The synthesis of novel 2-aminobenzothiazinone analogues and their evaluation as adenosine A_1/A_{2A} receptor antagonists.

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder that is characterised by a decrease in dopamine concentration in the striatum due to the degeneration of dopaminergic neurons in the substantia nigra. PD has a distinctive symptomatic footprint including bradykinesia as the hallmark symptom paired with tremor and muscle rigidity. PD also causes non-motor symptoms including depression and cognitive dysfunction. Current treatment options provide symptomatic relief by the manipulation of dopaminergic signaling, but fails to address disease progression. A new therapy is therefore urgently required to decrease disease progression, while providing symptomatic relief.

The adenosine A_1 and A_{2A} receptor subtypes have been recognised as possible drug targets for the treatment of PD. Selective adenosine A_1 receptor antagonists have the potential of treating cognitive deficits such as those associated with Alzheimer's disease and PD. Selective adenosine A_{2A} receptor antagonists on the other hand have the ability to improve motor dysfunction in PD, but they also have neuroprotective properties. Additionally, adenosine A_{2A} receptor antagonists have been shown to exhibit antidepressant activity in animal models and may be advantageous to treat PD associated depression. Dual antagonism of adenosine A_1 and A_{2A} receptors would thus be of great benefit to potentially treat both the motor as well as the non-motor (cognitive and depressive) symptoms associated with PD.

Recent research identified the benzothiazinone scaffold as a promising non-xanthine scaffold that may be used to design compounds with adenosine A_1 and A_{2A} receptor affinity. When compared to caffeine, 2-aminobenzothiazinone and benzoylaminobenzothiazinone showed a higher affinity for both the A_1 and A_{2A} adenosine receptors. Further research showed that chain elongation to phenylpropanamide-benzothiazinone lead to an increase in adenosine A_{2A} affinity, but a decrease in A_1 affinity. This higher affinity for the adenosine receptors displayed by the 2-acylaminobenzothiazinones makes it a promising scaffold for further exploration as a dual A_1/A_{2A} adenosine receptor antagonist. Furthermore, the triazolotriazine scaffold of ZM241385 has high affinity for the adenosine A_{2A} receptor with a phenylethylamine side-chain which comfortably fits into the binding cleft of the adenosine receptor.

Prompted by the above two scaffolds; an exploratory pilot study was undertaken where the N-acyl side-chain of the benzothiazinone scaffold was replaced by the

flexible N-alkyl side-chain of ZM241385, thus exploring the necessity of the CO-group for adenosine affinity. In addition, different *para* and *meta* substituents on the phenyl ring in the 2-alkylamino side-chain of the 2-phenlylalkylamino-benzothiazinone scaffold was also explored, as well as different chain lengths in the phenylalkyl side-chain.

A series of fourteen novel 2-phenylalkylaminobenzothiazinone derivatives were synthesised via N-alkylating using phenylhalides containing various chain lengths and *para* and *meta* phenyl substitutions. The 2-phenylalkylaminobenzothiazinones were evaluated by using a radioligand binding protocol described in literature to investigate the binding of the compounds to the adenosine A_{2A} and A_1 receptors.

The tested compounds were devoid of any A_1 and A_{2A} adenosine binding affinity. The poor adenosine A_1 and A_{2A} affinity exhibited by the compounds of this study can probably be attributed to the absence of the carbonyl group in the N-alkyl side-chain of the 2-phenylalkylaminobenzothiazinones, thereby emphasising the necessity of the carbonyl group for adenosine affinity. The phenylalkyl substitution offered an attractive substitution for a hybrid non-xanthine adenosine antagonist using the 2-aminobenzothiazinone scaffold and the phenylalkyl side-chain of ZM241385, but biological evaluation proved the 2-phenylalkylaminobenzothiazinone derivatives as ineffective adenosine A_1 and A_{2A} receptor antagonists.

In conclusion, this research made an important contribution showing that the carbonyl group in the 2-acylaminobenzothiazinone scaffold is a prerequisite for adequate A_1 and A_{2A} binding affinity which can be used for the designing of high affinity adenosine receptor antagonists for the treatment of PD in future.

Keywords: Parkinson's disease, 2-aminobenzothiazinones, 2-phenylalkylaminobenzothiazinones, adenosine A_1 antagonist, adenosine A_{2A} antagonist,

OPSOMMING

Parkinson se siekte (PS) is ‘n neurodegeneratiewe siekte wat gekarakteriseer word deur ‘n merkbare verlies in die dopamienkonsentrasie in die striatum as gevolg van die degenerasie van dopaminergiese neurone in die substantia nigra. Die vernaamste kenmerk van PS is bradikinesie gepaardgaande met tremore en spierrigiditeit. PS veroorsaak ook nie-motoriese simptome soos depressie en kognitiewe defekte. Huidige behandeling vir PS verskaf tans simptomatiese verligting deur dopamienvervanging, maar sonder dat die siekteverloop vertraag of gestop word. Die soeke na ‘n nuwe behandeling is dus nodig om die progressie van PS te stuit en terselfdertyd simptomatiese verligting te bied.

Adenosien A_1- en A_{2A}-reseptore is geïdentifiseer as belowende geneesmiddelteikens vir die behandeling van PS. Selektiewe adenosien A_1-antagoniste het die potensiaal om gebruik te word by die behandeling van kognitiewe defekte soos waargeneem in die geval van PS en Alzheimer se siekte. Verder kan selektiewe adenosien A_{2A}-antagoniste van waarde wees om motoriese funksie te verbeter en ook neurobeskermend te wees teen die progressie van PS. Hierbenewens het adenosien A_{2A}-antagoniste ook antidepressiewe effekte getoon in proefdiermodelle wat voordelig kan wees vir die behandeling van depressie wat met PS geassosieer word. Dualistiese antagonisme van beide adenosien A_1- en A_{2A}-reseptore kan dus van groot waarde wees om die motoriese sowel as die nie-motoriese (kognitief en depressief) simptome van PS te behandel.

Navorsing het onlangs getoon dat die bensotiasinoonkernstruktuur ‘n belowende nie-xantien leidraadverbinding is vir die ontwikkeling van verbindings met affiniteit vir die adenosien A_1- en A_{2A}-reseptore. 2-Aminobensotiasinoon en bensoïel-aminobensotiasinoon het beter adenosien A_1- en A_{2A}-reseptor affiniteit getoon in vergelyking met kafeïen. Verder het navorsing getoon dat kettingverlenging aan fenielpropanamied-bensotiasinoon tot ‘n verhoging in adenosien A_{2A}-affiniteit en ‘n verlaging in A_1-affintiteit lei. Die verhoogde affiniteit van die 2-asielaminobensotiasinone vir die adenosien reseptore maak hierdie verbindings belowende kernstrukture vir die ontwikkeling van dualistiese adenosien A_1\A_{2A}-antagoniste. Verder toon die triasolotriasien kernstruktuur van ZM241385 ook goeie A_{2A} affiniteit waar die fenieletielamien syketting gemaklik in die bindingsholte van die adenosien reseptor pas.

Na aanleiding van bogenoemde twee kernstrukture is ’n lootstudie onderneem waar die N-asielsyketting van die bensotiasinoonkernstruktuur vervang is met die buigbare N-alkielsyketting van ZM241385 om sodoende die noodsaaklikheid van die CO-groep vir adenosien affiniteit te ondersoek. Addisioneel is verskeie *para*- en *meta*-substitusies op die fenielring in die syketting van die 2-fenielalkielaminobensotiasinoon kernstruktuur verken, asook verskeie kettinglengtes in die fenielalkielsyketting.

‘n Reeks van veertien nuwe 2-fenielalkielaminobensotiasinoon derivate is gesintetiseer deur N-alkilering, waar fenielhaliede gebruik is met variërende kettinglengtes asook *para*- en *meta*-fenielsubstitusies. ‘n Radioligandbindingsprotokol, soos in die literatuur beskryf, is gebruik om die binding van die 2-fenielalkielaminobensotiasinone aan die adenosien A_1- en A_{2A}-reseptore te ondersoek.

Die toetsverbindings het geen adenosien A_1- of A_{2A}-affiniteit getoon nie. Die swak affiniteit kan toegeskryf word aan die afwesigheid van die karbonielgroep in die N-alkielsyketting van die 2-fenielalkielaminobensotiasinone. Laasgenoemde beklemtoon die noodsaaklikheid van die karbonielgroep vir adenosienreseptor affiniteit. Die kombinasie van die 2-aminobensotiasinoonkernstruktuur en die fenielalkielsyketting van ZM241385 het aanvanklik belowend gelyk om ’n hibridiese nie-xantien adenosien antagonis te ontwerp, maar ongelukkig het die biologiese evaluering aangetoon dat die 2-fenielalkielaminobensotiasinoonderivate oneffektiewe adenosien A_1- en A_{2A}-reseptor antagoniste is.

Ten slotte, die huidige lootstudie het ‘n waadevolle bydrae gelewer deur aan te toon dat die karboniel-groep in die 2-aminobensotiasinoon kernstruktuur ‘n voorvereiste is om adenosien A_1- en A_{2A}-aktiwiteit te bekom en dat hierdie bevinding gebruik kan word in die ontwerp van nuwe hoë affiniteit adenosienreseptorantagoniste vir die toekomstige behandeling van PS.

Sleutelwoorde: Parkinson se siekte, 2-aminobensotiasinone, 2-fenielalkielaminobensotiasinone, adenosien A_1-antagonis, adenosien A_{2A}-antagonis

ABBREVIATIONS

[^{3}H]DPCPX	1,3-[3H]-Dipropyl-8-cyclopentylxanthine
[^{3}H]NECA	[3H]5'N-Ethylcarboxamido-adenosine
5-HT	serotonin
6-OHDA	6-Hydroxydopamine
APCI	Atmospheric-pressure chemical ionization
AR	Adenosine receptors
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
br s	Broad singlet
CNS	Central nervous system
COMT	Catechol-O-methyl-transferase
CPA	N6-Cyclopentyladenosine
CPM	Counts per minute
CPX	8-Cyclopentyl-1,3-dipropylxanthine
CSC	8-(3-Chlorostyryl)-caffeine
d	Doublet
dd	Doublet of doublets
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d6	Deuterodimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
FDA	Food and Drug Administration
FST	Forced swim test
GPe	Globus pallidus externa
Gs	Stimulatory G-protein
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectra
IC50	Half maximal inhibitory concentration
iPD	Idiopathic Parkinson's disease
IR	Infrared spectroscopy
ITAs	N-alkyl- and N-acyl-(7-substituted-2-phenylimidazo[1,2-a][1,3,5]triazin-4-yl)amines
J	Coupling constant

Ki	Dissociation constant
KW6002	Istradefylline
L-AAD	L-amino acid decarboxilase
LB	Lewy body
LBs	Lewy bodies
L-DOPA	L-3,4-Dihidroxyphenylalanine / Levodopa
m	Multiplet
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase isoform B
MAO-I	Monoamine oxidase inhibitors
mp	Melting point
MPDP+	1-Methyl-4-phenyl-2,3-dihydropyridinium ion
MPPP	1-Methyl-4-phenyl-4-propionoxypiperidine
MPP+	1-Methyl-4-phenylpyridinium ion
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OCT3	Organic cation *transporter 3*
PCD	Programmed cell death
PD	Parkinson's disease
PK	Pharmacokinetic
ppm	Parts per million
PS	Parkinson se siekte
s	Singlet
SEM	Standard error of the mean
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNpc	Substantia nigra pars compacta
SNr/GPi	Substantia nigra pars reticulata/globus pars interna
SNRI	5-HT Noradrenaline reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
STN	Subthalamic nucleus
t	Triplet
TCA	Tricyclic antidepressants
TLC	Thin layer chromatography
UCHL-1	Ubiquitin C-terminal hydrolase L1
XAC	(8-[4-[[[[(2-Aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-l,3-

	dipropylxanthine
ZM241385	4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin- 5-yl amino]ethyl) phenol
α-syn	Alpha-synuclein
δ	Delta scale indicating chemical shift

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Parkinson's disease (PD) is an age related progressive neurodegenerative disorder that is characterised by a loss of dopamine concentration in the nigrostriatal pathway (Obeso *et al.*, 2010). It is estimated that more than 1% of people over the age of 60 has some form of the latter disease and it is speculated that the number will increase (Abdullah *et al.*, 2015; Miller & O'Callaghan, 2015) to approximately between 8.7 and 9.3 million people by 2030 (Dorsey *et al.*, 2007). As a consequence this disease is an economic and social burden (Miller & O'Callaghan, 2015). The chronic loss of nigrostriatal dopamine is the main cause for PD's clinical features, of which bradykinesia is the hallmark symptom (Dauer & Przedborski, 2003). The most effective treatment for PD to date is L-3,4-dihydroxyphenylalanine (L-DOPA), with the drug adding directly to the dopamine concentration in the striatum as it is dopamine's immediate precursor (Chen & Swope, 2007). The use of L-DOPA is still controversial because of its shortcomings as a neuroprotective agent and the fact that it leads to dyskinesia (Blunt *et al.*, 1993).

Other drug classes used for the treatment of PD include dopamine agonists, dopa-decarboxylation inhibitors, monoamine oxidation inhibitors and catechol-O-methyltranferase inhibitors (Factor, 2008). Most therapies focus on dopamine replacement, however, antagonism of the neurotransmitter adenosine has been indicated as another non-dopaminergic approach.

Various physiological processes have been shown to be modulated with adenosine. To date, four adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) have been identified. These belong to the G protein-coupled receptors. The adenosine A_1 and A_{2A} receptor subtypes have been recognised as possible drug targets for the treatment of neurological disorders, such as Parkinson's disease (Schwarzschild *et al.*, 2006). Adenosine A_{2A} receptor antagonists are involved in the interaction between corticostriatal glutaminergic neurons and the nigrostriatal dopaminergic neurons in the striatum spiny neurons of the brain (Schwarzschild *et al.*, 2006). The inhibition of A_{2A} adenosine receptors (AR) in the basal ganglia

potentiates neurotransmission mediated by the dopamine D_2 receptor, which causes the reduction of effects as seen in the loss of dopamine concentration (Fink *et al.*, 1992). While adenosine A_{2A} receptors are highly concentrated in the striatum, the adenosine A_1 receptors are widely expressed in the brain (Pinna *et al.*, 2005).

According to literature, selective adenosine A_1 receptor antagonists have been documented as potential treatment in cognitive impairment, such as found with PD (Ribeiro & Sabastio, 2010). Furthermore, A_1 AR antagonism may also prove usable in renal and cardiac failure (Shook & Jackson, 2011).

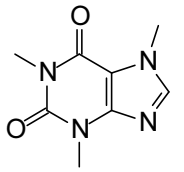
It is also suggested that selective adenosine A_{2A} receptor antagonists may find therapeutic value in the treatment of PD, not only for their ability to improve motor dysfunction, but also for their neuroprotective properties (Nobre *et al.*, 2010; Chen *et al.*, 2001). Adenosine A_{2A} receptor antagonists may be used as adjunct therapy to L-DOPA. This is beneficial as adenosine A_{2A} receptor antagonists may lower the risk of dyskinesia associated with long term treatment with L-DOPA (Kanda *et al.*, 2000). Additionally, adenosine A_{2A} receptor antagonists have been shown to exhibit antidepressant activity in animal models (Yamada *et al.*, 2013). This may be advantageous with regards to one of the non-motor symptoms namely PD associated depression.

The combination of adenosine A_1/A_{2A} receptors as a dual-target approach may find therapeutic value in PD. Dual antagonism of the A_1 and A_{2A} receptors may improve motor impairment and possess neuroprotective properties via adenosine A_{2A} receptor blockade and may enhance the cognitive function via adenosine A_1 receptor blockade (Kachroo & Schwarzschild, 2012).

Adenosine A_{2A} receptor affinities can be seen in compounds commonly belonging to two chemical classes, namely the xanthine derivatives and amino-substituted heterocyclic compounds (Klotz, 2000). Caffeine (**1**) is a well-known 1,3,7-trimethyl-substituted xanthine that acts as a non-selective A_1 and A_{2A} adenosine receptor antagonist (A_1K_i value = 55 μM; $A_{2A}K_i$ value = 50 μM) (Daly *et al.*, 1985). Substitution of caffeine (**1**) at the C8 position has previously shown to result in gained A_1 and A_{2A} adenosine receptor affinity. For example, 8-phenylcaffeine (**2**) was documented with dissociation constant (K_i) values of 17 μM and 27 μM for adenosine A_1 and A_{2A} receptors, respectively (Daly *et al.*, 1985).

1.2 Rationale

Generally, the drawback of the xanthine derivatives is low water solubility (Müller *et al.,* 2002) and this encouraged the exploration of non-xanthine and non-adenine related scaffolds to be investigated in the hope of identifying new adenosine receptor antagonists. Recently Gütschow and co-workers (2012) identified the benzothiazinone scaffold as a promising non-xanthine scaffold that may be used to design compounds with adenosine A_1 and A_{2A} receptor affinity. When compared to caffeine (**1**) and 8-phenylcaffeine (**2**), the 2-aminobenzothiazinone (**3**) (K_iA_1 = 2.39 µM and K_iA_{2A} = 1.58 µM) and benzoylaminobenzothiazinone (**4**) (K_iA_1 = 0.025 µM and K_iA_{2A} = 0.609 µM) showed lower K_i values and thus a higher affinity for both the A_1 and A_{2A} adenosine receptors. Further research in 2013 (Stöβel *et al.*, 2013) showed that chain elongation to phenylpropanamide-benzothiazinone (**5**) (K_iA_1 = 0.422 µM and K_iA_{2A} = 0.103 µM) lead to an increase in adenosine A_{2A} affinity, but a decrease in A_1 affinity. This higher affinity for the adenosine receptors displayed by the 2-acylaminobenzothiazinones makes it a promising scaffold for further exploration as a dual A_1/A_{2A} AR antagonist.



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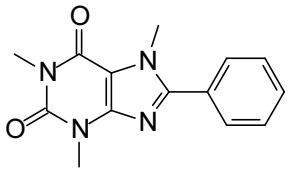

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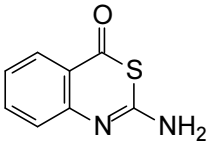

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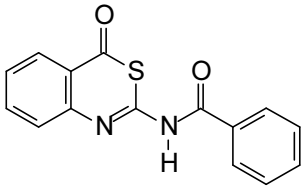

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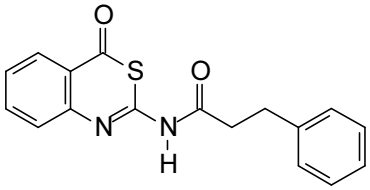

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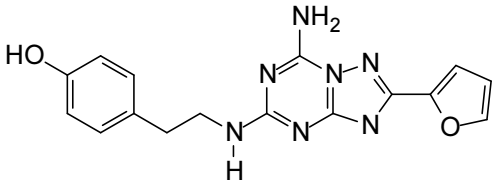
Furthermore, ZM241385 (4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin- 5-yl amino]ethyl) phenol, **6**), is a non-xanthine and a selective adenosine A_{2A} receptor antagonist with K_i values of 0.273 µM and 0.002 µM for adenosine A_1 and A_{2A} receptors, respectively (Van der Walt *et al.,* 2013). In a

modelling study by Zhukov and co-workers in 2011, the two ligands, XAC (8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-l,3-dipropylxanthine, **7**) and ZM241385 (**6**) shared a significant portion of the adenosine A_{2A} binding pocket in both position and interactions with residues lining the pocket. Both had *π–π* interactions with their central aromatic cores and Phe168, hydrophobic contacts with Leu249 and Met270, and hydrogen bonding contact with Asn253. At the entrance to the binding site, the model had an open arrangement that allows stacking of the flexible phenolic substituent of the ligand with Tyr271 in a cleft at the extracellular ends of helices 1, 2, and 7. In a similar docking study (Doré *et al.*, 2011) it showed that the phenol group of ZM241385 was found in a cleft formed by Glu13, Ala63, Ile66, Ser67, Leu267, Met270, Ile274, His278 and Tyr271 at the extracellular ends of TM1, 2 and 7 with Tyr271 displaying a rotation towards TM1 to facilitate the conformation of the phenolic moiety. This region of the receptor appeared quite flexible, and as such these tyrosine residues could adopt two different rotameric states depending upon the ligand in the complex.



