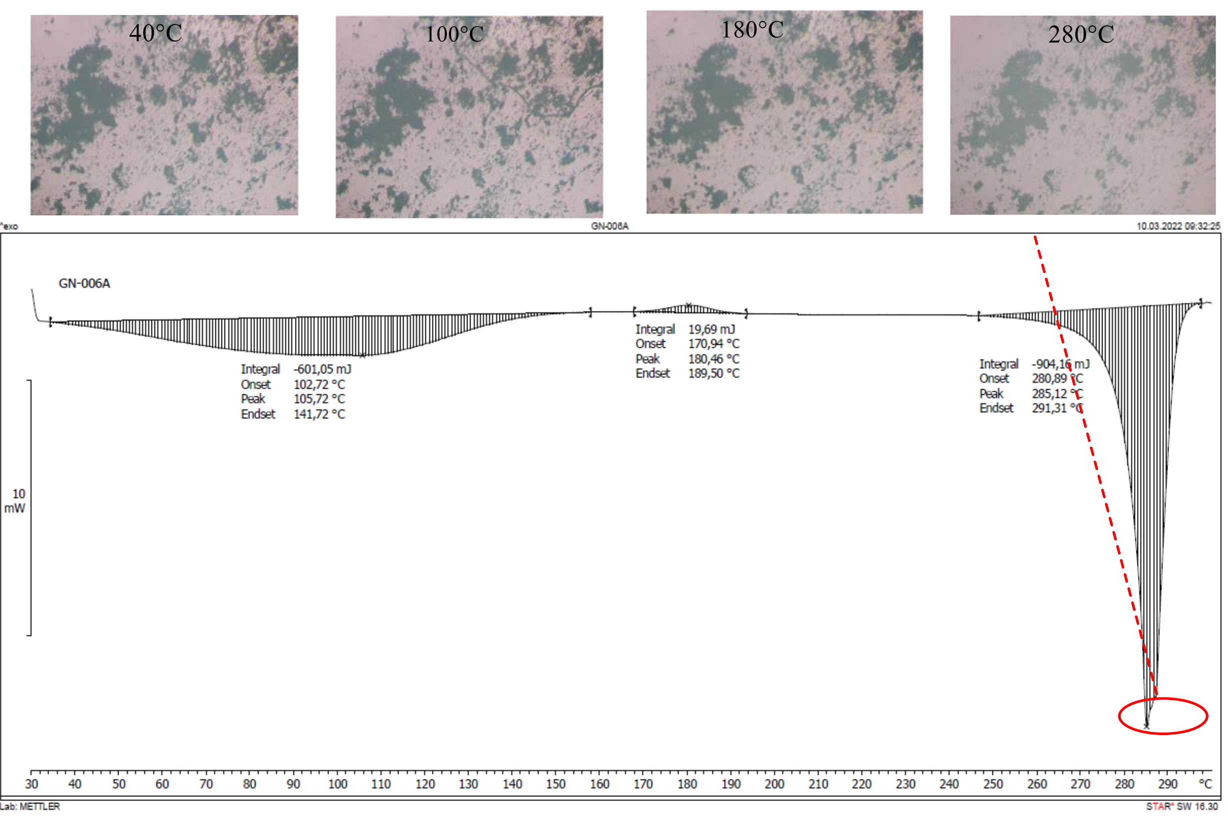
Compound displayed an endothermal event at 224.58°C. From hot-stage microscopy, we can see that that temperature was also associated with an onset of melting. Immediately after the endotherm at 224.58°C, there is another endothermal event, with an onset temperature of 230.39°C, followed by a large exothermal event. Such large exothermal events immediately after an endotherm is usually indicative of thermal decomposition. The results suggest that the melting point of this compound is 231.22°C.

Compound **7c**



Compound 7c displayed a broad endothermal event at low temperatures, peaking at 71.13°C. Hot-stage microscopy shows that this event was not melting. Its onset of melting was at 282.45°C, with a melting point of 296.82°C.

Compound **7d**



Compound displayed a broad endothermal event, peaking at 105.72°C, followed by a small exothermal event at 180.46°C. Its onset of melting was 280.89°C, as confirmed by hot-stage microscopy, with a melting point of 285.12°C.