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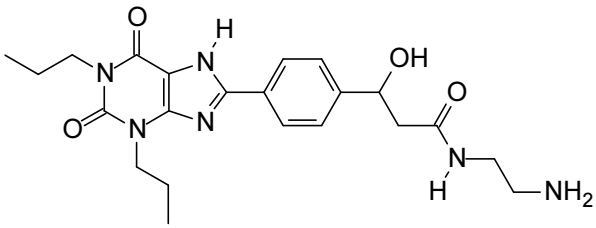

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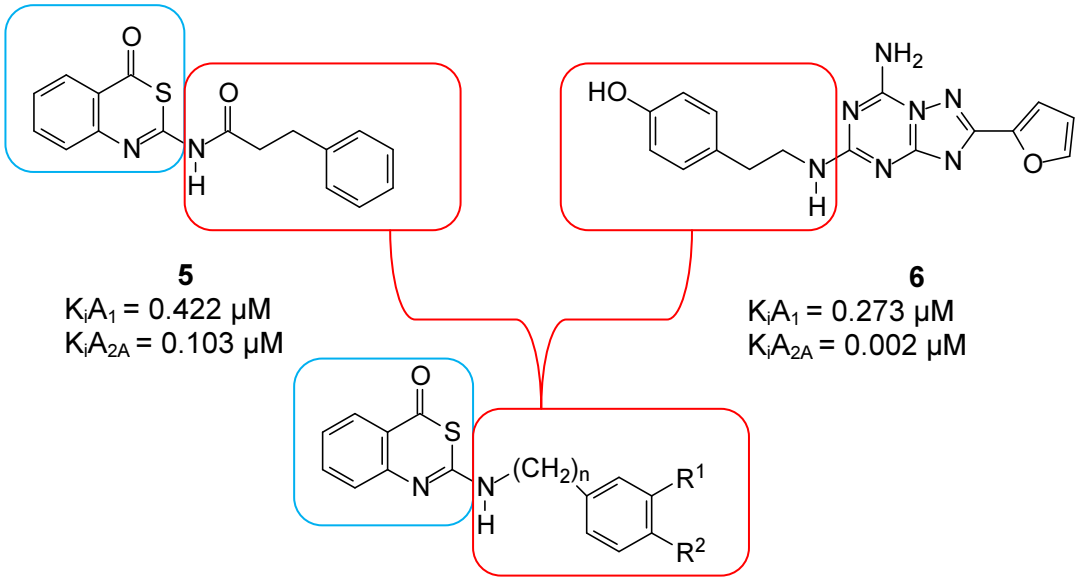
In a pilot study the acylamino side-chain of the benzothiazinone scaffold will be replaced by the flexible alkylaminophenyl side-chain of ZM241385 (**6**), thus exploring the necessity of the keto-group in the acyl side-chain. Since Gütschow and co-workers (2012) only explored the unsubstituted phenyl ring of the 2-acylaminobenzothiazinones, different *para* and *meta* substituents on the phenyl ring in the 2-alkylamino side-chain of the 2-phenylalkylaminobenzothiazinone scaffold will also be explored. In addition different chain lengths in the 2-amino side-chain will also be investigated.



2-phenylalkylaminobenzothiazinone scaffold

1.3 Hypothesis

Adenosine antagonists may be beneficial as a single drug therapy when compared to L-DOPA's symptomatic benefits and motor enhancement. As adjacent therapy, antagonism of adenosine A_{2A} receptors can improve the therapeutic action of dopamine agonists and L-DOPA. (Kanda *et al.*, 1998). It would improve the off-time caused by treatment of L-DOPA, and may improve dyskinesia as a result of the lower dose of L-DOPA needed for the same result. Effective dual antagonism of adenosine A_1 and A_{2A} receptors may also lead to improved therapy for the motor and non-motor symptoms associated with PD (Mihara *et al.*, 2007).

Because of the activity shown by the 2-acylaminobenzothiazinone scaffold, it is postulated that, if using the 2-phenylalkylaminobenzothiazinone scaffold with several chain lengths at the 2-amino side-chain and *para*- and *meta*-substitutions on the phenyl ring, a highly potent novel adenosine antagonist may be synthesised. This study will be conducting an investigation to establish if a novel compound can be found in this class that will possibly increase adenosine receptor affinity.

1.4 Objectives

- Series of 2-phenylalkylaminobenzothiazinone derivatives will be synthesised. For this purpose 2-aminobenzothiazinones will be reacted with the appropriate phenylhalide groups to provide a novel series of non-xanthine adenosine A_1/A_{2A} antagonists.
- Characterisation of the 2-phenylalkylaminobenzothiazinone derivatives through NMR, MS, melting points and IR.
- The *in vitro* evaluation of the 2-phenylalkylaminobenzothiazinones derivatives as antagonists of the adenosine A_1 and A_{2A} receptor. Values to be expressed via percentage of displacement, and compared to previous compounds documented by Gütschow and co-workers (2012).

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CHAPTER 2

LITERATURE STUDY

2.1 Introduction

Parkinson's disease (PD), the age-related motoric disease commonly observed in the 20th century (Mhyre, *et al.*, 2012), is a neuro-degenerative disease of unknown origin (Miller & O'Callaghan 2015). It is characterised by the degeneration of dopamine neurons in the substantia nigra *pars compacta* (SNpc) that result in the reduction of dopamine concentrations in the nigrostriatal pathway (Obeso *et al.*, 2010; Phani *et al.*, 2012). Idiopathic and sporadic PD rank second to Altzheimer's disease in prevalence and as a consequence is perceived as an economic and social burden (Miller & O'Callaghan, 2015).

The cause of PD is still unknown. Although the genetic factors that may have an effect on PD are being examined, interaction between genetic and environmental factors as cause for the degeneration of neurons is largely an unknown field of study (Abdullah *et al.*, 2015). Other factors also include aging and oxidative stress (Dauer & Przedborski, 2003). A more detailed discussion will follow pertaining to the epidemiology, pathophysiology, clinical overview as well as the etiology of PD.

2.2 Epidemiology

The number of individuals over 50 years of age with PD in the world's top 10 most populated countries was estimated between 4.1 and 4.6 million in 2005 and is projected to double by 2030 to 8.7 and 9.3 million (Dorsey *et al.*, 2007).

PD seems to occur more commonly in male subjects compared to females (Goetz & Pal, 2014) and new research showed a male to female ratio of 1.6:1 (Lubomski *et al.*, 2014). The prevalence of PD in individuals under the age of 50 is very rare (Goetz & Pal, 2014), but the number dramatically increases in people over the age of 60 (Abdullah *et al.*, 2015). Sporadic PD (not linked to genetics) is thought to be responsible for 95% of cases, with the other 5% being the result of inheritance (Dauer & Przedborski, 2003).

2.3 Clinical overview

The symptoms of PD are the consequence of two mechanisms, namely dopaminergic neuron degeneration and the subsequent reduction in dopamine concentration (Goetz & Pal, 2014). The most common and hallmark motor symptoms attributed to PD are rigidity (increased resistance to passive movement & stiffness), resting tremors, bradykinesia (slowness of movement), akinesia (loss of normal unconscious movements such as swinging of arms whilst walking), postural disabilities, and gait impairment (Dauer & Przedborski, 2003). These symptoms are a result of the deficiency of dopamine in the striatum (Miller & O'Callaghan, 2015; Goetz & Pal, 2014).

Although the diagnosis of PD is based on the latter motor symptoms; non-motor symptoms such as fatigue, depression and sleep disturbances form an integral part of PD (Goetz & Pal, 2014). These non-motor symptoms affect a large population of patients and can appear before the motor disorders and often have a larger negative effect on the life quality of a patient than the motor symptoms (Goetz & Pal, 2014).

2.4 Etiology

As mentioned above, even if the cause of PD is still unknown, remarkable advances have been made in understanding the possible underlying mechanisms which include environmental and genetic factors that underlie the loss of nigral dopaminergic neurons.

Ageing is also mentioned as an important risk factor for PD. In a review by Rodriguez and co-workers (2015), it is suggested that PD is the result of the slow neurodegenerative action of aging, which can be accelerated by repeated damage to dopamine neurons accumulated over a person's lifespan. When the degeneration of the dopamine neurons reaches a critical level where compensatory mechanisms are insufficient to maintain the basic functions of dopamine, the first motor disturbances appear and the diagnosis of PD can be made.

2.4.1 Genetic hypothesis

The role that genes have to play in PD has been noticed even a century ago when it was seen that family members of patients suffering from PD had a high

prevalence of acquiring the disease. Generally, the chance of a relative having the disease is 2 to 3 times higher than the norm (Pan-Montojo & Reichmann, 2014).

The idea behind studying the genetic abnormalities in common diseases is the expectation that similarities between the sporadic and genetic forms of the disease will help researchers focus on key biochemical pathways involved in both sporadic and genetic PD for cures in the future. The recognition of certain genetic abnormalities in patients suffering from PD is of great use, because now patients with the gene mutation can be used as novel models of PD and biomarkers for diagnosis of the disease in younger patients may prove vital in the management of PD (Dauer & Przedborski, 2003; Miller & O'Callaghan, 2015). A number of PD related genes have been researched and identified, which include α-synuclein, parkin and ubiquitin C-terminal hydrolase L1 (UCHL-1). These are only a few genes involved and many others have been identified as risk factors for PD (Pan-Montojo & Reichmann, 2014; Dauer & Przedborski, 2003).

Genetic mutations lead to accumulation and production of misfolded proteins that has been seen as a vital biochemical cause of the neurodegeneration observed in PD. Genes that underwent mutation as a result of a pathogen, may induce the direct production of misfolded proteins (believed to be the case with α-synuclein mutation) and can also damage the body's own process of eliminating and degrading of misfolded and incorrect proteins (UCHL-1 and Parkin) (Dauer & Przedborski, 2003).

Figure 2-1 illustrates where the mutation of these specific genes have their effect on the mechanisms of neurodegeneration. The conformation of abnormal proteins leads to toxicity and the formation of Lewy bodies (LBs), which have been shown to play key roles in the resulting neurodegeneration of dopaminergic cells (Dauer & Przedborski, 2003).

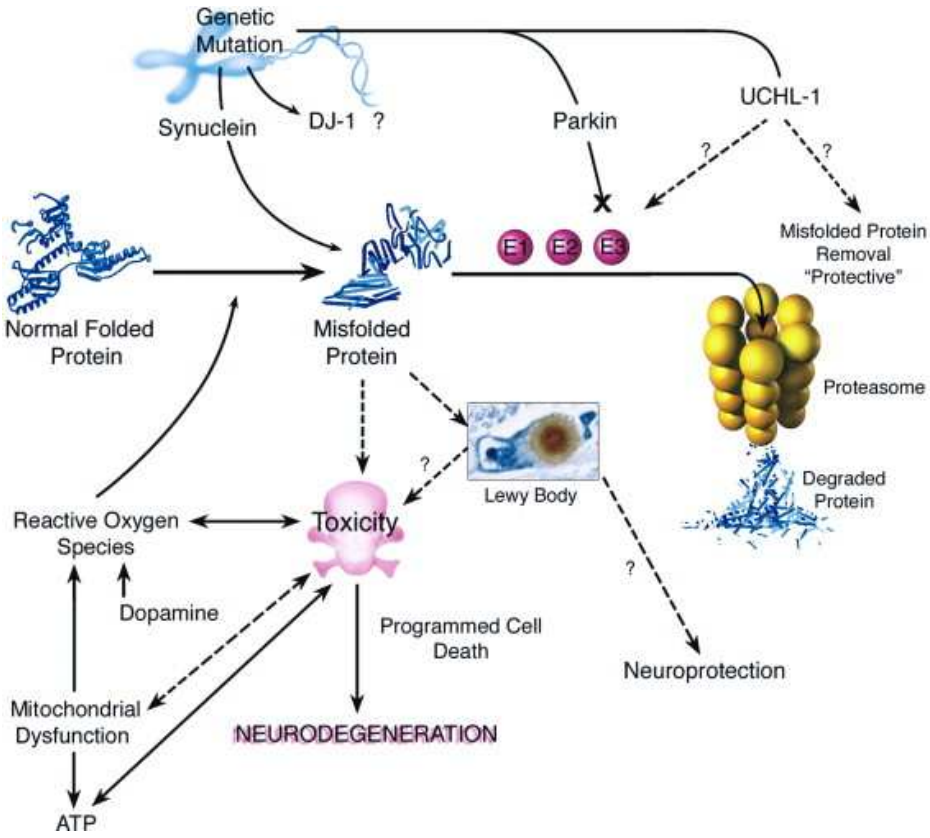
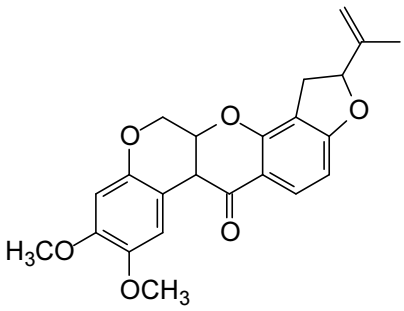


Figure 2-1: Genetic mutation and the resulting mechanisms of neurodegeneration (Dauer & Przedborski, 2003)

2.4.2 Environmental hypothesis

The environmental hypothesis proposes that PD-associated neurodegeneration is the result of exposure to a neurotoxin for dopaminergic cells. This effect can be the result of chronic exposure to a neurotoxin, which either causes the degeneration of neurons, or by acute exposure that activates a series of cascading events finally causing degeneration similar to that of PD (Dauer & Przedborski, 2003). Recent environmental studies have linked PD to factors like drinking well water, farming, living in rural areas and being exposed to chemicals used for agricultural means (Pan-Montojo & Reichmann, 2014). A study was performed to find a correlation between compounds that were known to be risk factors for PD, and it was found by Tanner and his team (2011) that compounds inhibiting mitochondrial complex 1 and increasing oxidative stress were prone to cause iPD (idiopathic Parkinson's disease) due to exposure (Tanner *et al.*, 2011). Certain metals and industrial compounds have also been linked to iPD in many studies done in the 90's and it was identified that exposure to metals such

as lead, copper, iron, zinc and manganese can be correlated with higher incidence of iPD (Pan-Montojo & Reichmann, 2014). Two compounds that were isolated in the studies done by Tanner and colleagues (2011) were rotenone **(8)** and paraquat **(9)**, with both showing inhibition of mitochondrial complex 1 and increases in oxidative stress, both of which are pathological attributes of PD, that make rotenone and paraquat plausible causes of PD (Tanner *et al*., 2011).



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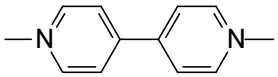

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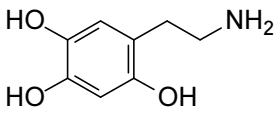
Another compound that was identified to induce symptoms and mechanisms similar to that of PD, was with the treatment of 6-hydroxydopamine (6-OHDA) (**10**), which is now known as the classical animal model of PD (Jackson-Lewis *et al*., 2012). It was found that treatment with 6-OHDA, by injecting it into the forebrain of rats; since it can't cross the blood-brain barrier, lead to the degeneration of dopaminergic neurons in the SNpc similar to that seen in PD. The 6-OHDA causes the formation of quinones within the neurons, which then results in the generation of free radicals that causes the deactivation of biochemical macromolecules (Pan-Montojo & Reichmann, 2014).



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By far the most common and extensively studied compound when discussing neurodegeneration as seen by environmental causes of PD, is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP, a non-toxic compound, is additionally produced during the manufacturing of desmethylprodine (MPPP), which is a synthetic opioid. There have been several cases in the 1980's of people ingesting MPTP, resulting in a syndrome described as parkinsonism (Singer & Ramsay, 1990). The cause of the neurodegeneration is the product of

MPTP metabolism in the body, which then forms MPP^+, a potent mitochondrial complex 1 inhibitor and dopamine neuron killer (Pan-Montojo & Reichmann, 2014). 1-methyl-4-phenylpyridinium (MPP^+) is structurally the same as paraquat, which was previously mentioned to cause PD like symptoms as a result of chronic exposure. MPTP is absorbed by the astrocytes, where it is then metabolised by monoamine oxidase type B (MAO-B), to form the active metabolite MPP^+. Once released from the astrocytes by the organic cation transporter (OCT3), MPP^+ is taken up into dopaminergic neurons via the dopamine transporter (Jackson-Lewis *et al.*, 2012). (**Figure 2-2)**.

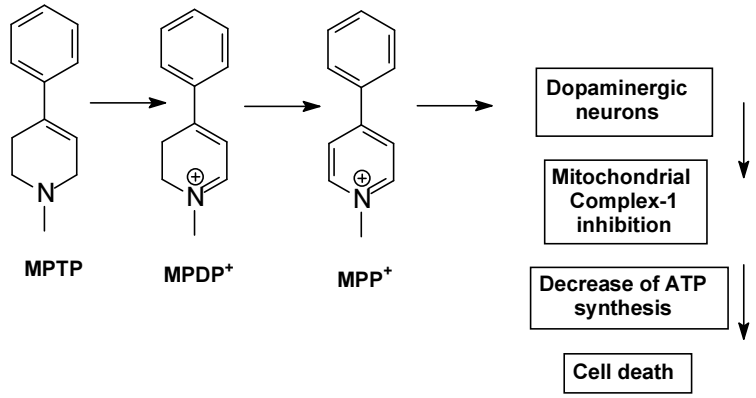


Figure 2-2: Cascading events that leads to neuron death as a result of MPTP (Singer & Ramsay, 1990)

Intracellularly MPP^+ causes the inhibition of mitochondrial complex 1 and leads to the initiation of other cellular reactions like the inhibition of nicotinamide adenine dinucleotide dehydrogenase that cause the decrease of ATP synthesis and eventually leads to the death of the neurons (Singer & Ramsay, 1990). MPP^+ is stored in vesicles in the dopaminergic neurons, which forces the release of dopamine into the extracellular space because of limited storage, which in turn leads to the metabolism of dopamine. MPTP may find value to generate an effective model for PD, with the only lacking biochemical hallmark of PD being the Lewy Body (LB) (Jackson-Lewis *et al.*, 2012).

2.5 Pathogenesis

The two major hypotheses involved in the neuronal degeneration (pathogenesis) of PD are the misfolding and aggregation of proteins, which have been found to be key participants in the degeneration of the SNpc dopamine neurons and

secondly, mitochondrial malfunction and the resulting oxidative stress, which also includes oxidised toxic dopamine species (Ebrahimi-Fakari *et al.*, 2011; Dauer & Przedborski, 2003) (**Figure 2-3**). Other hypothesis include mitochondrial dysfunction, excitotoxicity, trophic factor deficiency, inflammatory processes, genetic factors, environmental impact factors, toxic action of nitric oxide and apoptosis.

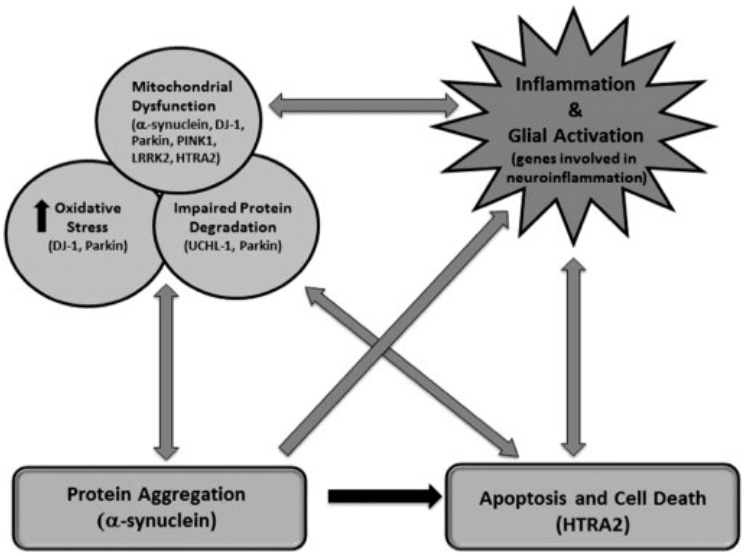


Figure 2-3: Causes of cell apoptosis and necrosis (Miller & O'Callaghan, 2015)

It is estimated that 4% of the original amount of neurons producing dopamine are lost every decade due to natural ageing. The symptoms of PD only appear after approximately 70% of dopaminergic cells have been destroyed which makes early interventions highly important (Youdim & Riederer, 1997).

The specific causes of cell death in neurodegenerative diseases such as PD still carry doubt and are poorly understood. It is suggested that the mechanism of PD neuronal damage leans towards programmed cell death (PCD) rather than passive cell death (necrosis). Apoptosis is a well characterised form of PCD and refers to the body's mechanism of removing damaged or unnecessary cells (Jellinger, 2000). Apoptosis though is not responsible for the total scope of cell death as seen in PD, other mechanisms recently discovered and researched also include PCD activated by autophagy in cells (Venderova & Park, 2012).

2.5.1 Neuropathology

The major pathological features of PD are loss of nigrostriatal neuromelanin containing dopaminergic neurons and also the presence of Lewy bodies (LBs) in the brain. This loss in neurons causes depigmentation (**Figure 2-4; Panel B**) of the SNpc and has been observed in postmortem studies performed on patients that suffered from PD (Schapira & Jenner, 2011).

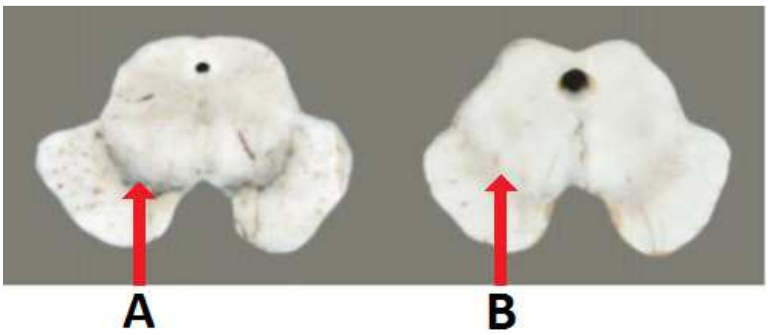


Figure 2-4: **Pigment loss in the SNpc as a result of the death of dopaminergic neurons, with A the SNpc of a healthy individual and B that of a patient that suffered from PD (Youdim & Riederer, 1997)**

LBs are identified as intraneuronal, eosinophyllic inclusions that can be found commonly in the substantia nigra. Lewy bodies concentrate in the cell soma and neurites of mostly the substantia nigra neuron population (Lotharius & Brundin, 2002; Dauer & Przedborski, 2003). In PD, LB pathology (**Figure 2-5**) first appears in the dorsal motor nucleus of the vagus and the olfactory system, then progressing to changes in the coeruleus complex, SNpc, basal forebrain magnocellular nucleus, subthalamic nucleus, and amygdala and finally the neocortex (Braak *et al*., 2002).

In addition to containing proteins such as ubiquitin, heat-shock proteins and neurofilaments, LB mostly consists of α-synuclein (α-syn) filaments, which are 200-600 nm in length and 5-10 nm in diameter. As can be seen from the pathogenesis of PD, the formation and accumulation of misfolded protein aggregates are the key participants in the degeneration of dopamine neurons in PD, indicating α-syn as a plausible cause of PD. Recently, several pieces of the puzzle have suggested that α-syn may self-propagate; thereby contributing to the initiation and the pathology of PD (Recasens & Dehay 2014). Supportive of this

suggestion is a recent study, demonstrating that immunotherapy with antibodies that specifically target misfolded α-syn was able to block the entrance and propagation of α-syn in neurons, and hence prevents the development of neuropathological abnormalities in the brain (Tran *et al.*, 2014).

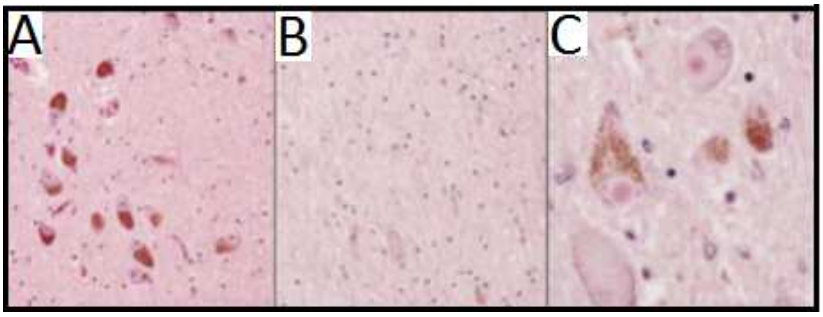


Figure 2-5: **DA neurons (A) in healthy brains as visible in the substantia nigra (SN) and how they are physically absent (B) in the SN brain tissue of PD patients in later stages of the disease. The pink spheres as seen in (C) are the above mentioned inclusions known as Lewy bodies (Youdim & Riederer, 1997)**

2.6 Current treatment

PD is still an incurable disease with a progressive nature (Lees *et al.*, 2009). The main focus and goal of PD treatment is the improvement of motor and non-motor symptoms (anxiety, cognitive impairment, constipation, depression, dysphagia, sleep disorders, etc.) to grant the patient the best possible quality of life. Many objectives are considered when choosing a suitable treatment option, for example the improvement of mobility and functionality to continue the normal quality of daily living, whilst restricting adverse effects of chronic treatment with PD medication (Chen & Swope, 2007). Currently treatment for PD can be categorised in three options, namely non-pharmacological, surgery and pharmacological treatment regimens (Kakkar & Dihiya, 2015).

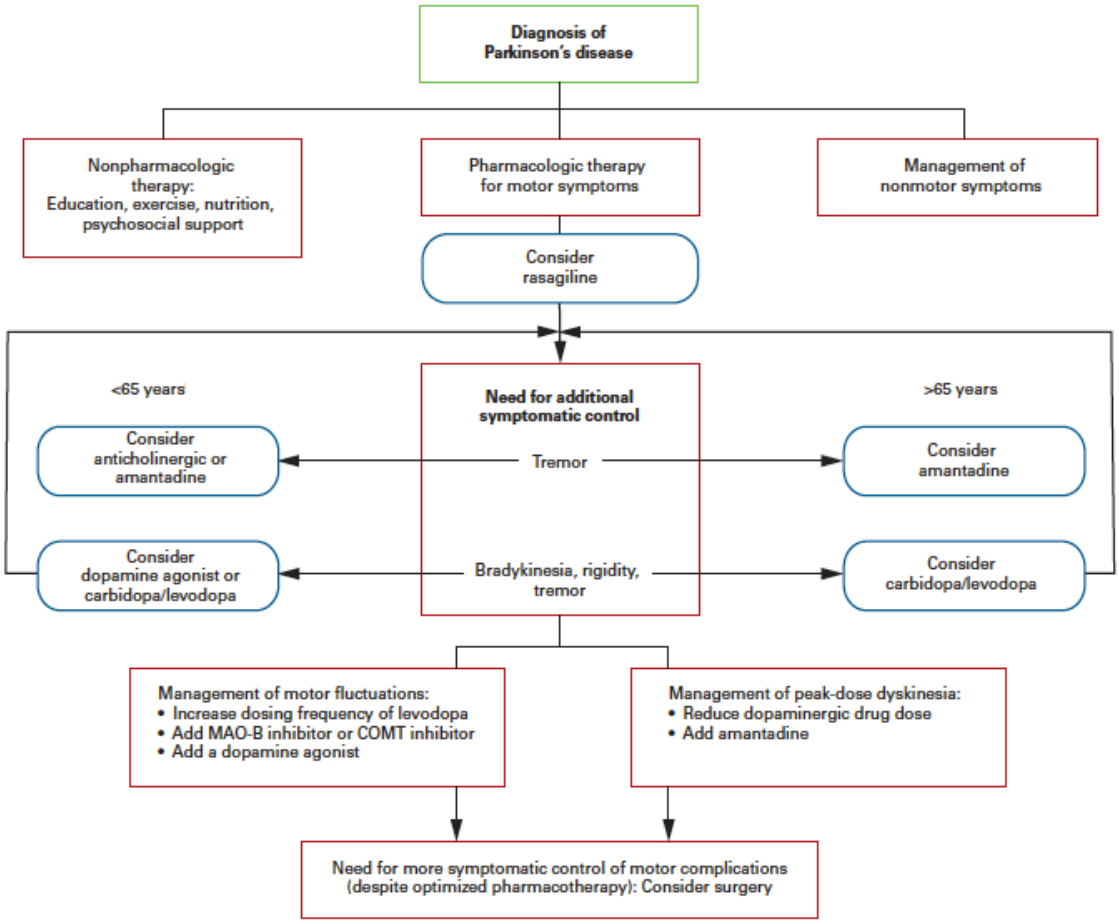
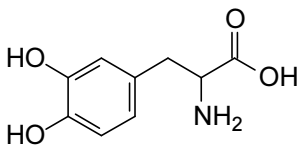


Figure 2-6: A schematic representation of a general approach to PD treatment (Chen, 2011)

With the diagnosis of PD, management strategies are implemented and based upon stage of the patient's diagnosis. In early stages of PD, monotherapy is effective in treatment of symptoms with limited adverse effects of the medication. As the disease progresses and medicine doses need to be increased, the monotherapy reaches a point where doses are too high and treatment is optimised. At this point, adjunctive combinations must be added to improve the disease management and symptomatic relief of this complex disorder. However, multicomponent therapy possesses some shortcomings; as it may cause drug intolerance and/or appearance of adverse symptoms. At this stage, agents are normally discontinued periodically to revert back to monotherapy that has a balance between disease management and least adverse effects, albeit not necessary the same strategy as initially used (Chen & Swope, 2007).

2.6.1 Levodopa (L-DOPA)



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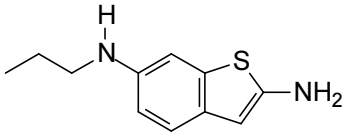
The discovery of dopamine loss in the brain led researchers to search for compounds that increased dopaminergic activity. L-3,4-Dihydroxyphenylalanine (L-DOPA, **11**), a direct precursor of dopamine, has been and remains the most effective treatment for PD since its approval for therapy in 1970, more than 10 years after it was realised that dopamine depletion was the main cause of PD (Factor, 2008; Kakkar & Dihiya, 2015). L-DOPA is decarboxylated by L-amino acid decarboxilase (L-AAD) to dopamine and directly supplements the dopamine concentration in the striatum. Although L-DOPA addresses the loss of dopamine as a result of neuron loss as seen in PD, it has not yet been reported to have an effect on disease progression (Goetz *et al.*, 2002).

L-DOPA is administered orally and absorbed in the gastrointestinal tract. Most of the L-DOPA ingested is metabolised to homovanillic acid and in smaller amounts to dopamine sulphate. Only a small amount of L-DOPA is delivered to the brain, as little as 5%. L-DOPA is usually administered in conjunction with carbidopa (see **2.6.3**) for optimal results and also to reduce adverse effects by allowing for a lower dose of L-DOPA needed (Kakkar & Dihiya, 2015). The use of COMT inhibitors also reduces the L-DOPA dose needed for symptomatic control by up to four-fold, and is therefore mostly added to the treatment strategy to reduce the appearance of adverse effects as a result of high doses of L-DOPA (Jankovic & Aguilar, 2008).

Evidence from various animal studies has shown that continual dopaminergic stimulation may be responsible for dyskinesia and other motor fluctuations. Postmortem studies on PD patients have shown signs of oxidative stress on the SNpc with the presence of oxidative damage to lipids, proteins and DNA. This leaves the SNpc in the brain vulnerable for oxidising agents such as L-DOPA to conflict more damage (Jankovic, 1989).

2.6.2 Dopamine agonists



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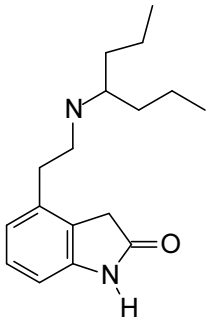

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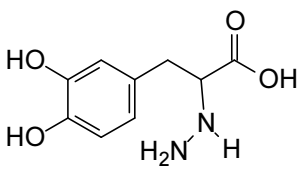
Dopamine agonists bypass damaged neurons and directly activate and stimulate the healthy postsynaptic receptors in the striatum, thereby making them superior to L-DOPA and with the increase in elimination half-life and stable blood levels some of the adverse effects associated with L-DOPA are eliminated (Factor, 2008). Dopamine agonists provide a wider therapeutic window, and therefore decrease the chance of experiencing dyskinesia (Factor, 2008).

Two groups of dopamine agonists have been in use since 1974, namely ergot- and non-ergot derivatives. The ergot derivatives include bromocriptine and pergolide, but these compounds were under scrutiny following research that indicated pulmonary and heart complications (Chen & Swope, 2007). Examples of non-ergot compounds are pramipexole (**12**) and ropinirole (**13**). Pramipexole acts as an autoreceptor agonist when presynaptic neurons are intact, and as a potent postsynaptic receptor stimulant when presynaptic neurons are damaged; making these properties ideal for PD therapy (Factor, 2008). Dopamine agonists are normally used as initial treatment of early stage PD usually for patients under the age of 55, however, three years after diagnosis L-DOPA is normally introduced (Lees *et al.*, 2009).

2.6.3 Dopa decarboxylase inhibitors



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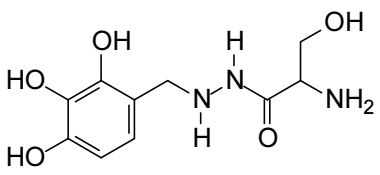

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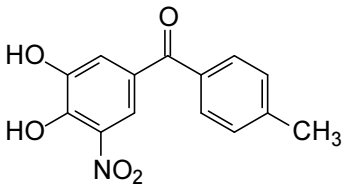
Carbidopa (**14**) and Benzeraside (**15**) are peripheral dopa decarboxylase inhibitors used in the preliminary treatment of PD in conjunction with L-DOPA (Lees *et al.*, 2009). These compounds were used in 1975 solely to improve L-DOPA related nausea (Hinz *et al.*, 2014). They inhibit peripheral L-AAD that convert L-DOPA to dopamine; thereby decreasing the adverse effects of L-DOPA in the peripheral space, resulting in a four-fold increase of L-DOPA entering the striatum (Chen & Swope, 2007; Goetz & Pal, 2014). A recent study showed that dopa decarboxylase inhibitors are the cause of irreversible dyskinesias, causing irreversible binding and inactivation of vitamin B_6 throughout the body (Hinz *et al.*, 2014).

2.6.4 COMT-inhibition



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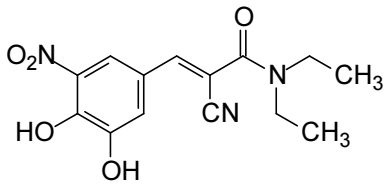

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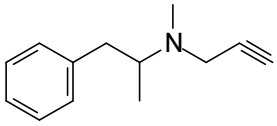
Catechol-O-methyltransferase (COMT) inhibitors were first introduced in 1997 with the release of tolcapone (**16**) and followed by entacapone (**17**) in 1999. Both these compounds were indicated for the treatment of advanced PD with fluctuations, and tolcapone as polytherapy in conjunction with L-DOPA in non-fluctuating patients (Factor, 2008). COMT-inhibitors peripherally decrease the metabolism of L-DOPA to 3-O-methyldopa, thereby increasing the bioavailability and half-life of L-DOPA.

Hepatic injury is rare but has been associated with chronic use of tolcapone (**16**), however with continual monitoring the drug can be prescribed safely (Factor, 2008). It is important to mention that COMT-inhibitors have no therapeutic value in the absence of dopamine, and are therefore only used as adjacent to other therapies (Chen & Swope, 2007).

2.6.5 MAO-inhibition



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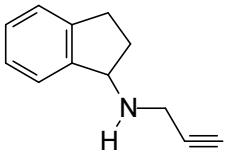

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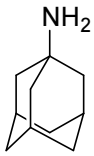
Monoamine oxidases (MAO) are enzymes that contribute to the oxidative deamination of amines such as dopamine. By the inhibition of these enzymes, the endogenous and exogenous dopamine effect is improved through prolonged activity (Chen & Swope, 2007; Factor, 2008).

Two compounds, selegenine (**18**) and rasagaline (**19**) are currently available for the treatment of PD. MAO-inhibitors also exhibit neuroprotective properties by decreasing the release of oxygen free radicals that contribute to the neuronal damage as seen in PD, and is used in early stages of the disease to delay disease progression (Chen & Swope, 2007, Lees *et al*., 2009).

The use of antidepressant drugs in conjunction with MAO-inhibitors may lead to patients experiencing serotonin syndrome. Therefore, careful monitoring should be introduced to lower this risk (Goetz & Pal, 2014). The use of MAO-inhibitors as dual therapy with L-DOPA may worsen pre-existing dyskinesias by increasing L-DOPA's peak effects (Chen & Swope, 2007).

2.6.6 Amantadine



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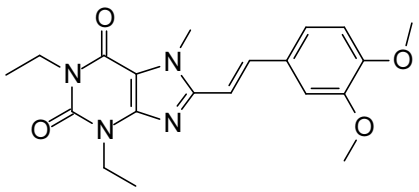
Amantadine (**20**) is known to change the release of dopamine from neuron terminals and decrease its reuptake with an unknown mechanism of action (Chen & Swope, 2007; Crosby, 2009). Amantadine was originally used for the antiviral treatment of influenza, but later it was realised that it improved PD related symptoms. (Crosby, 2009). Amantadine has been associated with severe side effects of a psychiatric nature, including dizziness, confusion and hallucinations (Chen & Swope, 2007; Crosby, 2009).

2.6.7 Surgery

Various surgical treatments have been researched as therapy for PD. These include thalamotomy (destroying portion of thalamus), pallidotomy (destroy small area of brain cells in globus pallidus), chronic intracerebral stimulation and/or deep brain stimulation of dopaminergic foetal tissue. The increased risk of thermolytic lesioning or haemorrhages of tissue near the target site add to the rate of mortality and morbidity associated with surgical treatment of PD (Obeso *et al*., 1997).

2.6.8 Adenosine receptor antagonists

While dopamine has long been the neurotransmitter most closely associated with PD; several other neurotransmitters also play a role in PD (Trevitt *et al*., 2009). Adenosine A_{2A} receptors have an important role in the modulation of dopamine-mediated responses and thus the control of motor behavior (Pinna *et al*., 2005). In the brain, adenosine A_{2A} receptors are almost exclusively expressed in the striatum of the basal ganglia (Tanganelli *et al.,* 2004, Pinna *et al*., 2005). Adenosine A_{2A} antagonists potentiate the motor benefit of L-DOPA in Parkinson's disease patients, without the potentiation of L-DOPA-associated dyskinesia (Bara-Jiminez *et al*., 2003). In addition, A_{2A} antagonists may also possess neuroprotective properties and provide antidepressant like effects in PD treatment (Hung & Schwarzschild., 2014). In contrast to adenosine A_{2A} receptors, adenosine A_1 receptors are widely expressed in the central nervous system (CNS). In the striatum, adenosine A_1 receptor stimulation inhibits dopamine release. Conversely, blockade of A_1 receptors facilitates dopamine release in the striatum and potentiates dopamine mediated responses. Additionally, it has been reported that adenosine A_1 receptor antagonists may improve learning and memory in animal models (Shook & Jackson, 2011) and may thus also find therapeutic application by enhancing cognitive functions in PD. Examples of A_{2A} antagonists that have progressed to clinical trials are istradefylline (**21**) (KW-6002) and tozadenant (**22**) (SYN115) (Pinna, 2014).



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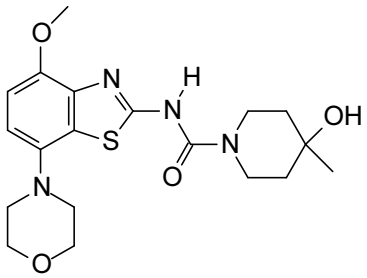

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2.7 Shortcoming in current treatment regimes

Current therapy of PD offer inadequate neuroprotection and disease progression is only partly stinted. These antiparkinsonian drugs also cause adverse effects that further add to their shortcomings (Xu *et al.*, 2005).

Antiparkinsonian therapy can cause multiple central nervous disturbances, with cognitive disturbances high on the list. L-DOPA has the highest potency in early and advanced disease states, but is accompanied by dyskinesia and other safety issues. COMT-inhibitors and DA-agonists are effective as additional or mono-therapy, but studies have shown that they can be associated with frequent gait disorders, orthostatic hypotension, psychosis, hallucinations, diarrhea etc. (Factor, 2008).

MAO-inhibitors are very sensitive to drug interaction with the possibility of a hypertensive attack as a result of excess tyramine or serotonin syndrome in conjunction with antidepressant agents (Factor, 2008). The limitations accompanied by DA altering treatment has motivated researchers to discover non-dopaminergic paths to PD treatment, thereby hopefully eliminating adverse effects such as dyskinesia and multiple drug interactions (Factor, 2008; Shook & Jackson, 2011). For the above reasons the adenosine receptor antagonists may provide an attractive drug target for developing novel treatment options for PD.