5. SWISSADME

Table S1 Physicochemical properties of selected test compounds (**6c**, **6d**, **6m**, **6o**, **6q** and **7c**)

Cmpd	*K_i (nM)*	*Molecular formula*	*Molecular Weight (g/mol)*	*Fraction CSp3 (> 0.25)*	*Num. of rotatable bonds*	*Num. of H-bond acceptors*	*Num. of H-bond donors*	*Molar Refractivity*	*TPSA <130 Å^2*	*Lipophilicity Consensus LogP o/W*	*Water solubility consensus LogS*
6c	0,076	$C_{21}H_{17}N_5OS$	387,46	0,14	5	5	1	108,97	133,91	3.18	-6,18
6d	10,3	$C_{16}H_{13}N_5O_2S$	339,37	0,12	5	5	2	89,43	164,11	1,33	-4,23
6m	0,179	$C_{20}H_{15}N_5OS$	373,43	0,10	4	5	2	104,50	144,91	2,83	-5,85
6o	1,64	$C_{22}H_{16}N_4O_3S$	416,45	0,09	6	6	2	113,17	158,32	3,10	-6,25
6q	0,383	$C_{25}H1_8ClN_5O_2S_2$	520,03	0,12	8	6	2	138,29	182,38	4,44	-8,03
7c	61,9	$C_{16}H_{11}N_5O_3S$	353,36	0,06	2	5	3	92,74	178,51	1,82	-4,74

Table S2 Pharmacokinetic properties of selected test compounds (**6c**, **6d**, **6m**, **6o**, **6q** and **7c**)

cmpd	*GI Absorption*	*BBB Permeation*	*P-gp substrate*	*CYP1A2 Inhibitor*	*CYP2C19 inhibitor*	*CYP2C9 Inhibitor*	*CYP2D6 inhibitor*	*CYP3A4 Inhibitor*
6c	low	No	No	Yes	Yes	Yes	No	Yes
6d	low	No	No	Yes	No	Yes	No	Yes
6m	low	No	No	Yes	Yes	Yes	No	Yes
6o	low	No	No	No	Yes	Yes	No	Yes
6q	low	No	Yes	No	Yes	Yes	Yes	Yes
7c	low	No	No	Yes	Yes	Yes	No	Yes

Table S3 Drug-likeness and medicinal chemistry friendliness of selected test compounds (**6c**, **6d**, **6m**, **6o**, **6q** and **7c**)

	Drug-likeness					*Medicinal Chemistry*		
	Num. of violations:					Number of violations:		
Cmpd	Lipinki	Ghose	Veber	Egan	Muegge	PAINS	Brenk	Leadlikeness
6c	0	0	0	1	0	0	0	2
6d	0	0	1	1	1	0	0	0
6m	0	0	1	1	1	0	0	1
6o	0	0	1	1	1	0	0	2
6q	1	2	1	1	2	0	0	3
7c	0	0	1	1	1	0	0	1