2.8 Conclusion

In this chapter, the current literature pertaining to Parkinson's disease as a neurodegenerative disease of the central nervous system was provided. The burden experienced by patients suffering from PD is clear. It also shows the current treatment options available and their shortcomings. It is important to note that all current treatment regimens have emphasis on alleviating disease symptoms, and do not focus on disease progression. Future research must be

concentrated towards lowering treatment associated side effects, and address disease progression by increasing factors such as neuroprotection.

Recently adenosine receptors have become an attractive class of promising new antiparkinsonian drugs for managing the symptoms of PD (Fredholm & Svenningsson, 2003; Fredholm *et al*., 2003; Golembiowska & Dziubina, 2004). Adenosine A_{2A} antagonists may be a valuable strategy in the symptomatic management of PD, by restoring motor behavior. Besides its symptomatic benefits, A_{2A} antagonists may also possess neuroprotective properties and may prevent the development of dyskinesias that are usually associated with L-DOPA treatment. In addition adenosine A_1 receptor antagonists have been documented as potential treatment in cognitive impairment, such as found within PD and Alzheimer's disease. The adenosine receptors as drug target for the treatment of PD will be discussed in chapter 3.

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CHAPTER 3

ADENOSINE RECEPTORS

3.1 Adenosine receptors

3.1.1 General background and tissue distribution

Adenosine receptors (ARs) are considered to be one of the human body's most important neuromodulators, in both the central and peripheral nervous systems (Nomoto, 2000). It modulates its effects via four receptor subtypes namely A_1, A_{2A}, A_{2B} and A_3, and are associated with the heterotrimetric guanine nucleotide-binding protein (G-protein) coupled receptors (Piirainen *et al.*, 2011). A_1 and A_3 AR are associated with inhibitory G-proteins, whilst A_{2A} and A_{2B} couple with stimulatory G-proteins (Shook & Jackson, 2011). Adenosine A_1 and A_3 receptors are widespread throughout the brain, whereas A_{2A} receptors are more restricted to the striatum (Svenningsson *et al.*, 1999), where they are co-expressed with dopamine D_2 receptors and are associated with motor behavior.

Furthermore, the adenosine A_1 receptors are associated with cognitive effects, and are also found in the hippocampus and prefrontal cortex, which are important brain areas for cognition (Onodera & Kogure, 1988). In turn the A_{2A} AR are associated with motor behavior and are co-expressed with dopamine D_2 receptors (Svenningsson *et al.*, 1999)

3.1.2 Adenosine receptors in Parkinson's disease

3.1.2.1 Motor symptoms

PD is mainly characterised by the motor symptoms presented, including bradykinesia, tremor and muscle rigidity (Dauer & Przedborski, 2003). These symptoms worsen following gradual disease progression (Chen & Swope, 2007). The intervention of A_{2A} AR on motor symptoms are currently based on our knowledge of basal ganglia anatomy; where antagonism of the adenosine A_{2A} receptors are seen as an ideal target for antiparkinsonian therapy because of its concentrated distribution in the striatum (Xu *et al.*, 2005). Antagonists of A_{2A} AR are suggested to be beneficial in PD (Schwarzschild *et al.*, 2006).

There are two main pathways (direct and indirect) in the striatum that contribute to opposite effects on motor movement. Adenosine A_1 and dopamine D_1 receptors are distributed throughout the direct pathway. The indirect pathway contains a small number of both dopamine D_1 and adenosine A_1 receptors, but mostly consist of adenosine A_{2A} and dopamine D_2 receptors. The direct pathway, also known as the nigrostriatal pathway, facilitates desired movement, whereas the indirect pathway (striatopallidal pathway) inhibits undesired movements. Adenosine A_{2A} receptors and dopamine D_2 receptors act in an antagonistic manner and it is believed that dopamine, via D_2 receptors, antagonise adenosine A_{2A} receptor mediated signalling (Tanganelli *et al.*, 2004; Vortherms & Watts, 2004). Dopamine depletion in PD would lead to unopposed adenosine signalling, resulting in over activity of the striatopallidal pathway, and excess inhibition of movement (Fredholm & Svenningsson, 2003). Animal studies with rodents have also shown that A_{2A} AR antagonists effectively reduce catalepsy and reverse locomotor activity suppressed by dopamine D_2 receptor antagonists (Salamone *et al.*, 2008; Antoniou *et al.*, 2005; Correa *et al.*, 2004; Moo-Puc *et al.*, 2003; Malec, 1997). The rationale for A_{2A} AR antagonists in the therapy of PD is thus based upon the co-localisation of A_{2A} AR with D_2 dopamine receptors on the striatopallidal neurons, which is an instrumental tool to restoring motor behavior (Ferré *et al.*, 1993).

This interaction may be explained in short as follows: during normal **physiological conditions** (**Figure 3-1; Panel-A**), striatal neurons receive dopaminergic inputs from the SNpc. Dopamine stimulates the direct pathway via D_1 stimulatory receptors which then sends GABAergic projections to the substantia nigra *pars reticulata*/globus *pars interna* (SNr/GPi) and increase motor activity (**Figure 3-1)**.

At the same time, dopamine inhibits the indirect pathway via dopamine D_2 inhibitory receptors which sends GABAergic projection to the SNr/Gpi via globus *pars externa* GPe and subthalamic nucleus (STN) and causes a removal of motor inhibition (motor activation). Dopamine thus excites the direct pathway and inhibits the indirect pathway, leading to increased motor activity (Kase *et al.*, 2004). On the other hand adenosine via A_{2A} AR in the striatum and GPe excites the indirect pathway, consequently opposing the activation of dopamine D_2 receptors. The latter results in balanced levels of activity of the direct and indirect pathways for correct processing of motor information and movement execution.

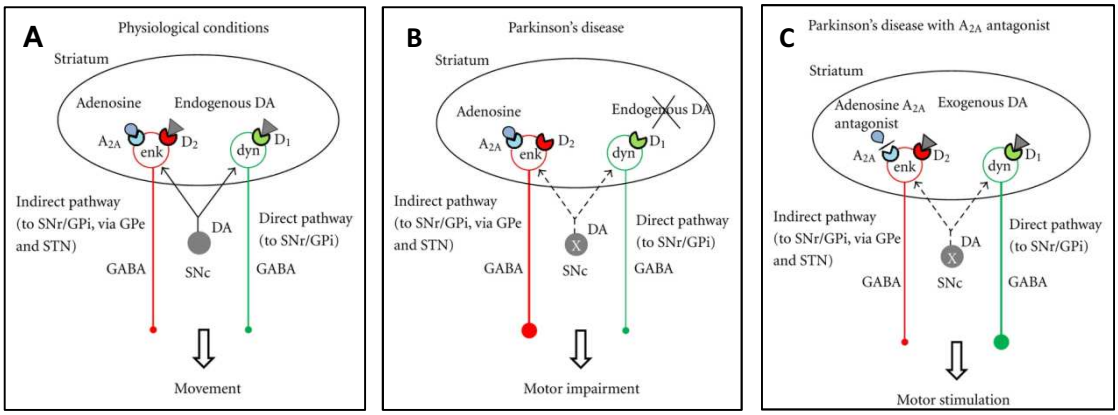


Figure 3-1: **Illustration of the activity (indicated by the thickness of the arrows) of the main pathways of the basal ganglia under (A) physiolgical conditions, (B) Parkinson's disease and (C) Parkinson's disease treated with A_{2A} antagonists (Morelli *et al.*, 2012)**

In PD (**Figure 3-1; Panel-B**), degeneration of the SNpc cause loss of dopamine, which lead to a reduction of striatal dopamine D_1 and D_2 mediated dopamine transmission. This loss of dopamine leads to a reduced activity in the direct pathway, while there is increased inhibitory GABA-mediated effects on GPe in the indirect pathway, causing a marked GPe suppression accompanied by disinhibition of excitatory glutamate mediated STN transmission. This imbalance between the direct and the indirect pathways leads to a reduction of movements, e.g. bradykinesia and akinesia in PD (Fuxe *et al.*, 2007; Schwarzschild *et al.*, 2006).

Adenosine via A_{2A} receptors in the striatum and GPe excites the indirect pathway thus opposing the activation of dopamine D_2 receptors. In contrast, adenosine A_{2A} antagonists will increase dopamine D_2 signalling (A_{2A} antagonists increase dopamine D_2 affinity for dopamine). Treatment with **A_{2A} antagonists** (**Figure 3-1 Panel-C**) will block the indirect striatopallidal GABA pathway, resulting in the recovery of GPe activity which in turn leads to the relief of over activity of STN neurons and subsequently, restore/normalise some balance between the direct and indirect pathways (Kase *et al.*, 2004).

Initially A_1 AR were recognised as the main mediator of the motor-inducing effects of caffeine, with Snyder and co-workers (1981), using displacement of radiolabeled A_1 AR agonists to show significant correlation between different

methylxanthine potencies and fluctuating motor activation in rats (Karcz-Kubicha *et al.,* 2003). However, discovery of the high concentration of A_{2A} AR in the striatum and using more selective compounds, several experiments proved that the A_{2A} AR subtype is the main target for the motor activating effects of caffeine in rats (Karcz-Kubicha *et al.,* 2003). Jacobson and co-workers (1993) also found evidence of the occurrence of synergism between the motor-activating effects of adenosine A_1 and A_{2A} AR antagonists.

Although an A_1 AR antagonist will have no direct influence on the postsynaptic striatopallidal neurons; it can still enhance the release of dopamine in the striatum, affecting striatonigral and striatopallidal dopaminergic pathways (Jacobson *et al.,* 1993).

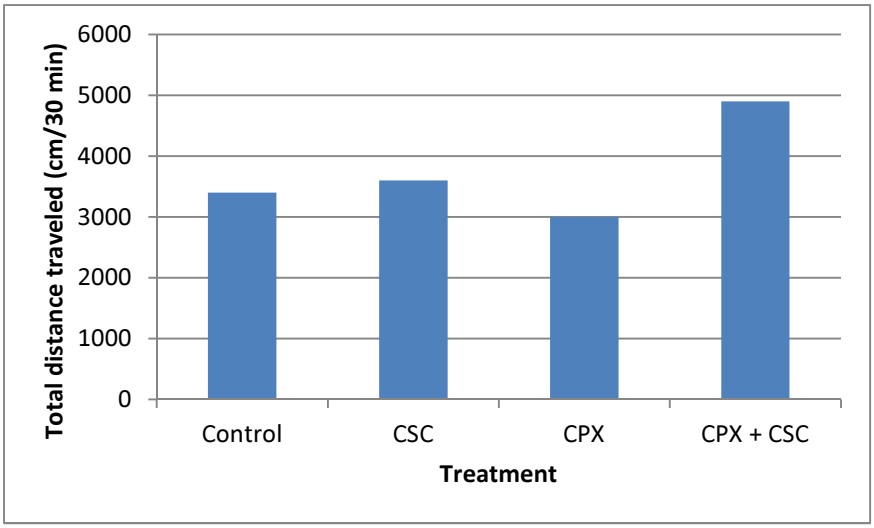


Figure 3-2: **Synergistic effects of a selective A_1 antagonist (CPX) and a selective A_{2A} antagonist (CSC) on locomotor effects in rodents (adapted from Jacobson *et al.*, 1993)**

3.1.2.2 Non-motor symptoms

Non-motor symptoms in PD patients are often neglected and the burden of non-motor symptoms can define a patient's health-related quality of life. The non-motor symptoms of PD range from cognitive problems such as apathy, depression, anxiety and hallucinations to fatigue, gait and balance disturbances, sleep disorders, sexual dysfunction, bowel problems, drenching sweats, and pain (Bartels & Leenders, 2008).

3.1.2.2.1 Cognition

Evidence from epidemiological studies indicates a strong inverse relationship between coffee drinking and a reduced risk of PD within many populations (Martyn & Gale, 2003). Additionally, it was found that patients with PD who drank coffee regularly had less pronounced symptoms of the disease compared to those with PD who did not. Caffeine is a non-selective antagonist of adenosine A_1 and A_{2A} receptors and selective A_1 AR antagonists have furthermore been shown to improve the cognitive symptoms of PD (Schwarzchild *et al.,* 2002).

The effects evoked by caffeine include stimulatory actions on motor activity, alertness, attention, cognitive performance and reduced sleep. The cognitive effects of caffeine are mostly due to its ability to antagonise adenosine A_1 receptors in the hippocampus and prefrontal cortex (Ribeiro & Sebastião, 2010). Adenosine depresses synaptic transmission and the release of various neurotransmitters acting through A_1 receptors (Fredholm & Dunwiddie, 1988).

Selective A_1 AR antagonists depolarise postsynaptic neurons and presynaptically enhance the release of a number of neurotransmitters, e.g. acetylcholine, glutamate, serotonin and norepinephrine. These transmitters have been implicated in learning and memory, and are also reduced in Alzheimer's disease (Rahman, 2009). Several studies have also shown a reduced density of A_1 receptors along with reduced binding sites for adenosine agonists and antagonists in the molecular layer of the dentate gyrus of postmortem samples of AD patients (Kalaria *et al.,* 1990; Ulas *et al.*, 1993). Several studies have proposed adenosine A_1 receptor antagonists for the treatment of memory disorders (Rahman, 2009; Mihara *et al.*, 2007; Maemoto *et al.*, 2004; Schingnitz *et al.,* 1991). A selective A_1 AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) has been used in a scopolamine-induced animal model of memory impairment and showed to be selectively engaged in cholinergic-driven memory impairment (Pagnussat *et al.,* 2015).

3.1.2.2.2 Neuroprotection

Since caffeine, present in coffee, mediates its action by antagonising adenosine receptors, it has led to the evaluation of adenosine receptor antagonists as potential neuroprotective agents (Schwarzchild *et al.,* 2006). Caffeine has been shown to have neuroprotective effects on the dopaminergic nigrostriatal system

in a MPTP induced mouse model (Xu *et al.*, 2002; Chen *et al.*, 2001). It protects against both neurotoxicity and degeneration of MPTP induced dopaminergic system.

The selective A_{2A} AR antagonists, CSC and ZM241385, as well as the less selective ones, CGS15943 and CP66713, were able to reduce cerebral ischemic damage in animal models of ischemia (Gao & Phillis, 1994). A neuroprotective effect of A_{2A} AR antagonist, KW6002 (**21**, section 2.6.8), was also shown in animal models of PD (MPTP, 6-OHDA, methamphetamine) (Ikeda *et al.*, 2002).

The blockade of A_{2A} AR may afford neuroprotection after ischemia due to a reduced glutamate release and induced excitotoxicity (**Figure 3-3**). There are several other mechanisms that mediate adenosine A_{2A} AR antagonist neuroprotection. One of them is inhibition of microglia and astrocyte cell activation, which in turn decrease the release of toxic cytokines, reduce inflammation and oxidative stress.

Despite the uncertainty regarding the mechanism of neuroprotection offered by A_{2A} AR antagonists in a variety of PD animal models, these findings strengthen the therapeutic, epidemiological and pathophysiological significance of A_{2A} AR antagonists. The combined symptomatic effects and neuroprotective properties greatly increases its therapeutic potential, and is a basis for further research into A_{2A} AR antagonism for future PD treatment (Xu *et al.*, 2005).

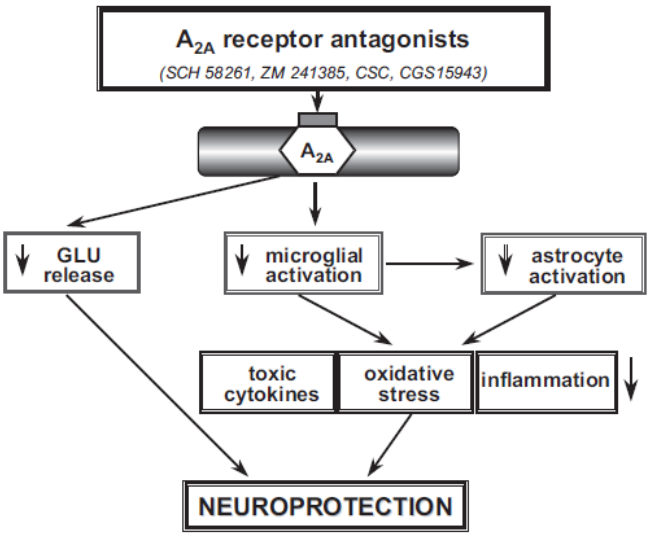


Figure 3-3: **Mechanisms relevant to the neuroprotection of A_{2A} AR antagonists (Wardas, 2002)**

3.1.2.2.3 Depression

Depression is a common non-motor feature of PD (Lemke, 2008), and not only contributes to long-term morbidity of the illness but reduces quality of life, as well as life expectancy (Shulman *et al.,* 2002). Depression in PD (Lemke, 2008; Reijnders *et al*., 2008) is not effectively managed by tricyclic antidepressants (TCAs), selective serotonin (5-HT) reuptake inhibitors (SSRIs), or 5-HT noradrenaline reuptake inhibitors (SNRIs).

To some extent, this relates to their adverse effects, some of which exacerbate the motor symptoms of PD and worsen non-motor signs, such as orthostatic hypotension and cognitive impairment (Lemke, 2008). Morelli and co-workers (2011) found that increased 5-HT signaling through chronic blockade of 5-HT function reduces the pool of readily releasable dopamine (DA) in the striatum. Reduced striatal 3,4-dihydroxyphenylacetic acid (DOPAC) levels after chronic 5-HT blockade further suggest reduced striatal DA signaling as a consequence. Consistent with this conclusion, they found that the basal ganglia-dependent motor behaviors are impaired after chronic 5-HT blockade and demonstrated reversibility of motor deficits through L-DOPA treatment (Morelli *et al*., 2011).

Adenosine and its analogues tend to produce ‘depressant'-like effects in animal models, and is believed to be relevant to human conditions. Stimulation of ARs or increasing adenosine levels induces a state of ‘learned helplessness' similar to that observed in humans with depression (Minor *et al*., 1994; Woodson *et al*., 1998). Furthermore, adenosine and 2-chloroadenosine increase immobility time in the forced swim test (FST) in mice, a widely used animal model of depressive-like behavior (Porsolt *et al*., 1977). Classic antidepressants have been found to reverse adenosine-mediated immobility (Kulkarni & Mehta, 1985).

KW6002 (**21**), a selective A_{2A} AR antagonist, is effective in improving motor function in L-DOPA treated patients with PD and this shows motor response complications (Bara-Jimenez *et al*., 2003; LeWitt *et al*., 2008; Mizuno *et al*., 2010). Yamada and co-workers (2013) confirmed the antidepressant-like activity of KW6002, using the FST and the tail suspension test. They also performed a pharmacological analysis of KW6002’s effects on a rat learned helplessness model of depression in order to determine whether dopaminergic, serotonergic, or noradrenergic transmission is involved. The results showed that KW6002 administered in addition to antidepressants provided a promising therapeutic tool

for depression (Yamada *et al.,* 2013). They concluded that KW6002 exerts antidepressant-like effects via modulation of the A_{2A} AR activity which is independent of monoaminergic transmission in the brain (Yamada *et al.*, 2014). These findings support the claim that KW6002 may represent a novel treatment option for depression in PD, as well as for the associated motor symptoms, and that these actions are specific to its inhibition of A_{2A} AR.

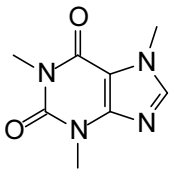
3.2 Adenosine A_{2A} antagonists

Many efforts have been made searching for potent and selective A_{2A} AR antagonists. Various xanthines and non-xanthine heterocyclic compounds consisting of a monocyclic, bicyclic or tricyclic nucleus have been synthesised to possess very good A_{2A} AR affinity with a broad range of selectivity.

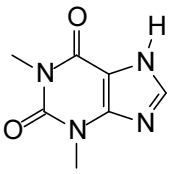
3.2.1 Xanthine derivatives

23

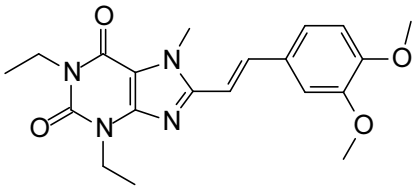
Xanthine is a chemical structure that consists of a six-and-five-membered ring system known as 2,6-dioxypurine (**23**) and derivatives have been well researched for their various therapeutic uses (Van der Walt & Terre'Blanche, 2016). Caffeine (**1**) and theophylline (**24**) are currently used for their pharmacological effects as antiasthmatics, analeptics, vasodilators, diuretics and antihypertensives (Fhid *et al.*, 2012). Numerous derivatives have been categorised and researched for their antagonistic effects on A_{2A} AR, with KW6002 (**21**) presenting with high efficacy in several preclinical models of PD (Shook & Jackson, 2011) (**Figure 3-4**).



1
rK_i A_1 = 48000 nM
rK_i A_{2A} = 29000 nM



24
rK_i A_1 = 25000 nM
rK_i A_{2A} = 8500 nM



21
rK_i A_1 = 12 nM
rK_i A_{2A} = 9600 nM

Figure 3-4: **The structures of caffeine (1), theophylline (24) and KW6002 (21), and their relevant binding affinities for adenosine A_{2A} receptors (Shook & Jackson, 2011)**

For bicyclic-xanthine structures to have binding affinity and interactions with A_{2A} AR, optimisation of substitutions at the 1-, 3-, and 8-positions led to the discovery of potent and selective A_{2A} antagonists. **Figure 3-5** presents the main structural requirements for A_{2A} AR interaction of xanthine derivatives (Azam *et al.*, 2009).

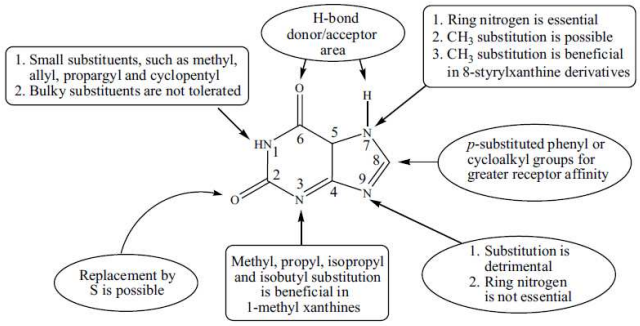
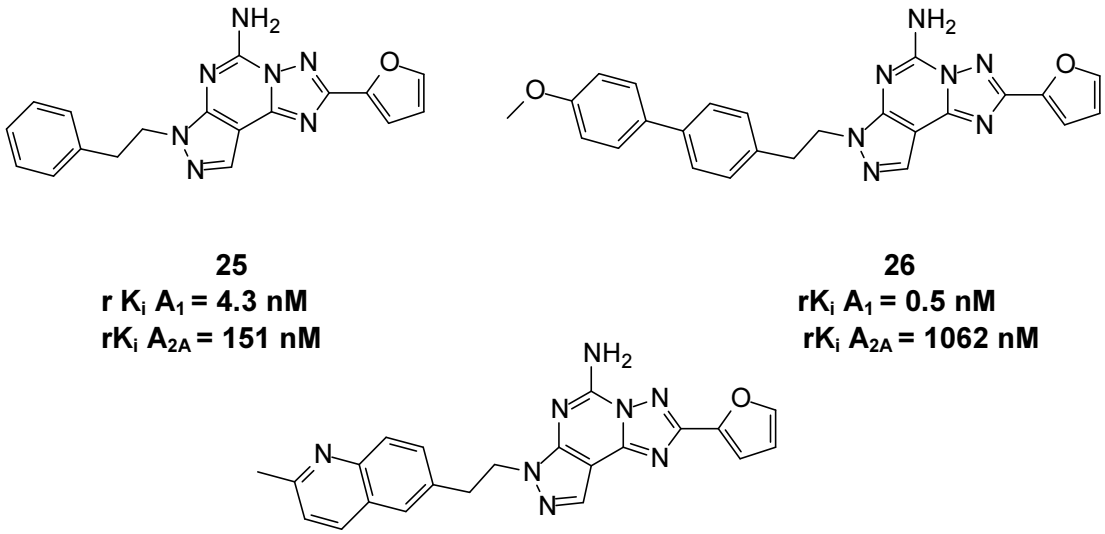


Figure 3-5: **The structural requirements needed for bicyclic-xanthine derivatives to have affinity (Azam *et al.*, 2009)**

Generally, the drawback of the xanthine derivatives is low water solubility (Müller *et al.*, 2002) and this encouraged the exploration of non-xanthine and non-adenine related scaffolds to be investigated in the hope of identifying new AR antagonists. The Schering-Plough group identified SCH58621 (**25**) with a high A_{2A} AR antagonistic potency and increased selectivity observed over the A_1 AR (**Figure 3-6**).

3.2.2 Non-xanthine derivatives

Recent focus has been shifted to non-xanthine compound scaffolds, also known as amino-substituted heterocyclic compounds. These can be categorised into monocyclic, bicyclic and tricyclic antagonists (Chang *et al*., 2004). In literature, various examples exist, for instance, the above mentioned SCH-58621 (**25**) identified by the Schering-Plough group possesses a high A_{2A} AR antagonistic potency with a 35-fold selectivity observed over the A_1 AR. SCH-58621 performed well with intraperitoneal rodent tests, but lacked solubility and has no activity when orally dosed (Shook & Jackson, 2011). The latter scaffold was used to synthesise a variety of biaryl substituted pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines, including a methoxy biaryl derivative (**26**) that showed a 2124-fold higher affinity for A_{2A} AR than A_1 AR. Efforts to replace the methoxyaryl-group with pyridines and pyrazines caused compounds with retained efficacy, but still lacked ideal pharmacokinetic (PK) properties. The search for a compound with an effective PK model resulted in the identification of a quinoline derivative (**27**) that possess good selectivity for A_{2A} AR, and presented with improved PK characteristics when compared to **25** and **26** (Shook & Jackson, 2011) (**Figure 3-6**).



25
r K_i A_1 = 4.3 nM
rK_i A_{2A} = 151 nM

26
rK_i A_1 = 0.5 nM
rK_i A_{2A} = 1062 nM

27
rK_i A_1 = 2.4 nM
rK_i A_{2A} = 406 nM

Figure 3-6: SCH-58621 and substituted derivatives (Shook & Jackson, 2011)

Various compounds have been entered into clinical trials for A_{2A} antagonism in PD; examples of these are explored below (**Figure 3-7**). High potency has been obtained, but the search for compounds with idyllic solubility, selectivity and satisfactory PK and pharmacodynamic properties still eludes researchers (Shook & Jackson, 2011).

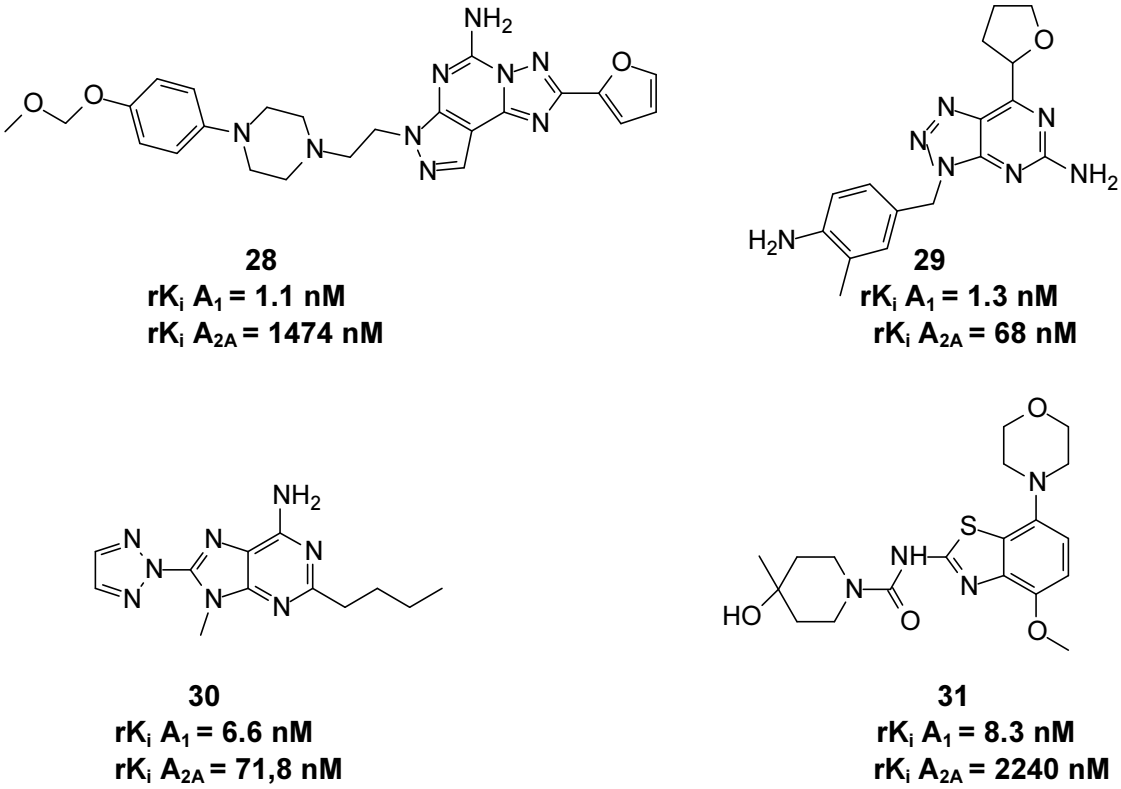
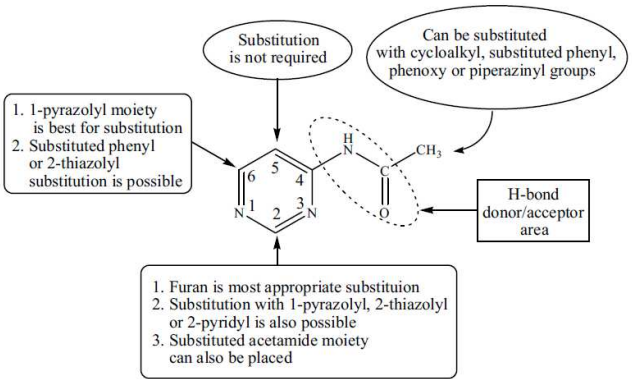


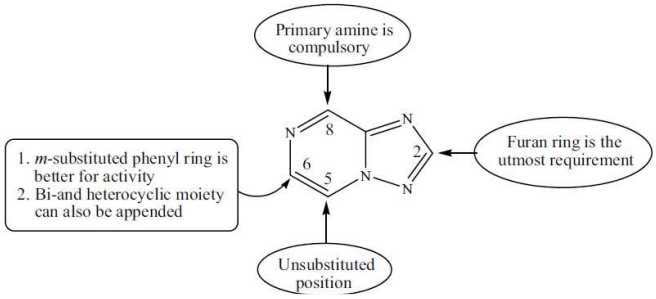
Figure 3-7: **Structures of preladenant (28), vipadenant (29), ST-1535 (30) and SYN-115 (31) that have been entered into clinical trials (Shook & Jackson, 2011)**

Azam and co-workers (2009) presented an overview of structure-activity relationships of several non-xanthine classes for A_{2A} AR affinity. In **Figure 3-8** the requirements for monocyclic, bicyclic and tricyclic non-xanthine derivatives for the A_{2A} AR affinity are given as proposed by Azam and colleagues (2009).

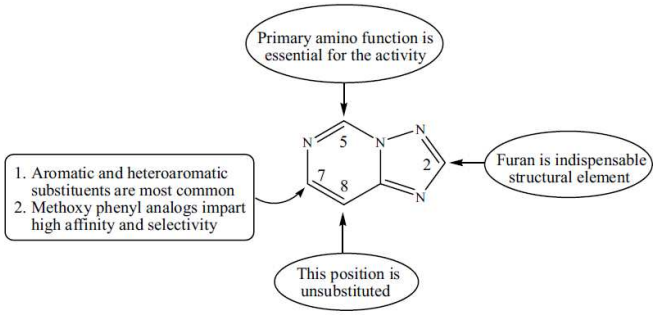
Although none of the A_{2A} AR antagonists that were evaluated clinically have been approved by the Food and Drug Administration (FDA), the possible advantageous effects and acceptable side effect profiles motivate researchers to continue exploring these compounds for future therapeutic uses.



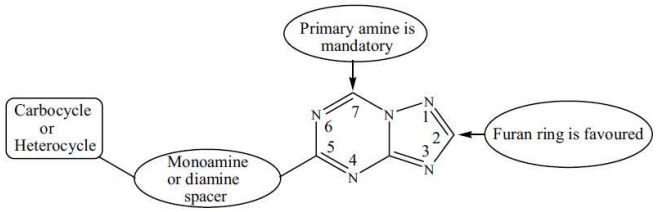
pyrimidineacetamide derivatives



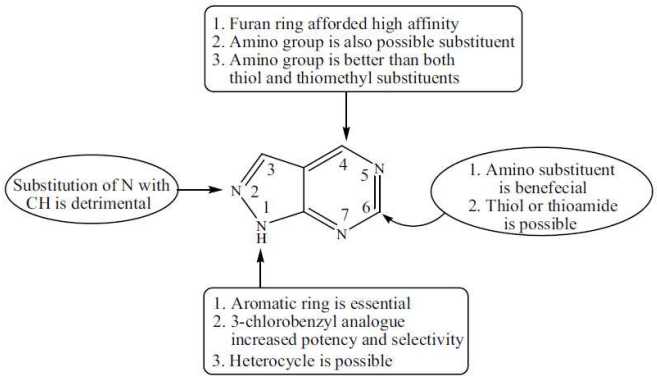
[1,2,4]triazolo[1,5- *c*]pyrazine derivatives



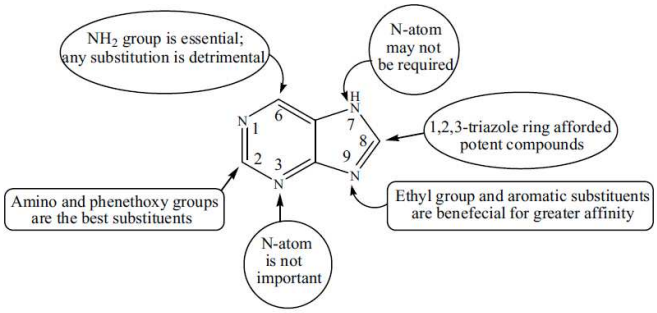
[1,2,4]triazolo[1,5-*c*]pyrimidine derivatives



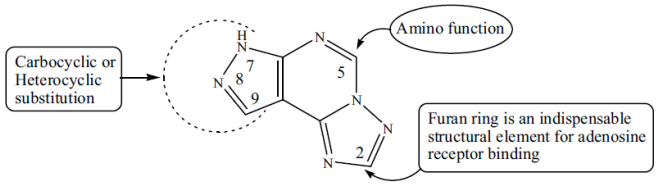
[1,2,4]triazolo[1,5- *c*][1,3,5]triazine



pyrazolo pyrimidines



purine derivatives



pyrazolo[4,3-e][1,2,4]triazolo[1,5-*c*]pyrimidines

Figure 3-8: Requirements for monocyclic, bicyclic and tricyclic non-xanthine derivatives for the A_{2A} AR affinity (Azam *et al.*, 2009)

3.3 Adenosine A_1 antagonists

3.3.1 Xanthine derivatives

Various A_1-selective AR antagonists are xanthine based derivatives with bulky C8-substituents (Van der Walt & Terre'Blanche, 2015). The main requirements for xanthine based A_1 AR antagonist activity are given in **Figure 3-9** (Müller, 2001). Increasing the chain length at position N1 and N3, lead to an increase of A_1 AR affinity. Dipropyl substitution is optimal. In the N3-position, more bulky groups are tolerated. Phenyl or cycloalkyl substitution on the 8-position lead to highly selective A_1 AR antagonists (**Figure 3-9**).

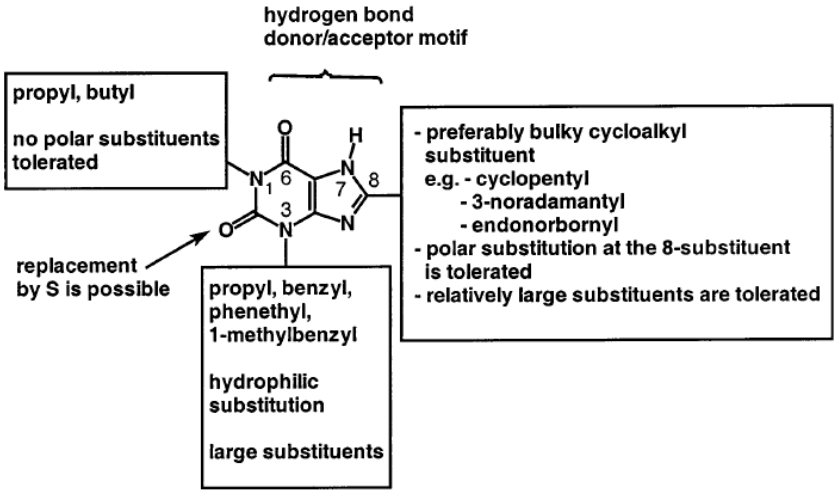
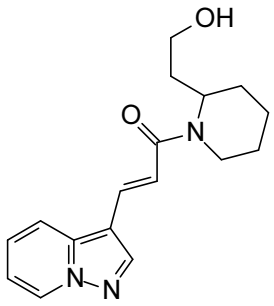


Figure 3-9: Structure-activity relationships of xanthine based adenosine A_1 antagonists (Müller, 2001)

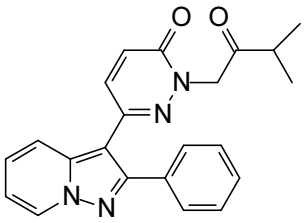
3.3.2 Non-xanthine derivatives

Numerous other classes of amino-substituted heterocyclic derivatives are also known to bind to the A_1 AR. The pyrazolo[1,5-α]pyridine derivatives are structurally related to the xanthine core and show good affinity and selectivity for the A_1 AR (**32**). Modifications to the pyrazolo[1,5-α]pyridines included constraining of the acryloyl amide into a pyridazinone nucleus and substituting the nitrogen with an isobutyryl moiety (**33**) that lead to increased potency and selectivity for the A_1 AR (Kuroda *et al.*, 2001; Kuroda *et al.*, 2000; Kuroda *et al.*, 1999). The naphthyridine nucleus (**34**) showed promising antagonist activity in the nanomolar range and high selectivity in a bovine model, but unfortunately it

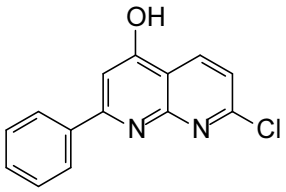
lost potency and selectivity in the human model (Ferrarini *et al.,* 2004; Ferrarini *et al.,* 2000). Pyrimidine derivatives, compound (**35**), also showed good potency and selectivity in human A_1 AR (Chang *et al.*, 2004) (**Figure 3-10**).



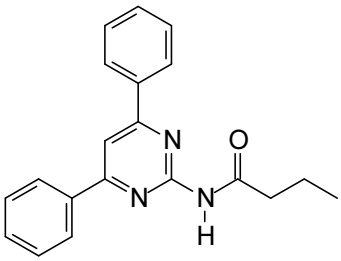
32
rK_i A_1 = 17 nM
rK_i A_{2A} = 11000 nM



33
hK_i A_1 = 0.026 nM
hK_i A_{2A} = 140 nM



34
hK_i A_1 = 300 nM
hK_i A_{2A} = 450 nM



35
hK_i A_1 = 3.7 nM
hK_i A_{2A} > 1000 nM

Figure 3-10: The structures of non-xanthine derivatives and their relevant binding affinities for A_1 and A_{2A} ARs (Kuroda *et al.*, 2000; Chang *et al.*, 2004)

Furthermore, the adenine nucleus was also explored (**Figure 3-11**). The N9-position was primarily explored to introduce substituents with the capacity for radiolabelling. Moreover, to increase A_1 AR affinity, the researchers focussed on substitution at the 8-position; since other positions had already been examined closely. The N^6-cyclopentyl is known to induce high A_1 AR affinity and selectivity (Moos *et al.*, 1985; Thompson *et al.*, 1991). Furthermore, small substituents, for example a chlorine atom, at the 2-position of adenines (Moos *et al.*, 1985) only have limited effects on A_1 AR affinity.