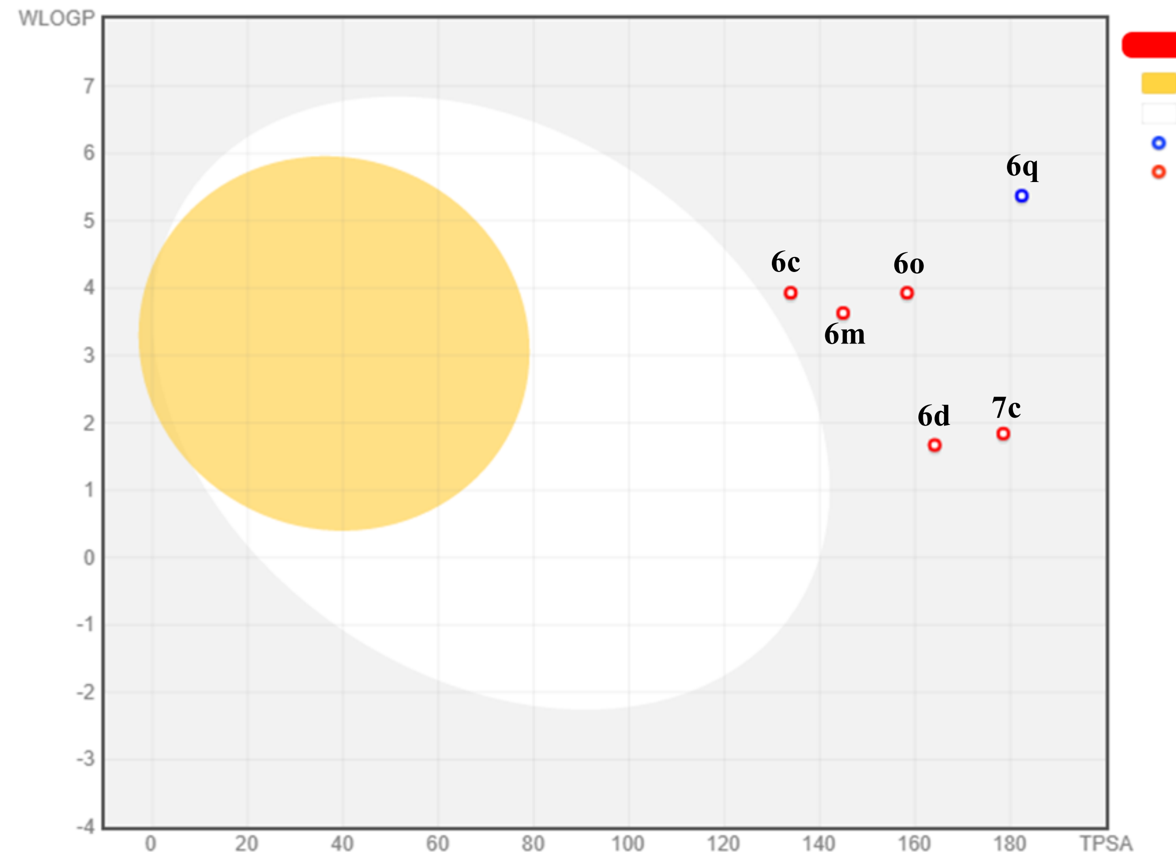


Fig. S1 BBB permeability and GI passive absorption properties of compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c.** Compounds which are not substrate for Pgp (PGP-) are represented by the red circles and those which are substrates (PGP+) are indicated by blue circles. The white region is for high probability of passive absorption by the gastrointestinal tract (HIA), and the yellow region (yolk) is for high probability of brain penetration (BBB). Compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c** were predicted to lack both BBB permeability and GI passive absorption.