Additionally, larger and bulkier substituents at position 2 seem to favour A_{2A} and/or A_3 AR selectivity of adenines (Cristalli *et al.*, 1998). The C8-position of adenines was explored and it was found that nitrogen-containing groups at this position enhance A_1 AR affinity (Martin et al., 1996). It was also shown that substitution at N9 with a methyl and propyl group was well tolerated without loss of A_1 AR affinity (De Ligt *et al.*, 2004). A broad range of substituents was also introduced at the 8-position and tertiary aliphatic and cyclic amines showed good A_1 AR affinity (De Ligt *et al.*, 2004).

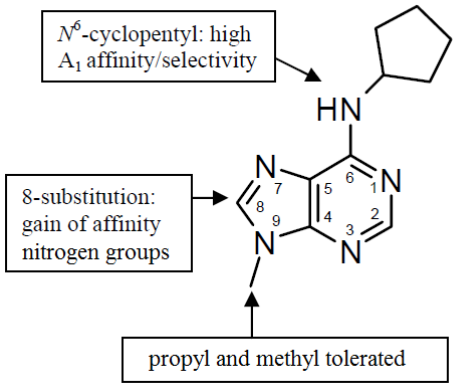
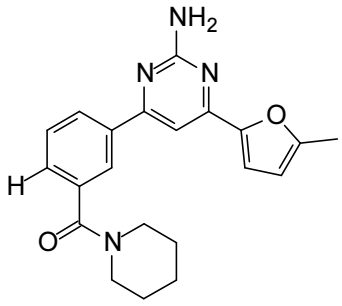


Figure 3-11: Structure-activity relationships of adenine based adenosine A_1 antagonists (De Ligt *et al.*, 2005)

3.4 Dual targets

As discussed previously, dual antagonism of the adenosine A_1 and A_{2A} receptors would result in highly effective treatment of PD. The simultaneous antagonism of A_{2A} AR (improving motor function) and A_1 AR (improving cognitive effects) are more beneficial than only blocking A_{2A} AR (Shook *et al.*, 2010). Various compounds have been discovered and tested for dual potency, with promising results. Arylindenopyrimidines presented with high *in vitro* affinity for A_1 and A_{2A} AR subtypes (Shook *et al.*, 2010). The 2-aminopyrimidines (**36**), related to arylindenopyrimidines, also proved effective dual antagonists (Robinson *et al.*, 2015) (**Figure 3-12**).

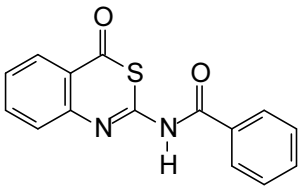


36

rK_i A_1 = 9.54 nM
rK_i A_{2A} = 6,34 nM

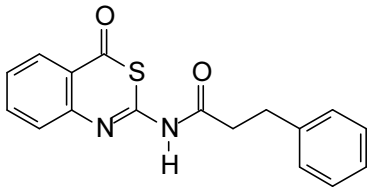
Figure 3-12: **The structure of a 2-aminopyrimidine and binding affinities for A_1 and A_{2A} AR (Robinson *et al.*, 2015)**

Recently Gütschow and co-workers (2012) identified the benzothiazinone scaffold (**4**) as a promising non-xanthine amino-substituted heterocyclic scaffold that may be used to design compounds with adenosine A_1 and A_{2A} receptor affinity. Further, research in 2013 (Stöβel *et al.*, 2013) showed that chain elongation to the phenylpropanamide-benzothiazinone (**5**) (K_iA_1 = 0.422 μM; K_iA_{2A} = 0.103 μM) lead to an increase in adenosine A_{2A} AR affinity, but a decrease in A_1 AR affinity. This higher affinity for the adenosine receptors displayed by the 2-acylaminobenzothiazinone makes it a promising scaffold for further exploration as a dual A_1/A_{2A} AR antagonist (**Figure 3-13**) (see section 1.2).



4

K_i A_1 = 25 nM
K_i A_{2A} = 609 nM



5

K_i A_1 = 422 nM
K_i A_{2A} = 103 nM

Figure 3-13: **The structures of 2-aminobenzothiazinones and their binding affinities for A_1 and A_{2A} AR (Gütschow *et al.*, 2012; Stöβel *et al.*, 2013)**

3.5 Conclusion

Shortcomings in current treatment of PD have led to researchers exploring new treatment possibilities. The antagonism of adenosine A_1/A_{2A} receptors provide an interesting course of action, with several models showing possibility of alleviating disease symptoms and slowing disease progression. The synthesis of selected benzothiazinones as potential adenosine A_1 and A_{2A} antagonists will be discussed in the following chapter.

3.6 References

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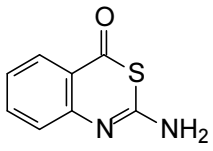
CHAPTER 4

SYNTHESIS

4.1 Introduction

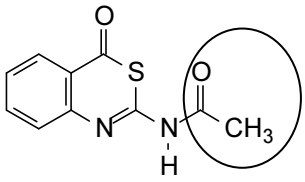
As previously discussed in Chapter 1, the first aim of this study was to synthesise a series of 2-phenylalkylaminobenzothiazinone derivatives in order to evaluate their affinity towards A_1 and A_{2A} ARs. Compounds with high potency antagonism of AR may prove beneficial in the treatment of PD.

The basic structure 2-aminobenzothiazinone (**3**) showed moderate affinity at rat A_1 and A_{2A} AR, with K_i values in the low micromolar range of 2.39 µM and 1.58 µM, respectively (Gütschow *et al.,* 2012). The acetylation (**37**) of the exocyclic-2-amino group of compound **3** reduced affinity of the A_1 AR, with a slight decrease in the A_{2A} AR affinity. Furthermore, acylation with a benzoyl group (**4**) showed a large increase in A_1 AR affinity (K_i = 0.025 µM) and in A_{2A} AR affinity (K_i = 0.609 µM).



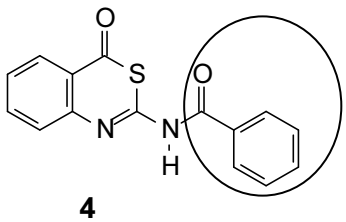
3

rK_i A_1 = 2391 nM
r K_i A_{2A} = 1580 nM



37

rK_i A_1 = >10000 nM
rK_iA_1 = 2390 nM



4

rK_i A_1 = 25 nM
rK_i A_1 = 609 nM

In the current study the acylamino side-chain of the benzothiazinone scaffold will be replaced by the flexible alkylaminophenol side-chain of ZM241385, thus exploring the necessity of the CO-group for adenosine receptor affinity. Since Gütschow and co-workers (2012) only explored the unsubstituted phenyl ring of the 2-acylaminobenzothiazinones, different *para* and *meta* substituents on the phenyl ring in the 2-alkylamino side-chain of the 2-phenylalkylaminobenzothiazinone scaffold will also be explored. In addition different chain lengths in the 2-amino side-chain will also be investigated (**Figure 4-1**).

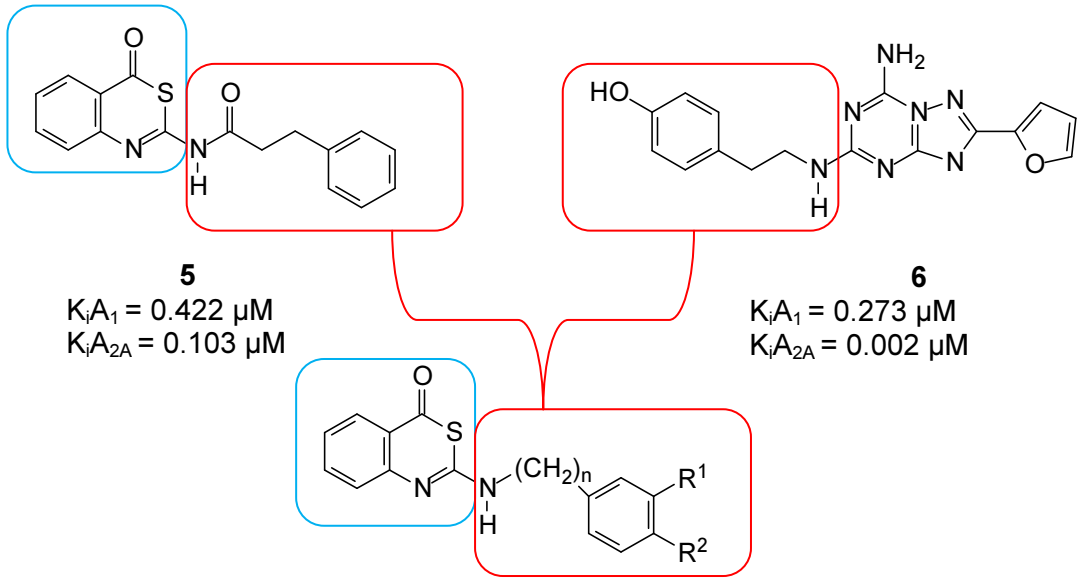


Figure 4-1: The scaffold structure of the 2-phenylalkylaminobenzothiazinones derived from 2-acylaminobenzothiazinones and ZM241385

4.2 Synthetic procedure

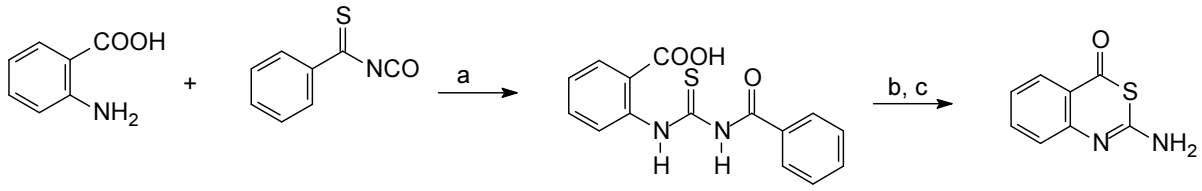
All the 2-phenylalkylaminobenzothiazinone test compounds of this study were synthesised under basic conditions using commercially available phenylhalides and 2-aminobenzothiazinone (**3**) as starting materials. **Table 4-1** illustrates the proposed series of compound derivatives that were synthesised during this study. Previous compounds with phenylacetyl substitution on the secondary amine presented with moderate adenosine affinity (Gütschow *et al.*, 2012), therefore compounds with the phenylalkyl substitutions were synthesised, to produce a series of novel phenylalkylaminobenzothiazinone derivatives.

*Synthesis of 2-aminobenzothiazinone (**3**)*

In the investigation of the 2-phenylalkylaminobenzothiazinones, 2-aminobenzothiazinone (**3**) was used as the key starting material for the preparation of the novel test compounds (**41**-**50**). 2-Aminobenzothiazinone was synthesised in good yields using a modified method previously described by Leistner and co-workers (1990) and Gütchow and co-workers (2012) (**Scheme 4-1**).

In short, anthranillic acid (**38**) (6.8 g; 49 mmol) was dissolved in 100 mL acetone. Subsequently, benzoyl isothiocyanate (**39**) (8 g; 49 mmol) was added dropwise to the above stirring solution. The reaction was monitored via TLC with petroleum ether : ethyl acetate (1:1). The reaction was stirred for 4h at room temperature. The resulting intermediate (**40**) was obtained via filtration as a yellow power and dried overnight.

The intermediate (**40**) was used without further purification (4 g, 13.3 mmol) and stirred in 25 mL concentrated H_2SO_4 at 100 ºC for 4h. The resulting solution was poured onto ice containing $NaHCO_3$ and yielded a precipitate that was collected via filtration. The filtrate was subjected again to acidic conditions via concentrated H_2SO_4, stirred for 4h, then poured into water and boiled until a precipitate formed. The product (**3**) was filtered and dried.



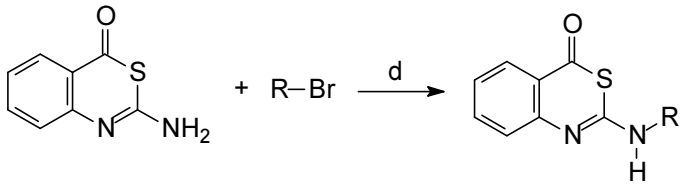
38 **39** **40** **3**

Scheme 4-1: Synthetic pathway to 2-aminobenzothiazinone (3). Reagents and conditions: (a) Acetone, r.t., 4h, (b) H_2SO_4, 100 ºC, 4h, ice, (c) H_2SO_4, r.t. 4h, H_2O

*Synthesis of 2-phenylalkylaminobenzothiazinone derivatives (**41-50**)*

A modified method from Bhattacharyya and co-workers (2014) was used in order to achieve N-alkylation of the 2-aminobenzothiazinone (**3**) and ultimately synthesise the desired test compounds (**41-50**) (**Scheme 4-2**).

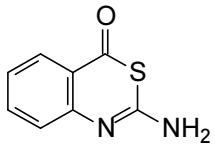
The key stating material (**3**; 0.5 g; 2.8 mmol) was suspended in 5 mL DMF at room temperature. Triethylamine (645 mg; 6.4 mmol) was added dropwise to form a yellow solution. The appropriate phenylhalide (**51a-m**) (5.6 mmol) was added dropwise to the solution, stirred for 4h and then poured into 50 mL H_2O. TLC using petroleum ether : ethyl acetate (1:1) was used to monitor the reaction until completion. The phenylalkylaminobenzothiazinone derivatives were obtained via filtration, dried and recrystallised from ethanol to obtain white crystals (See **Table 4-2** for commercially available phenylhalides).



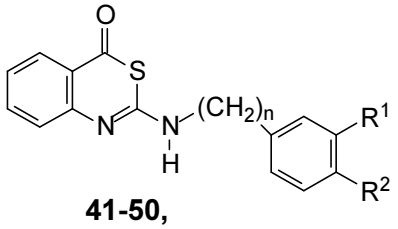
3 **41-50**

Scheme 4-2: **Synthetic pathway to 2-phenylalkylaminobenzothiazinone derivatives. Reagents and conditions: (d) Et_3N, DMF, r.t, 4h, H_2O**

Table 4-1: **Synthesised compounds**



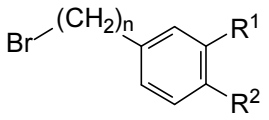
3



41-50,

Compd	R^1	R^2	*n*
41	-	-	1
42	-	-	2
43	-	-	3
44a	F	H	1
44b	H	F	1
45a	Cl	H	1
45b	H	Cl	1
46a	Br	H	1
46b	H	Br	1
47	CF_3	H	1
48a	CH_3	H	1
48b	H	CH_3	1
49	OCH_3	H	1
50	H	NO_2	1

Table 4-2: Commercially available phenylhalides used as starting materials



Compd	R^1	R^2	*n*	Compd	R^1	R^2	*n*
51a	-	-	1	**51h**	Br	H	1
51b	-	-	2	**51i**	H	Br	1
51c	-	-	3	**51j**	CF_3	H	1
51d	F	H	1	**51k**	CH_3	H	1
51e	H	F	1	**51l**	H	CH_3	1
51f	Cl	H	1	**51m**	OCH_3	H	1
51g	H	Cl	1	**51n**	H	NO_2	1

4.3 Experimental – Materials and methods

Materials: Starting materials that were not prepared were obtained from Sigma-Aldrich and used without further purification.

Thin layer chromatography (TLC): TLC was carried out to determine the completeness of the reaction. Silica gel 60 (Merck) containing UV_{254} fluorescent indicator were employed with a mobile phase consisting of 1 part petroleum ether and 1 part ethyl acetate. The developed TLC sheets were observed under an UV-lamp at a wavelength of 254 nm.

Melting points (mp): A Büchi B-545 melting point apparatus was used to measure the mp of all of the synthesised compounds (**3, 41-50**).

Mass spectra (MS): High resolution mass spectra (HRMS) and nominal mass spectra (MS) were obtained with a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionisation (APCI) mode.

Infrared spectroscopy (IR): A Bruker Alpha (platinum ATR) infrared spectrometer was used to record the IR and the Opus® mentor software platform used to process the data.

Nuclear magnetic resonance (NMR): A Bruker Avance III 600 spectrometer, at frequencies of 600 MHz and 150 MHz, were used to record proton (^1H) and carbon (^{13}C) NMR spectra, respectively. NMR measurements were conducted in

DMSO and the chemical shifts reported in parts per million (δ) downfield and were referenced to the residual solvent signal (DMSO 2.5 and 39.5 ppm for ^{1}H and ^{13}C, respectively and a H_2O chemical shift of 3.3 for ^{1}H). Spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet). The coupling constants (*J*) are given in Hz.

4.4 Interpretation of physical data

4.4.1 Physical data of compounds

2-aminobenzothiazinone (3)

Title compound was synthesised from anthranillic acid (**38**) and benzoyl isothiocyanate (**39**) in a yield of 17%, mp 178.0 ^{o}C (H_2O), white powder. IR cm^{-1} 3032, 2898 (NH_2), 1685 (C=O), 1567 (C=N). ^{1}H NMR (DMSO-d6) *δ* 7.29 (t, 1H, *J*=7.2 Hz), 7.34 (d, 1H, *J*=7.9 Hz), 7.70 (t, 1H, *J*=7.2 Hz), 7.91 (d, 1H, *J*=7.5 Hz), 12.45 (s, 1H), 12.69 (s, 1H). ^{13}C NMR (DMSO-d6) *δ* 115.9, 116.2, 124.4, 126.8, 135.4, 140.4, 159.7, 174.3. APCI-HMRS *mz* calc. for $C_8H_7N_2OS$ (MH^+), 179.0301, found 179.0280 (Pazdera *et al.,* 1991).

2-(benzylamino)-4H-3,1-benzothiazin-4-one (41)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and benzyl bromide **(51a)** in a yield of 30.6%, mp 213.4 ^{o}C, (Ethanol), white powder. IR cm^{-1} 2862 (NH), 1665 (C=O), 1560 (C=N). ^{1}H NMR (DMSO-d6) *δ* 4.50 (s, 2H), 7.24–7.27 (m, 1H), 7.31–7.34 (m, 2H), 7.42–7.45 (m, 1H), 7.49 (d, 2H, *J*=8.6 Hz), 7.60 (d, 1H, *J*=8.0 Hz), 7.77–7.80 (m, 1H), 8.04 (dd, 1H, *J*=7.9, 1.3 Hz), 12,60 (s, 1H). ^{13}C NMR (DMSO-d6) *δ* 34.0, 120.5, 126.2, 126.5, 127.8, 129.0, 129.7, 135.4, 137.8, 148.7, 155.7, 161.7. APCI-HRMS *m/z*: calc. for $C_{15}H_{13}N_2OS$ (MH^+), 269.0770, found 269.0735.

2-[(2-phenylethyl)amino]-4H-3,1-benzothiazin-4-one (42)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 2-bromoethylbenzene **(51b)** in a yield of 18.4%, mp 190.9 ^{o}C, (Ethanol), white powder. IR cm^{-1} 2867 (NH), 1662 (C=O), 1590 (C=N). ^{1}H NMR (DMSO-d6) *δ* 3.0 (t, 2H, J=7.9 Hz), 3.44 (t, 2H, *J*=7.9 Hz), 7.20–7.22 (m, 1H), 7.31 (d, 4H, *J*=4.5 Hz), 7.41 (t, 1H, *J*=7.6 Hz), 7.56 (d, 1H, *J*=7.9 Hz), 7.76 (t, 1H, *J*=7.9 Hz), 8.02 (d, 1H, *J*=7.2 Hz) 12,54 (s, 1H). ^{13}C NMR (DMSO-d6) *δ* 31.0, 34.8, 120.0, 125.6,

125.9, 126.0, 126.4, 128.4, 128.6, 134.6, 140.0, 148.4, 155.6, 161.3. APCI-HRMS *m/z*: calc. for $C_{16}H_{15}N_2OS$ (MH^+), 283.0927, found 283.0901.

2-[(3-phenylpropyl)amino]-4H-3,1-benzothiazin-4-one (43)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 3-bromopropylbenzene **(51c)** in a yield of 20.2%, mp 160.4 °C, (Ethanol), white powder. IR cm^{-1} 2907 (NH), 1671 (C=O), 1574 (C=N). ^1H NMR (DMSO-d6) δ 1.9–72.02 (m, 2H), 2.72 (t, 2H, *J*=7.5 Hz), 3.19 (t, 2H, *J*=7.2 Hz), 7.18 (t, 1H, *J*=7.5 Hz), 7.22 (d, 2H, *J*=7.2 Hz), 7.28 (t, 2H, *J*=7.5 Hz), 7.39 (t, 1H, *J*=7.5 Hz), 7.45 (d, 1H, *J*=8.3 Hz), 7.74 (t, 1H, *J*=7.2 Hz), 8.01 (d, 1H, *J*=7.2 Hz), 12.55 (s, 1H,). ^{13}C NMR (DMSO-d6) δ 29.1, 30.4, 34.2, 120.0, 125.6, 125.9, 126.0, 128.3, 128.4, 134.6, 141.1, 148.4, 155.6, 161.3. APCI-HRMS *m/z*: calc. for $C_{17}H_{17}N_2OS$ (MH^+), 297.1083, found 297.1060.

2-{[(3-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44a)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 3-fluorobenzyl bromide **(51d)** in a yield of 26.6%, mp 212.9 °C, (Ethanol), white powder. IR cm^{-1} 2871 (NH), 1664 (C=O), 1575 (C=N). ^1H NMR (DMSO-d6) δ 4.49 (s, 2H), 7.05–7.08 (m, 1H), 7.32–7.37 (m, 3H), 7.41–7.43 (m, 1H), 7.59 (d, 1H, *J*=8.3 Hz), 7.76–7.78 (m, 1H), 8.02 (dd, 1H, *J*=7.9 Hz), 12.6 (s, 1H). ^{13}C NMR (DMSO-d6) δ 32.9, 114.1 (s, $J_{C,F}$ = 21 Hz), 116.0 (s, $J_{C,F}$ = 22Hz), 120.0, 125.4 (d, $J_{C,F}$ = 2.2 Hz), 125.8, 126.1, 130.4 (d, $J_{C,F}$ = 8.8 Hz), 134.8, 140.6 (d, $J_{C,F}$ = 7.7 Hz), 148.3, 155.1, 161.3, 162.0 (s, $J_{C,F}$ = 244 Hz). APCI-HRMS *m/z*: calc. for $C_{15}H_{12}FN_2OS$ (MH^+), 287.0676, found 287.0681

2-{[(4-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44b)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 4-fluorobenzyl bromide **(51e)** in a yield of 5.6%, mp 186.2 °C, (Ethanol), white powder. IR cm^{-1} 2855 (NH), 1665 (C=O), 1579 (C=N). ^1H NMR (DMSO-d6) δ 4.47 (s, 2H), 7.13 (t, 2H, *J*=8.7 Hz), 7.40–7.43 (m, 1H), 7.52 (d, 1H, *J*=8.7 Hz), 7.53 (d, 1H, *J*=8.7Hz), 7.60 (d, 1H, *J*=8.3 Hz), 7.76–7.78 (m, 1H) 8.02 (dd, 1H, *J*=7.9, 1.3 Hz), 12,6 (s, 1H). ^{13}C NMR (DMSO-d6) δ 32.7, 115.2 (s, $J_{C,F}$ = 22.0 Hz), 120.0, 125.7, 126.1, 131.2 (d, $J_{C,F}$ = 7.7 Hz), 133.8 (d, $J_{C,F}$ = 3.3 Hz), 134.7, 148.3, 155.2, 161.3, 161.3 (s, $J_{C,F}$ = 243 Hz). APCI-HRMS *m/z*: calc. for $C_{15}H_{12}FN_2OS$ (MH^+), 287.0676, found 287.0650

2-{[(3-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45a)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 3-chlorobenzyl bromide **(51f)** in a yield of 14.2%, mp 217.2 °C, (Ethanol), white powder. IR cm^{-1} 2808 (NH), 1665 (C=O), 1584 (C=N). ^{1}H NMR (DMSO-d6) δ 4.47 (s, 2H), 7.28–7.34 (m, 2H), 7.40–7.43 (m, 1H), 7.45 (d, 1H, *J*=7.5 Hz), 7.57–7.59 (m, 2H), 7.76–7.78 (m, 1H), 8.02 (dd, 1H, *J*=7.9, 1.3 Hz), 12.6 (s, 1H). ^{13}C NMR (DMSO-d6) δ 32.8, 120.0, 125.8, 126.1, 127.2, 127.9, 129.1, 130.3, 132.8, 134.7, 140.4, 148.2, 155.0, 161.3. APCI-HRMS *m/z*: calc. for $C_{15}H_{12}ClN_2OS$ (MH^+), 303.0381, found 303.0346.

2-{[(4-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45b)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 4-chlorobenzyl bromide **(51g)** in a yield of 10.3%, mp 236.8 °C, (Ethanol), white powder. IR cm^{-1} 2865 (NH), 1677 (C=O), 1574 (C=N). ^{1}H NMR (DMSO-d6) δ 4.47 (s, 2H), 7.36 (d, 2H, *J*=8.7 Hz), 7.41 (t, 1H, *J*=7.9 Hz), 7.51 (d, 2H, *J*=8.3 Hz), 7.59 (d, 1H, *J*=7.9 Hz), 7.75–7.77 (m, 1H), 8.01 (d, 1H, *J*=7.9 Hz) 12,6 (s, 1H). ^{13}C NMR (DMSO-d6) δ 33.1, 120.5, 126.2, 126.5, 128.8, 131.5, 132.3, 135.1, 137.2, 137.3, 148.7, 155.5, 161.7. APCI-HRMS *m/z*: calc. for $C_{15}H_{12}ClN_2OS$ (MH^+), 303.0381, found 303.0354.

2-{[(3-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46a)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 3-bromobenzyl bromide **(51h)** in a yield of 3.9%, mp 223.2 °C (Ethanol), white powder. IR cm^{-1} 2805 (NH), 1664 (C=O), 1580 (C=N). ^{1}H NMR (DMSO-d6) δ 4.46 (s, 2H), 7.26 (t, 1H, *J*=7.9 Hz), 7.40–7.43 (m, 2H), 7.49 (d, 1H, *J*=7.9 Hz), 7.58 (d, 1H, *J*=7.9 Hz), 7.71 (br s, 1H), 7.76–7.78 (m, 1H), 8.01 (dd, 1H, *J*=7.9, 1.5 Hz), 12,6 (s, 1H). ^{13}C NMR (DMSO-d6) δ 32.8, 120.1, 121.5, 125.9, 126.2, 128.4, 130.2, 130.7, 132.1, 134.9, 140.7, 148.3, 155.1, 161.4. APCI-HRMS *m/z*: calc. for $C_{15}H_{11}BrN_2OS$ (MH^-), 346.9875, found 346.9839.

2-{[(4-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46b)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 4-bromobenzyl bromide **(51i)** in a yield of 12.6%, mp 250.1 °C, (Ethanol), white powder. IR cm^{-1} 2865 (NH), 1678 (C=O), 1606 (C=N). ^{1}H NMR (DMSO-d6) δ 4.47 (s, 2H), 7.42–7.45 (m, 1H), 7.46–7.47 (m, 2H), 7.50–7.52 (m, 2H), 7.60 (d,

1H, *J*=8.1 Hz), 7.77–7.80 (m, 1H), 8.03 (d, 1H, *J*=7.9 Hz), 12,61 (s, 1H). ^{13}C NMR (DMSO-d6) *δ* 33.2, 120.5, 120.8, 126.2, 126.5, 131.7, 131.9, 135.1, 137.7, 138.8, 148.7, 155.5, 161.6. APCI-HRMS *m/z*: calc. for $C_{15}H_{12}BrN_2OS$ (MH^+), 346.9875, found 346.9841.

2-{[[3-(trifluoromethyl)phenyl]methyl}amino)-4H-3,1-benzothiazin-4-one (47)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 3-(trifluoromethyl)benzyl bromide **(51j)** in a yield of 8.1%, mp 217.9 °C, (Ethanol), white powder. IR cm^{-1} 2866 (NH), 1664 (C=O), 1584 (C=N). ^{1}H NMR (DMSO-d6) *δ* 4.51 (s, 2H), 7.40–7.43 (m, 1H), 7.54 (t, 1H, *J*=7.9 Hz), 7.58–7.59 (m, 2H), 7.76–7.79 (m, 1H), 7.81 (d, 1H, *J*=7.9 Hz), 7.91 (br s, 1H), 8.02 (dd, 1H, *J*=7.9, 0.75 Hz), 12.61 (s, 1H),. ^{13}C NMR (DMSO-d6) *δ* 32.8, 120.0, 123.3, 123.9, 125.1, 125.8, 126.1, 128.8, 129.0, 129.4, 133.4, 134.7, 139.5, 148.2, 154.9, 161.2. APCI-HRMS *m/z*: calc. for $C_{16}H_{12}F_3N_2OS$ (MH^+), 337.0644, found 337.0632g.

2-{[(3-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48a)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 3-methylbenzyl bromide **(51k)** in a yield of 13.7%, mp 208.7 °C, (Ethanol), white powder. IR cm^{-1} 2804 (NH), 1659 (C=O), 1579 (C=N). ^{1}H NMR (DMSO-d6) *δ* 2.26 (s, 3H), 4.44 (s, 2H), 7.05 (d, 1H, *J*=7.5 Hz), 7.18 (t, 1H, *J*=7.5 Hz), 7.26 (d, 1H, *J*=7.9 Hz), 7.27 (br s, 1H) 7.41 (t, 1H, *J*=7.5 Hz), 7.59 (d, 1H, *J*=7.9 Hz), 7.75–7.78 (m, 1H) 8.02 (dd, 1H, *J*=7.9, 0.75 Hz), 12.57 (s, 1H). ^{13}C NMR (DMSO-d6) *δ* 20.9, 33.6, 120.0, 125.7, 126.1, 126.3, 128, 128.4, 129.8, 134.7, 137.1, 137.6, 148.3, 155.3, 161.3. APCI-HRMS *m/z*: calc. for $C_{16}H_{15}N_2OS$ (MH^+), 283.0927, found 283.0904.

2-{[(4-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48b)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 4-methylbenzyl bromide **(51l)** in a yield of 25.5%, mp 214.2 °C, (Ethanol), white powder. IR cm^{-1} 2863 (NH), 1679 (C=O), 1607 (C=N). ^{1}H NMR (DMSO-d6) *δ* 2.26 (s, 3H), 4.45 (s, 2H), 7.12 (d, 2H, *J*=7.7 Hz) 7.36 (d, 2H, *J*=7.9 Hz), 7.41–7.44 (m, 1H), 7.6 (d, 1H, *J*=8.1 Hz), 7.77–7.79 (m, 1H), 8.04 (dd, 1H, *J*=7.9, 1.3 Hz), 12.57 (s, 1H). ^{13}C NMR (DMSO-d6) *δ* 21.2, 33.9, 120.5, 126.1, 126.4, 126.5, 129.5, 129.6, 134.6, 135.1, 137.0, 148.8, 155.8, 161.7. APCI-HRMS *m/z*: calc. for $C_{16}H_{15}N_2OS$ (MH^+), 283.0927, found 283.0904.

2-{[(3-methoxyphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (49)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 3-methoxybenzyl bromide **(51m)** in a yield of 5.2%, mp 177.1 ^{o}C, (Ethanol), white powder. IR cm^{-1} 2834 (NH), 1670 (C=O), 1578 (C=N). ^{1}H NMR (DMSO-d6) δ 3.7 (s, 3H), 4.45 (s, 2H), 6.81 (dd, 1H, *J*=7.9, 2.6 Hz), 7.03 (d, 1H, *J*=7.5 Hz), 7.07–7.08 (m, 1H), 7.22 (t, 1H, *J*=7.9 Hz), 7.40–7.43 (m, 1H), 7.59 (d, 1H, *J*=7.9 Hz), 7.75–7.78 (m, 1H), 8.02 (dd, 1H, *J*=7.9 1.1 Hz), 12,6 (s, 1H). ^{13}C NMR (DMSO-d6) δ 33.5, 55.0, 112.8, 114.8, 120.0, 121.4, 125.7, 126.0, 126.1, 129.5, 134.7, 138.9, 148.3, 155.3, 159.2, 161.3. APCI-HRMS *m/z*: calc. for $C_{16}H_{15}N_2O_2S$ (MH^+), 299.0876, found 299.0860.

2-{[(4-nitrophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (50)

Title compound was synthesised from 2-aminobenzothiazinone **(3)** and 4-nitrobenzyl bromide **(51n)** in a yield of 7.2%, mp 231.8 ^{o}C, (Ethanol), white powder. IR cm^{-1} 2871 (NH), 1662 (C=O), 1583 (C=N). ^{1}H NMR (DMSO-d6) δ 4.59 (s, 2H), 7.41 (t, 1H, *J*=7.9 Hz), 7.60 (d, 1H, *J*=7.9 Hz), 7.75–7.78 (m, 3H), 8.01 (d, 1H, *J*=7.5 Hz), 8.16 (d, 2H, *J*=8.7 Hz), 12.64 (s, 1H). ^{13}C NMR (DMSO-d6) δ 32.7, 120.0, 123.4, 125.8, 126.0, 126.0 130.5, 134.7, 146.2, 146.5, 154.7, 161.2. APCI-HRMS *m/z*: calc. for $C_{15}H_{12}N_3O_3S$ (MH^+), 314.0621, found 314.0589.

4.5 Summary

Fourteen novel 2-phenylalkylaminobenzothiazinone (**41-50**) derivatives were successfully synthesised in good yields according to general synthetic procedures acquired through relevant literature. Compound structures were confirmed using ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy. Analytical data corresponds to calculated and predicted results. In this chapter, the synthetic procedure and analytic evaluation of the derivatives listed in **Table 4.1** is discussed.

4.6 References

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CHAPTER 5

RADIOLIGAND BINDING STUDIES

5.1 Introduction

The second aim of this study was to determine if the synthesised 2-phenylalkylaminobenzothiazinones (**41-50**) possess affinity for the adenosine A_1 and/or A_{2A} receptors. For this purpose adenosine A_1 and A_{2A} receptor radioligand binding experiments were performed *in vitro* as described in the literature (Bruns *et al*., 1986; Bruns *et al*., 1987; Van der Walt & Terre'Blanche, 2015). Thus, in this chapter, the aforementioned methods used for the *in vitro* biological evaluation of the novel test compounds will be provided, as well as a discussion relating to the obtained experimental results. The adenosine A_1 and A_{2A} receptor radioligand binding assay results are documented in **Table 5-2**.

5.2 Experimental procedure – Radioligand binding studies

With the adenosine A_1 and A_{2A} receptor radioligand binding assays a test compound's binding affinity towards the adenosine A_1 and A_{2A} receptor are determined, respectively. Furthermore, the degree of selectivity towards either of the adenosine receptors (A_1 and A_{2A}) may subsequently be calculated. The basic concept of these assays comprises of a radioligand that possess high affinity for a specific receptor. Thus, the radioligands are used to measure the affinity a test compound possesses for a specific receptor. For example, 1,3-[^{3}H]-dipropyl-8-cyclopenthylxanthine ([^{3}H]DPCPX) is used as radioligand to determine a compounds' affinity for the adenosine A_1 receptor subtype. The unlabelled DPCPX (**52**) has been reported with K_i values of 0.55 nM and 530 nM towards the adenosine A_1 and A_{2A} receptor, respectively (Van der Walt & Terre'Blanche, 2015). In turn, 5'-N-ethylcarboxamido[^{3}H]adenosine ([^{3}H]NECA) is used as radioligand in order to measure a compounds' binding affinity for the adenosine A_{2A} receptor. Previously, unlabelled NECA (**53**) has been documented to exhibit a K_i value of 10.3 nM for the adenosine A_{2A} receptor and 6.26 nM for the adenosine A_1 receptor (Bruns *et al*., 1986). In order to minimise the binding of [^{3}H]NECA to the adenosine A_1 receptor, N^6-cyclopentyladenosine (CPA) is added to each incubation. CPA is a known adenosine A_1 receptor agonist and possess

a higher affinity towards the adenosine A_1 receptor than [^{3}H]NECA. The degree of selectivity towards the adenosine A_1 or A_{2A} receptor is determined by calculating a selectivity index. The selectivity index is defined as the ratio between the recorded K_i values of the adenosine A_1 and A_{2A} receptor binding assays. In the case of unlabelled DPCPX a selectivity index of 958 was obtained for the adenosine A_1 receptor isoform by calculating the ratio as K_i (A_{2A})/ K_i (A_1) (Van der Walt & Terre'Blanche, 2015).

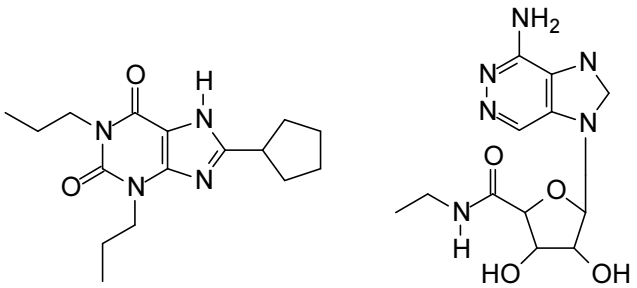
The adenosine A_1 receptor radioligand binding assays were performed with rat whole brain membranes that express the adenosine A_1 receptor subtype, while rat striatal membranes (expressing the adenosine A_{2A} receptor) were used for the adenosine A_{2A} receptor binding experiment (**Table 5-1**) (Van der Walt & Terre'Blanche, 2015).

Table 5-1: A list of the various rat brain membranes and appropriate radioligands used with the adenosine A_1 and A_{2A} receptor radioligand binding experiments

	Rat tissue used	Expressing adenosine receptor subtype	Radioligand used
Adenosine A_1 receptor radioligand binding assay	Whole brain membranes	A_1	[^{3}H]DPCPX
Adenosine A_{2A} receptor radioligand binding assay	Striatal membranes	A_{2A}	[^{3}H]NECA

5.2.1 Reagents and materials

Unless otherwise noted, all the commercially available reagents used for the radioligand binding assays were obtained from Sigma-Aldrich® and included: Sigma-cote® (silicone solution); adenosine deaminase (8.3 mg protein/mL, 141 units/mg protein); anhydrous magnesium chloride ($MgCl_2$); Trizma-base; Trizma-hydrochloride and N^6-cyclopentyladenosine (CPA). In turn, [^{3}H]NECA (specific activity 25 Ci/mmol) and [^{3}H]DPCPX (specific activity 120 Ci/mmol) were obtained from Amersham Biosciences and PerkinElmer, respectively. The scintillation vials and Filter-count scintillation fluid were purchased from PerkinElmer, while the Whatman GF/B 25 mm diameter filters and dimethyl sulfoxide (DMSO) solvent were sourced from Merck®. The radio activities were determined with a Packard Tri-CARB 2810 TR liquid scintillation counter.



52 **53**

Figure 5-1: The chemical structures of unlabelled DPCPX (52) and NECA (53)

5.2.2 Membrane preparations

Before the collection of tissue samples, ethics approval was obtained from the North-West University's Animal Research Ethics Committee (application number NWU-0035-10-A5). Male Sprague-Dawley rats, weighing between 250 g and 500 g, were obtained from the Vivarium of the North-West University. These rats were dissected in order to obtain rat whole brain (excluding cerebellum and brain stem) and striatal membranes for the adenosine A_1 and A_{2A} receptor radioligand binding assays, respectively (**Table 5-1**). The brain tissues were kept on ice during dissection, immediately snap frozen with liquid nitrogen after collection and stored at -70 °C.

On the day of the membrane preparation, the rat whole brains and striatal membranes were prepared according to a previously documented protocol (Bruns *et al*., 1986, Van der Walt *et al.,* 2015; Van der Walt & Terre'Blanche, 2015). Briefly, the rat whole brain membranes were thawed on ice, weighed and subsequently suspended in 10 volumes of original tissue weight with ice cold 50 mM Tris.HCl (pH 7.7 when prepared at 25 °C). The membranes were disrupted for 90 seconds using a Polytron PT-10 homogenizer (Brinkman) on setting 5. The resulting suspension was centrifuged at 50 000 x g for 10 min at 4 °C. The supernatant was decanted and the remaining pellets were resuspended in 50 mM Tris.HCl as mentioned before. The resulting suspension was again homogenised and centrifuged as before. Finally, the obtained pellets were suspended in ice cold 50 mM Tris.HCl to yield 1 g / 5 mL original tissue weight and stored at -70 °C until needed. The rat striatal membranes were prepared similarly as described above, except that the membranes were disrupted for

30 sec using the Polytron PT-10 homogenizer (Brinkman). The protein content of all membrane preparations were determined with Bradford reagent via a protocol described previously by Bradford (1979).