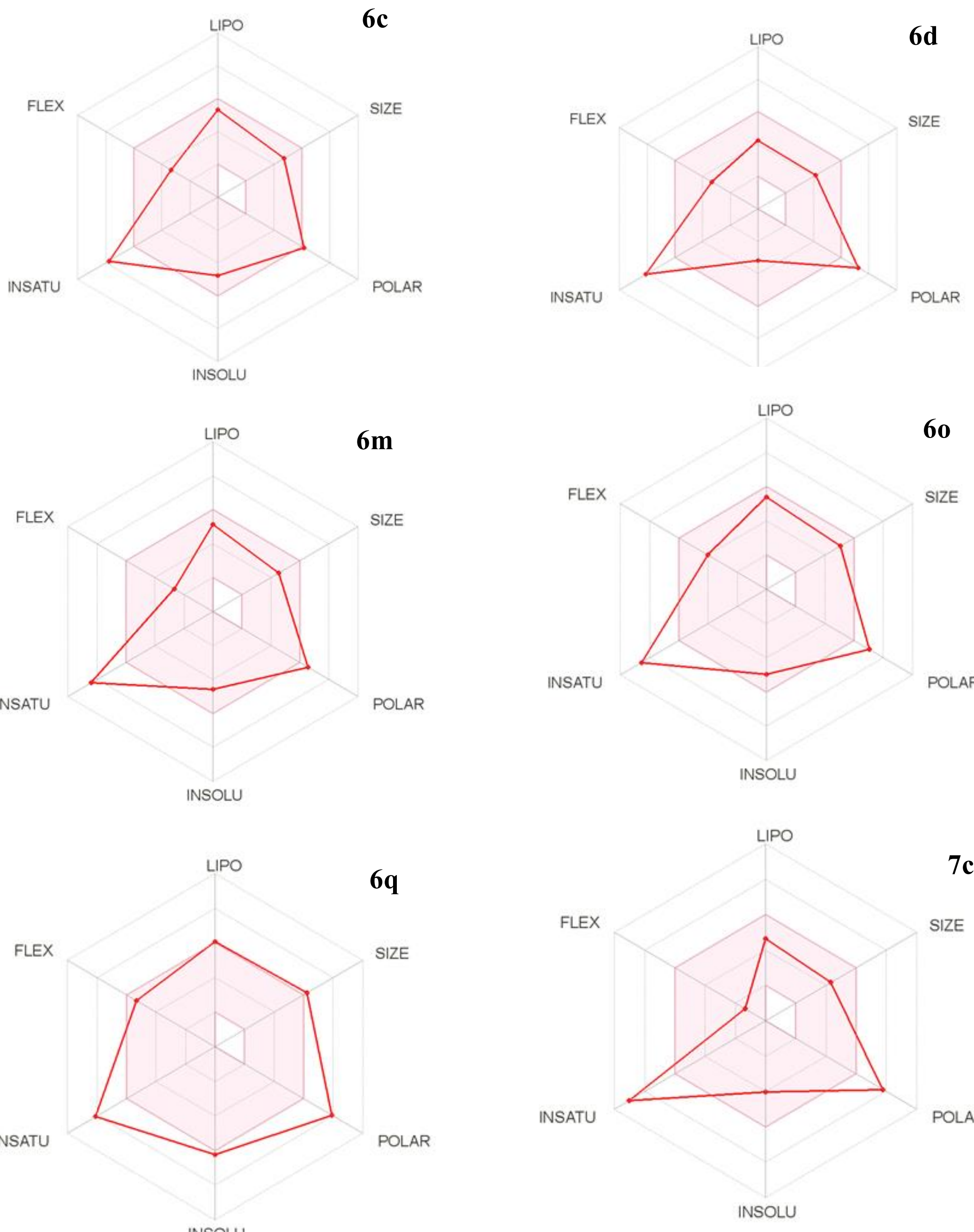


Fig. S2 GI Bioavailability radar for compounds **6c**, **6d**, **6m**, **6o,6q** and **7c**. The pink area represents the optimal range for lipophilcity (LIPO:− 0.7 < XLOGP3 < + 5.0), size (SIZE: 150 < MW < 500), polarity (POLAR: 20 < TPSA < 130), solubility (INSOLU: log*S* < 6), saturation (INSATU: fraction Csp3 > 0.25) and flexibility (FLEX: num. rotatable bonds < 9). The red lines must fall completely within the pink area for a compound to be considered drug-like; therefore, compounds **6c, 6d, 6m, 6o, 6q & 7c** are predicted not orally bioavailable.

ANNEXURE B: AUTHORS' GUIDELINES

Medicinal Chemistry Research:

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Medicinal Chemistry Research
Editor-in-Chief: Hu, L.
ISSN: 1054-2523 (print version)
ISSN: 1554-8120 (electronic version)
Journal no. 44

Instruction to authors

1. Introduction

Medicinal Chemistry Research is a journal for the prompt disclosure of novel experimental achievements in many facets of drug design, drug discovery, and the elucidation of mechanisms of action of biologically active compounds. Articles are sought which emphasize research in chemical biological relationships, especially with respect to: structure-activity relationships, investigations of biochemical and pharmacological targets of drug action, and correlations of structures with the mode of action of biologically active compounds. Studies will be welcomed that increase our understanding of biochemical interactions between drug molecules, ions, free radicals, and sterically important sections of macromolecular targets. The Journal is also dedicated to medicinal plants and to bioactive natural products of plant, fungal, mammalian, and aquatic origin. The Journal publishes original contributions in the following major areas:

- Design, synthesis, and structure-activity relationships of bioactive compounds
- Docking, molecular modeling, and computational studies of bioactive interactions
- Characterization of active ingredients of medicinal plants and identification of bioactivity in plant extracts
- Identification of targets and mechanism of activity of bioactive compounds
- Chemistry and biochemistry of bioactive natural products of plant origin
- Critical reviews of the historical, clinical, and legal status of medicinal agents, and accounts on topical issues.

Contributions reporting the following are not normally considered for publication:

- Biological activity on crude extracts that have not been characterized by analysis of their secondary metabolites (HPLC, ^{1}H and ^{13}C NMR including 2D NMR).
- Unexceptional and predictable bioactivity (e.g. antioxidant properties of phenolic or antibacterial activity of essential oils or antioxidant properties of metals such as iron, copper, etc.).
- Uncritical ethnopharmacological investigations, where a list of plants and their use are simply reported.
- Synthetic work in which the spectroscopic characterization is not complete (e.g., ^{1}H and ^{13}C NMR, HRMS, CHN, UV, IR, etc.).
- Computational work that simply discusses the docking, molecular modeling, QSAR, SAR, and computational studies of bioactive interactions without validation of the method (with experimental data).
- Biological activity that is low and insufficient to generate meaningful structure activity relationship.

Violation of any of the following rules will result in an immediate rejection:

RULE 1: The manuscript does not fall into any of the areas of interest of the Journal.
RULE 2: The manuscript is too preliminary (e.g., data without comparison to a reference, or without a positive control).
RULE 3: The botanical source is not clearly identified, authenticated, or documented (voucher specimen).
RULE 4: The manuscript is too focused on a non-chemical subject (e.g., pharmacology, analytical studies of active ingredients, analytical studies of drug concentrations (ADME is suitable), etc.

RULE 5: Manuscripts that simply discuss antioxidant properties of phenols or other compounds known to possess antioxidant effects.
RULE 6: Computer QSAR/modeling manuscripts that lack experimental biological validation of the proposed model(s).
RULE 7: The manuscript does not follow the formatting provided in this document.
RULE 8: The manuscripts contains poor English or difficult to read language.

2. General Consideration

Authors are strongly encouraged to provide their manuscript in an electronic format. The text must be in a single-column format and lines with double space. Use plain font 12 point Times New Roman and symbols (use internationally accepted signs and symbols for units, SI units). Use the automatic page numbering function to number all the pages. Ensure that all special characters are presented in the body of the text and do not use graphics. Abbreviations, except for very common ones, must be defined the first time they are used and a list supplied with the manuscript.

Using clear and concise English will help the editors and the reviewers concentrate on the scientific merit of the manuscript and thus smooth the peer review process. We reject manuscripts with good science that are poorly written.

The text of a research manuscript should be divided into the following sections: **Introduction**, **Results and Discussion**, **Conclusions**, **Materials and Methods/Experimental**, **Acknowledgements** (Funding), **Conflict of Interest**, and **References**. Tables, figures, and schemes should be embedded in the text or be included right after the references on separate pages (one each per page). Do not upload tables, figures, and schemes that are to be published in the manuscript into the electronic supplementary material. Authors are encouraged to provide supplementary material to keep the manuscript to a reasonable length.

3. Manuscript Organization

3.1. Title Page. A concise and informative title should appear on a separate page and avoid abbreviations and formulas, and followed by the authors' first name, middle initial(s) and last name. Each name is followed by the digit(s) of the author's affiliation in superscript. For example:

Michael G. Mueller[1,2] · Gregory C. Vain[1] ·Alexander B. Smith[2] · Diamond A. Club[2]

Each corresponding author's name is preceded by an envelope icon (✉)and the e-mail address should be indented by 5 mm. Authors with a supplied e-mail but who are not corresponding should have their name and e-mail content listed beneath the corresponding author's details, but without the envelope symbol. Each subsequent author and email will be separated by a blank line.

The affiliation list follows the corresponding authors and e-mail details and is separated from the email address by a blank line. It lists all affiliations within the author group. Each affiliation starts with a superscript number, which corresponds to the digit from the respective author names. Each subsequent affiliation will be separated by a blank line.

✉ Michael G. Mueller
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Diamond A. Club
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2

[1] Discovery Chemistry, ABC Pharmaceutical Research Institute, 1500 Hastings Street, Pharm City, NJ 08854, USA

[2] Department of Medicinal Chemistry, XYZ University School of Pharmacy, 100 University Road, New York, NY 10019, USA

3.2. Abstract. This should be presented as one paragraph detailing the purpose, experimental results and major conclusions, in a finding oriented format. This must be on the second page and is limited to 200, ideally less than 150 words. The abstract should not contain any undefined abbreviations or unspecified references.

Graphical Abstract. The author(s) are strongly encouraged to provide a graphical abstract that is a single schematic image which visually represents the main findings of the article, allowing readers to easily capture the content of the article at a single glance. The graphical abstract must meet the same quality and permissions standards as any other figure in the article. A caption is not needed, while compound numbers can be given in the graphical abstract. The use of color is encouraged and there is no charge for the graphical abstract. The graphical abstract should be legible at a size of 5 × 2 inches (130 × 50 mm, w × h)) using a regular screen resolution of 96 dpi. Graphical abstracts can be selected as the front cover art.

Immediately after the abstract paragraph/graphical abstract, 4 to 6 keywords, should be provided for indexing purposes under the heading Keywords.

3.3. Introduction. The manuscript should start with an introduction where the rationale and aims of the research are discussed. Be sure to include and reference similar investigations in support of the work.

3.4. Results and Discussion. This section should concisely present the chemistry and medicinal/biological results. Tables, figures, and schemes help to present the experimental data and design to maximize the comprehension and clarity of the results. The discussion should interpret the results, and significantly analyze the data.

3.5. Conclusion. This is an optional section where authors can highlight their results.

3.6. Experimental/Material and Methods. The author(s) are encouraged to be as concise as possible in the experimental description. Specific details about instruments used and sources of the reagents used should be incorporated in the text headed by the word "experimental." In a separate paragraph experimental biological material should be used to describe the work and may include herbarium, voucher number, authenticated by, date of collection or cultivation, etc. Scientific names should be in italics (in manuscripts reporting natural product isolation) and the description of the isolation process, as well as other relevant data, should be provided in one paragraph. For synthetic papers all methodology used must be described.

The characterization of compounds should be presented in a separate paragraph. Generally, a listing of ^{1}H or ^{13}C NMR peaks is sufficient. However, when the NMR data is used as the basis for structural identification, the peaks from the ^{13}C NMR must be assigned to the corresponding carbon atom (i.e, if C-1 (carbon in position #1) has a NMR peak at 170.1 then the data should show that C-1 has the 170.1 peak (one decimal: do not use a range). There are a couple of ways to represent this information: ^{13}C-NMR (DMSO-d_6): 170.1 (C=O) or 170.1 (C-1)).

Under the material and methods section - compounds should be identified by IUPAC nomenclature and written using the following example:

*Compound (*or IUPAC name*)* (3a): Yellowish needles (MeOH) (This compound was prepared by.... It was obtained as a white solid, color, yield, etc); mp 85-86 °C; $[\alpha]^{25}_{D}$ + 92 (*c* 0.003, Py); UV (EtOH) λ_{max} (log ε) 240 (4.15), 278 (4.30) nm; IR (KBr) ν_{max} 3382, 2877, 2925, 1736, 1701, 1630, 1606, 1517, 1445, 1374, 1276, 1165, 1117, 1070 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.84 (2H, d, *J* = 7.4 Hz, H-2′, H-6′), 6.78 (2H, d, *J* = 7.4 Hz, H-3′, H-5′), 5.15 (1H, d, *J* = 4.4 Hz, H-1), 4.60 (1H, dd, *J* = 2.4, 12.0 Hz, H-6a), 4.50 (1H, dd, *J* = 5.0, 12.0 Hz, H-6b), 4.38 (1H, dd, *J* = 1.2, 4.4 Hz, H-2), 4.24 (1H, dd, *J* = 1.6, 10.0 Hz, H-4), 3.92 (1H, ddd, *J* = 5.2, 7.4, 10.0 Hz, H-5), 3.49 (1H, dq, *J* = 6.8, 9.0 Hz, O-$C\underline{H_2}CH_3$), 3.68 (1H, dq, *J* = 6.8, 9.0 Hz, O-$C\underline{H_2}CH_3$), 1.12 (3H, t, *J* = 6.8, Hz, O-$CH_2C\underline{H_3}$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 205.4 (C, C-3), 166.6 (C, <u>C</u>OBz), 161.6 (C, C-4′), 131.8 (CH, C-2′, C-6′), 120.8 (C, C-1′), 115.2 (CH, C-3′, -5′), 100.8 (CH, C-1), 74.7 (CH, C-2), 73.2 (CH, C-5), 72.7 (CH, C-4), 64.3 (CH_2, O-$\underline{CH_2}CH_3$), 63.4 (CH_2, C-6), 14.5 (CH_3, O-$CH_2\underline{CH_3}$); EIMS *m/z* 326 $[M]^+$ (5), 308 (100); HRMS (ESI^+) *m/z* calcd for $C_{15}H_{18}O_8Na$ 349.0899, found 349.0898 ($M+Na^+$); Anal. Calcd. for $C_{15}H_{15}N_5$: C, 67.90; H, 5.70; N, 26.40. Found: C, 67.84; H, 5.39; N, 26.12.

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A paragraph with the pharmacological assays must be described in sufficient detail; positive and negative controls must be evaluated at the same concentration(s) to compare the effectiveness of the test compounds. With respect to the biological data, the concentration and doses must be presented as molar units, and presented as $IC_{50,}$ $EC_{50,}$ etc. References to statistical methods of calculation must be included in the manuscript. Also, the tested compounds, regardless if they are isolated as secondary metabolites, synthesized or purchased, must range between 95-100 % purity (TLC is not a reliable procedure for analysis). Materials and methods must include statements of human and animal welfare. Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should also be provided.

Theoretical calculations (docking, molecular modeling, QSAR, SAR, computational studies, etc.), software used, etc. should be included in the material and methods section. All models must be validated with biological experimental data.

<u>3.7. Acknowledgments.</u> (Funding information) Acknowledgment of people, grants, funds, etc. should be placed in a separate section before the references. The complete names of funding organizations should be provided. In addition, please provide funding information, which includes a separate step in the submission process of the peer-review system. Funding providers should be selected from the standardized list provided during the submission of the manuscript. If the funding institution is not listed, it can be entered as free text. Funding information will be published as a searchable metadata for all accepted articles. Even so, acknowledgements of funding support should be described within the paper.

<u>3.8. Conflict of Interest.</u> Authors must disclose all relationships or interests that could have direct or potential influence or impart bias on the work. Although an author may not feel there is any conflict, disclosure of relationships and interests provides a more complete and transparent process, leading to an accurate and objective assessment of the work. Awareness of real or perceived conflicts of interest is a perspective to which readers are entitled. This is not meant to imply that a financial relationship with an organization that sponsored the research or compensation received for consultancy work is inappropriate. Examples of potential conflicts of interests **that are directly or indirectly related to the research** may include but are not limited to the following:

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- Financial support for educational programs
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In addition, interests that go beyond financial interests and compensation (non-financial interests) that may be important to readers should be disclosed. These may include but are not limited to personal relationships or competing interests directly or indirectly tied to this research, or professional interests or personal beliefs that may influence your research.

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Conflict of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stock in Company Y. Author C is a member of committee Z.

If no conflict exists, the authors should state: Conflict of Interest: The authors declare that they have no conflict of interest.

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For Journal articles:

1. Obeng S, Kamble SH, Reeves ME, Restrepo LF, Patel A, Behnke M et al. Investigation of the adrenergic and opioid binding affinities, metabolic stability, plasma protein binding properties, and functional effects of selected indole-based kratom alkaloids. J Med Chem. 2020;63(1):433-9. doi:10.1021/acs.jmedchem.9b01465.
2. Anifowose A, Agbowuro AA, Yang X, Wang B. Anticancer strategies by upregulating p53 through inhibition of its ubiquitination by MDM2. Med Chem Res. 2020;29(7):1105-21. doi:10.1007/s00044-020-02574-9.

Articles published online through early access by DOI:

3. Klus NJ, Kapadia K, McDonald P, Roy A, Frankowski KJ, Muma NA et al. Discovery of sultam-containing small-molecule disruptors of the huntingtin–calmodulin protein–protein interaction. Med Chem Res. 2020:[Online first article]. doi:10.1007/s00044-020-02583-8.

Books:

4. Youssef JA, Badr MZ. Peroxisome Proliferator-Activated Receptors: Discovery and Recent Advances. New York: Humana Press, Springer; 2013.

Book chapters:

5. Gogineni V, Leon F, Avery BA, McCurdy CR, Cutler SJ. Phytochemistry of Mitragyna speciosa. In: Raffa RB, editor. Kratom and Other Mitragynines: the Chemistry and Pharmacology of Opioids from a Non-Opium Source. Boca Raton: CRC press; 2015. p. 77-94.

Online documents:

6. National Institute on Drug Abuse Press Office. Teen Prescription Opioid Abuse, Cigarette, and Alcohol Use Trends Down. 2014. http://www.drugabuse.gov/news-events/news-releases/2014/12/teen-prescription-opioid-abuse-cigarette-alcohol-use-trends-down. Accessed May 19 2020.

Dissertations:

7. Abed DA. Structure-activity relationships of small molecule direct inhibitors of Keap1-Nrf2 interaction [Dissertation]: Rutgers, The State University of New Jersey; 2018.

Patents:

8. Hu L, Sahota A, inventors; Cystine diamide analogs for the prevention of cystine stone formation in cystinuria patient. US9428453. 2016 August 30.

3.10. Tables. All tables are to be numbered using Arabic numerals. Tables should always be cited in the text and in consecutive numerical order. For each table, please supply a table caption (title) explaining the components of the table. Identify any previously published material by giving the original source in the form of a reference at the end of the table caption. Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

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- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

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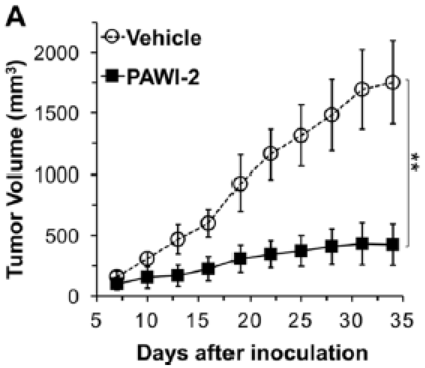

Reference: Cheng J, Cashman JR. PAWI-2: A novel inhibitor for eradication of cancer. Med Chem Res. 2020;29(7):1147-59. doi:10.1007/s00044-020-02575-8.~~Cheng, J.; Cashman, J.R. PAWI-2: A novel inhibitor for eradication of cancer. *Med. Chem. Res.* **2020**, *29*, 1147-1159.~~

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- All lines should be at least 0.1 mm (0.3 pt) wide.
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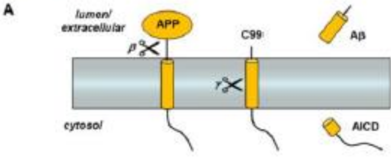

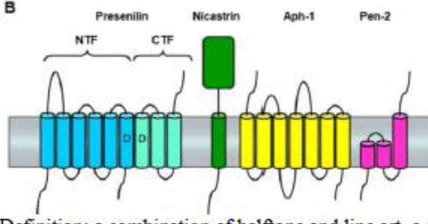

Reference: Wolfe MS. Substrate-based chemical probes for Alzheimer's γ-secretase. Med Chem Res. 2020;29(7):1122-32. doi:10.1007/s00044-020-02565-w.Wolfe, M.S. Substrate-based chemical probes for Alzheimer's γ-secretase. *Med. Chem. Res.* **2020**, *29*, 1122-1132.

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- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

Color art is free of charge for online publication while the print version has a charge for color. If black and white will be used in the print version, make sure that the main information will be visible. Many colors are not distinguishable from one another when converted to black and white format. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent. If the figures will be printed in black and white, do not refer to color in the captions of figures, schemes, etc. Color illustrations should be submitted as RGB (8 bits per channel).

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Submission:

- Supply all supplementary material in standard file formats.
- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author, table of contents.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.

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- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be converted to a PDF if no interaction with the data is intended.
- If the readers are encouraged to make their own calculations, spreadsheets should be submitted as .xls files (MS Excel).

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- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

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Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
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In addition:

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- Requesting to add or delete authors at revision stage, proof stage, or after publication is a serious matter and may be considered when justifiably warranted. Justification for changes in authorship must be compelling and may be considered only after receipt of written approval from all authors and a convincing, detailed explanation about the role/deletion of the new/deleted author. In case of changes at revision stage, a letter must accompany the revised manuscript. In case of changes after acceptance or publication, the request and documentation must be sent via the Publisher to the Editor-in-Chief. In all cases, further documentation may be required to support your request. The decision on accepting the change rests with the Editor-in-Chief of the journal and may be turned down. Therefore authors are strongly advised to ensure the correct author group, corresponding author, and order of authors at submission.
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- The author's institution may be informed.

Compliance with Ethical Standards: To ensure objectivity and transparency in research and to ensure that accepted principles of ethical and professional conduct have been followed, authors should include information regarding sources of funding, potential conflicts of interest (financial or non-financial), informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals.

Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" before the References when submitting a paper:

- Disclosure of potential conflicts of interest
- Research involving human participants and/or animals
- Informed consent

The corresponding author should be prepared to collect documentation of compliance with ethical standards and send if requested during peer review or after publication.

The Editors reserve the right to reject manuscripts that do not comply with the above-mentioned guidelines. The author will be held responsible for false statements or failure to fulfill the above-mentioned guidelines.

5. Research involving human participants and/or animals

5.1 Statement of human rights. When reporting studies that involve human participants, authors should include a statement that the studies have been approved by the appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that the independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study.

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For retrospective studies, please add the following sentence:

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For studies with animals, the following statement should be included in the text before the References section:

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If articles do not contain studies with human participants or animals by any of the authors, please select one of the following statements:

"This article does not contain any studies with human participants performed by any of the authors."

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"This article does not contain any studies with human participants or animals performed by any of the authors."

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6.2. Authors should cite those publications that are relevant to the manuscript.

6.3. Fragmentation of manuscripts should be avoided, and extensive work on a system or group of related systems should be combined in a single manuscript.

6.4. Authors must not submit manuscripts describing essentially the same research to more than one journal.

6.5. Everyone who has made significant scientific contributions to the work should be included in the list of authors. Those who have made minor contributions should be indicated as an "Acknowledgment".

6.6. The editors reserve the right to reject manuscripts that do not comply with the above-mentioned requirements. The author will be held responsible for false statements or failure to fulfill the above-mentioned requirements.

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ANNEXURE C: ETHICS

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

1 November 2021

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 1 November 2021, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Design, synthesis and evaluation of thienopyridines as ligands of adenosine receptors
Principal Investigator/Study Supervisor/Researcher: Prof G Terre'Blanche
Student: G Nkomba - 22358595

Ethics number:

N	W	U	-	0	0	4	1	8	-	2	1	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 01/11/2021
Expiry date: 30/11/2022

Risk: **Category 0**

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of November annually until completion of the study.

In process requirements: None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*

- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

Please note: Due to the nature of the study i.e. (laboratory work involving the investigation of a specific pharmaceutical agent in previously collected brain tissue), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Christiaan B Brink (Tiaan)
Date: 2021.11.02 12:45:11 +02'00'

Chairperson: NWU-AnimCareREC

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18 Ocotber 2021

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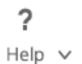
ANNEXURE D: PERMISSIONS

Figure 2-1



Adenosine and Epilepsy: From Therapeutic Rationale to New Therapeutic Strategies

Author: Detlev Boison

Publication:
The Neuroscientist: Reviews at the Interface of Basic & Clinical Neurosciences

Publisher: SAGE Publications

Date: 02/01/2005

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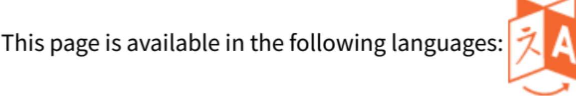

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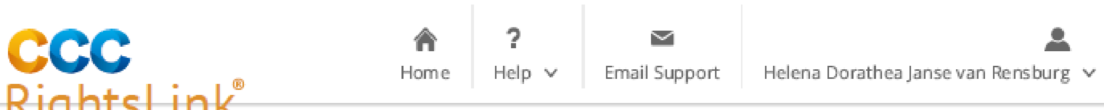
Figure 2-5 and Figure 2-8



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Adenosine receptors as therapeutic targets

Author: Kenneth A. Jacobson et al
Publication: Nature Reviews Drug Discovery
Publisher: Springer Nature
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