5.2.3 Determination of the adenosine A_1 receptor affinity

The adenosine A_1 binding affinity of each test compound was determined with the adenosine A_1 radioligand binding protocol that was documented previously (Bruns *et al.*, 1987, Van der Walt & Terre'Blanche, 2015). This assay is routinely performed in our laboratory. The day before the radioligand binding assay, 50 mM Tris.HCl (pH 7.7 at 25 °C) buffer was prepared and stored at 4 °C. The test compounds were weighed and DMSO solvent was used to prepare all stock solutions. These stock solutions were used to obtain a final concentration range between 0 and 300 µM for each test compound. The polypropylene vials, caps and tips used with the radioligand binding assay were coated with Sigma-cote® (to prevent adhesion) and dried overnight at room temperature.

On the day of the assay, the previously prepared rat whole brain membranes (see section 5.2.2) were thawed on ice. The final volume of each incubation contained 1 mL 50 mM Tris–HCl buffer; 120 µg membrane protein suspension (rat whole brain membranes), 0.1 nM [^{3}H]DPCPX as radioligand, 0.1 units/mL adenosine deaminase, the test compound and 1% DMSO (Bruns *et al.*, 1987; Van der Walt & Terre'Blanche, 2015). The order of additions were as follow: test compound (10 µL), [^{3}H]DPCPX (100 µL) and membrane suspension (890 µL).

Furthermore, control incubations received 1% DMSO, indicating that DMSO had no effect on specific binding (Bruns *et al.*, 1987). The order of addition for the control was as follow: DMSO (10 µL), [^{3}H]DPCPX (100 µL) and membrane suspension (890 µL). Non-specific binding was defined by the addition of a final concentration of 100 µM CPA. Each non-specific binding experiment contained 1 mL 50 mM Tris–HCl buffer; 120 µg membrane protein suspension (rat whole brain membranes), 0.1 nM [^{3}H]DPCPX as radioligand, 0.1 units/mL adenosine deaminase, 1% DMSO and 100 µM CPA (Bruns *et al.*, 1987; Van der Walt & Terre'Blanche, 2015). The order of addition was as follow: 10 mM CPA (10 µL), [^{3}H]DPCPX (100 µL) and membrane suspension (890 µL).
After all the additions were added, each vial was incubated as follow: each vial was first vortexed and then incubated for 60 minutes at 25 °C in a shaking water

bath. Half an hour after incubation started; the incubations were vortexed again. Each filter was soaked in 50 mM Tris.HCl buffer before filtration. Subsequently, the incubations were terminated with filtration through a 2.5 cm GF/B filter under reduced pressure. Every incubation vial was then rinsed twice with 50 mM Tris.HCl buffer (4 mL) followed by washing each filter with 50 mM Tris.HCl buffer (4 mL). Hereafter the filters were placed into a scintillation vial with Filter-count (4 mL). Each vial was shaken and left to stand for two hours before the radio activities (retained on the filters) were counted with a Packard Tri-CARB 2810 TR liquid scintillation counter (Bruns *et al.*, 1986; Van der Walt & Terre'Blanche, 2015). Each competition experiment was performed in duplicate for screenings and in triplicate for full assays. CPA and ZM241385 were used as reference compounds to validate the adenosine A_1 radioligand binding assay and the results were found to be in accordance with literature values (**Table 5-2**).

5.2.4 Determination of the adenosine A_{2A} receptor affinity

The adenosine A_{2A} binding affinity of the synthesised test compounds were determined with the adenosine A_{2A} radioligand binding protocol previously described in the literature (Bruns *et al.*, 1986; Van der Walt & Terre'Blanche, 2015). This assay is routinely performed in our laboratory. In short, the day before the assay, 50 mM Tris.HCl (pH 7.7 at 25 ^{o}C) buffer was prepared and stored at 4 ^{o}C. The test compounds were weighed and stock solutions was prepared with DMSO as described in section 5.2.3. Similarly to the adenosine A_1 radioligand binding assay polypropylene vials, caps and tips used with the adenosine A_{2A} binding assay were coated with Sigma-cote® (to prevent adhesion) and dried overnight at room temperature.

On the day of the assay, the previously prepared rat striatal membranes (see section 5.2.2) were thawed on ice. As mentioned above, [^{3}H]NECA is used as the radioligand for the adenosine A_{2A} receptor binding experiment and is known to bind non-selectively to both the adenosine A_1 and A_{2A} receptor. Therefore, in order to minimise the binding of [^{3}H]NECA to the adenosine A_1 receptor, N^6-cyclopentyladenosine (CPA) is added to each incubation in a final concentration of 50 nM. Furthermore, it was found that a final concentration of 10 mM $MgCl_2$ increased adenosine A_{2A} binding, as well as decrease non-specific binding (Bruns *et al.*, 1986). Therefore, each incubation contained 1 mL 50 mM Tris–HCl buffer; 120 µg membrane protein suspension (rat striatal membranes), 0.2 units/mL adenosine deaminase, 10 mM $MgCl_2$, 50 nM CPA, 4 nM [^{3}H]NECA

as radioligand, the test compound and 1% DMSO (Van der Walt & Terre'Blanche, 2015). The order of additions were as follow: test compound (10 µL), membrane suspension (790 µL), 500 nM CPA (100 µL) and [^{3}H]NECA (100 µL).

Moreover, control incubations received 1% DMSO, indicating that DMSO had no effect on specific binding (Bruns *et al.*, 1986). The order of additions were as follow: DMSO (10 µL), membrane suspension (790 µL), 500 nM CPA (100 µL) and [^{3}H]NECA (100 µL). Non-specific binding was defined by the addition of a final concentration of 100 µM CPA (Bruns *et al.*, 1986; Van der Walt & Terre'Blanche, 2015). Thus, each non-specific binding experiment contained 1 mL 50 mM Tris–HCl buffer; 120 µg membrane protein suspension (rat striatal membranes), 0.2 units/mL adenosine deaminase, 10 mM $MgCl_2$, 50 nM CPA, 4 nM [^{3}H]NECA as radioligand, 1% DMSO and 100 µM CPA. The order of addition was as follow: 10 mM CPA (10 µL), membrane suspension (790 µL), 500 nM CPA (100 µL) and [^{3}H]NECA (100 µL).

After all the additions were added, each incubation vial was vortexed and then incubated for 60 minutes at 25 ^{o}C in a shaking water bath. Half an hour after incubation started, the incubations were vortexed again. The incubations were terminated with filtration and the radio activities (retained on the filters) were subsequently counted with a Packard Tri-CARB 2810 TR liquid scintillation counter (Bruns *et al.*, 1986; Van der Walt & Terre'Blanche, 2015). Each competition experiment was performed in duplicate (screenings) and triplicate (full assays). CPA and ZM241385 was used as reference compounds to validate the adenosine A_{2A} radioligand binding assay and the results were found to be in accordance with literature values (**Table 5-2**).

5.2.5 Data analysis

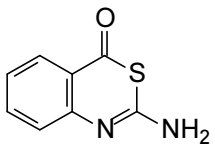
The protein content of all membrane preparations were determined with Bradford reagent according to the method described by Bradford (1979). Non-specific binding of [^{3}H]DPCPX and [^{3}H]NECA was defined as binding in the presence of 100 µM CPA (Bruns *et al.*, 1986; Van der Walt *et al.*, 2015). In turn, specific binding was defined as the total binding minus the non-specific binding (Bruns *et al.*, 1986; Van der Walt & Terre'Blanche, 2015).

Each test compound was initially screened at a final concentration of 1 µM, 10 µM, 100 µM and 300 µM and finally expressed as a calculated percentage displacement of the control. The percentage binding was calculated by dividing the specific binding of the test compound by the specific binding of the control (1% DMSO) times 100. The higher the specific binding percentage obtained, at the various tested concentrations, the weaker the exhibited A_1 or A_{2A} adenosine receptor affinity. Furthermore, test compounds with a calculated binding percentage greater than 30% (at 100 µM) was not tested at a final concentration range between 0 and 100 µM (full assay). The obtained percentage binding of each test compound at 300 µM final concentration is provided in **Table 5-2**.

If full assays were warranted the data analyses of the test compounds were performed as follow: the IC_{50} values were determined by plotting the specific binding (nine concentrations ranging between 0 and 100 µM) vs. the logarithm of the test compounds' concentrations to obtain a sigmoidal dose–response curve via the Prism software package (GraphPad Software Inc.) (Van der Walt *et al.*, 2015). The dissociation constant (K_i) values for the competitive inhibition of [^{3}H]DPCPX (K_D = 0.36 nM) (Bruns *et al.*, 1987) or [^{3}H]NECA (K_D = 15.3 nM) (Bruns *et al.*, 1986) by the test compounds were calculated from the IC_{50} values. The calculated K_i values are expressed as mean ± standard error of the mean (SEM) after triplicate determinations (Van der Walt & Terre'Blanche, 2015).

5.3 Results

Table 5-2: Dissociation constant (K_i) values and percentage displacement for the binding of the 2-phenylalkylaminobenzothiazinones analogues to rat A_1 and A_{2A} AR



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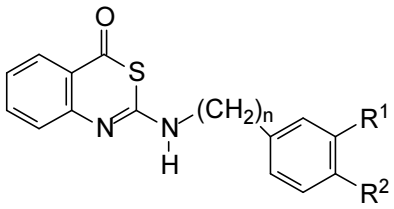

41-50

				K_i ± SEM µM[a] (% displacement)[c]	
Compd	R^1	R^2	*n*	A_1 vs [^{3}H]DPCPX	A_{2A} vs [^{3}H]NECA
3	-	-	-	1.56 ± 0.35[a]	[nd]
				2.39[b]	1.58[b]
41	-	-	1	>300 (92%)[c]	>300 (50%)[c]
42	-	-	2	>300 (100%)[c]	>300 (56%)[c]
43	-	-	3	>300 (45%)[c]	>300 (53%)[c]
44a	F	H	1	>300 (100%)[c]	>300 (38%)[c]
44b	H	F	1	>300 (44%)[c]	>300 (53%)[c]
45a	Cl	H	1	>300 (78%)[c]	>300 (75%)[c]
45b	H	Cl	1	>300 (100%)[c]	>300 (42%)[c]
46a	Br	H	1	>300 (57%)[c]	>300 (70%)[c]
46b	H	Br	1	>300 (100%)[c]	>300 (63%)[c]
47	CF_3	H	1	>300 (58%)[c]	>300 (45%)[c]
48a	CH_3	H	1	>300 (88%)[c]	>300 (54%)[c]
48b	H	CH_3	1	>300 (100%)[c]	>300 (69%)[c]
49	OCH_3	H	1	>300 (61%)[c]	>300 (62%)[c]
50	H	NO_2	1	>300 (94%)[c]	>300 (63%)[c]
ZM241385 (6) A_{2A} AR antagonist				0.273[d]	0.002[d]
CPA A_1 AR agonist				0.015[e]	0.331[e]

[a] All K_i values determined in triplicate and expressed as mean ± SEM.

[b] Literature value obtained from reference (Gütschow *et al.*, 2012)

[c] Percentage displacement of the radioligand at a maximum tested concentration (300 µM).

[d] Literature value obtained from reference (Van der Walt *et al.*, 2013)

[e] Literature value obtained from reference (Van der Walt & Terre'Blanche, 2015)

[nd] not determined

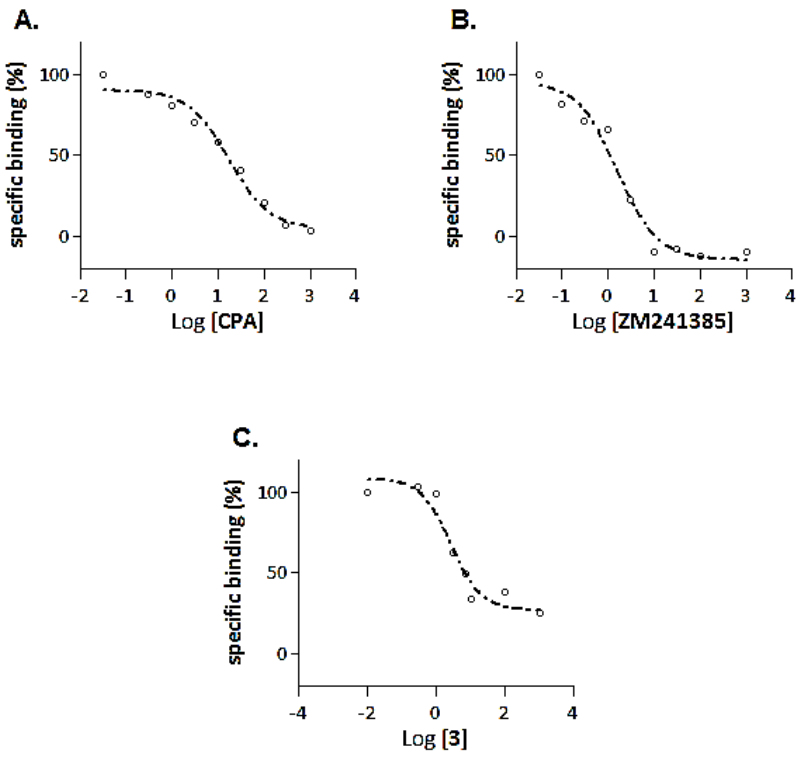

Figure 5-2: **The sigmoidal dose-response curves of CPA (Panel A), ZM241385 (Panel B) and 2-aminobenzothiazinone (3) (Panel C) displaying the binding affinity of CPA and 2-aminobenzothiazinone to adenosine A_1 receptors and ZM241385 to adenosine A_{2A} receptors**

5.4 Conclusion

The 2-phenylalkylaminobenzothiazinones synthesised in **Chapter 4** were tested as potential adenosine A_1 and A_{2A} receptors antagonists by using a radioligand binding assay with [^{3}H]DPCPX as radioligand for the A_1 AR assay and [^{3}H]NECA for the A_{2A} AR assay. The 2-phenylalkylaminobenzothiazinones in this study did not display A_1 and A_{2A} AR affinity when compared to the reference compounds, ZM241385 (**6**) and CPA and the lead scaffold, 2-aminobenzothiazinone (**3**). The poor adenosine A_1 and A_{2A} affinity can probably be attributed to the absence of the amidic acyl group. The results of the radioligand binding study **(Table 5-2)** will be further discussed in detail in **Chapter 6**.

5.5 References

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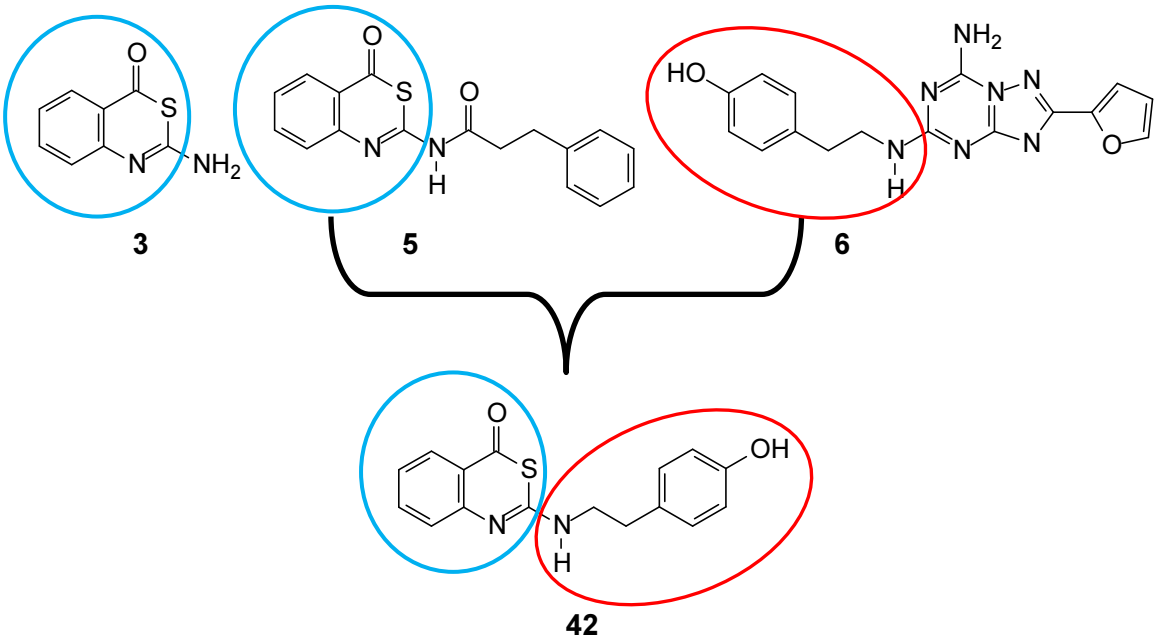
CHAPTER 6

DISCUSSION AND CONCLUSION

The A_1 and A_{2A} AR subtypes have been recognised as possible drug targets for the treatment of neurological disorders such as PD. In PD treatment it is suggested that selective A_1 AR antagonists have the potential to treat cognitive impairments, whereas selective A_{2A} AR antagonists may not only improve motor dysfunction, but also possess neuroprotective properties. Additionally A_{2A} AR antagonists may be used as adjunct therapy to L-DOPA to lower the risk of dyskinesia associated with long term treatment with L-DOPA. A_{2A} AR has also been implicated to exhibit antidepressant activity in animal models, which may be advantageous in PD depression. To date an ideal drug candidate remains elusive and several research groups are working toward an effective treatment for PD, especially focusing on adenosine receptor antagonists.

Recently the benzothiazinone scaffold was deemed an appropriate amino-substituted heterocyclic scaffold to design A_1 and A_{2A} AR antagonists. Gütschow and co-workers (2012) identified 2-aminobenzothiazinone (**3**) with good A_1 and A_{2A} AR affinity (K_iA_1 = 2.39 µM; K_iA_{2A} = 1.58 µM). Several 2-acylaminobenzothiazinone derivatives showed good adenosine A_1 and A_{2A} receptor affinity, with various acyl substitutions on the 2-amino group. Further research in 2013 (Stöβel *et al.*, 2013) showed that chain elongation to phenylpropanamide-benzothiazinone (**5**) (K_iA_1 = 0.422 µM and K_iA_{2A} = 0.103 µM) lead to an increase in adenosine A_{2A} AR affinity, but a decrease in A_1 AR affinity. These results prompted the study to investigate if the flexible phenylalkyl group derived from ZM241385 (**6**) could increase the affinity towards AR subtypes A_1 and A_{2A}, and if the CO group of the acyl substitution is required for AR affinity. ZM241385 (**6**) is a non-xanthine selective adenosine A_{2A} receptor antagonist with K_i values of 0.273 µM for A_1 and 0.002 µM for A_{2A} receptors (Van der Walt *et al.*, 2013).



Fourteen 2-phenylalkylaminobenzothiazinone derivatives were synthesised by combining the methods from Leistner and co-workers (1990), and Bhattacharyya and co-workers (2014). The binding affinities at the A_1 and A_{2A} AR of each of the synthesised benzothiazinone derivatives were determined in competitions assays to rat whole brain (A_1) and striatal (A_{2A}) membranes, respectively. The results of the binding assays are summarised in **Table 5-2.** As stated in the rational our research focused on the lead 2-aminobenzothiazinone (**3**) synthesised by Gütschow and co-workers (2012), which showed good A_1 and A_{2A} AR affinity (K_iA_1 = 2.39 µM; K_iA_{2A} = 1.58 µM). Fourteen compounds were synthesised where the N-acyl group was replaced by the phenylalkyl group of ZM241385 (**6**). In addition chain elongation was also investigated. The test compounds showed a dramatic decrease (>300 µM) in both A_1 and A_{2A} AR affinities, thus showing the importance of the CO moiety for A_1 and A_{2A} affinity in the benzothiazinone scaffold.

In line with our results another study by Novellino and co-workers (2002) explored the adenosine A_1 affinity of novel N-alkyl- and N-acyl-(7-substituted-2-phenylimidazo[1,2-a][1,3,5]triazin-4-yl)amines (ITAs) and investigated if these compounds fulfill the pharmacophore requirements for A_1 AR antagonism. In a pharmacophore- and docking-based modeling study with six well-known A_1 AR antagonists (**Figure 6-1**), it was suggested that these compounds interact with three H-bond sites and three lipophilic pockets (L1, L2, L3) within the A_1 receptor binding site. In helix IV the Asn254 has a CO and NH_2 moiety (HB1 and HB2),

which can form two H-bonds and Ser277 helix VII has a hydroxyl group that forms a H-bond (HB3). **Figure 6-2** illustrates the interactions of the six A_1 antagonists as well as a pharmacophoric scheme for the ITAs interaction.

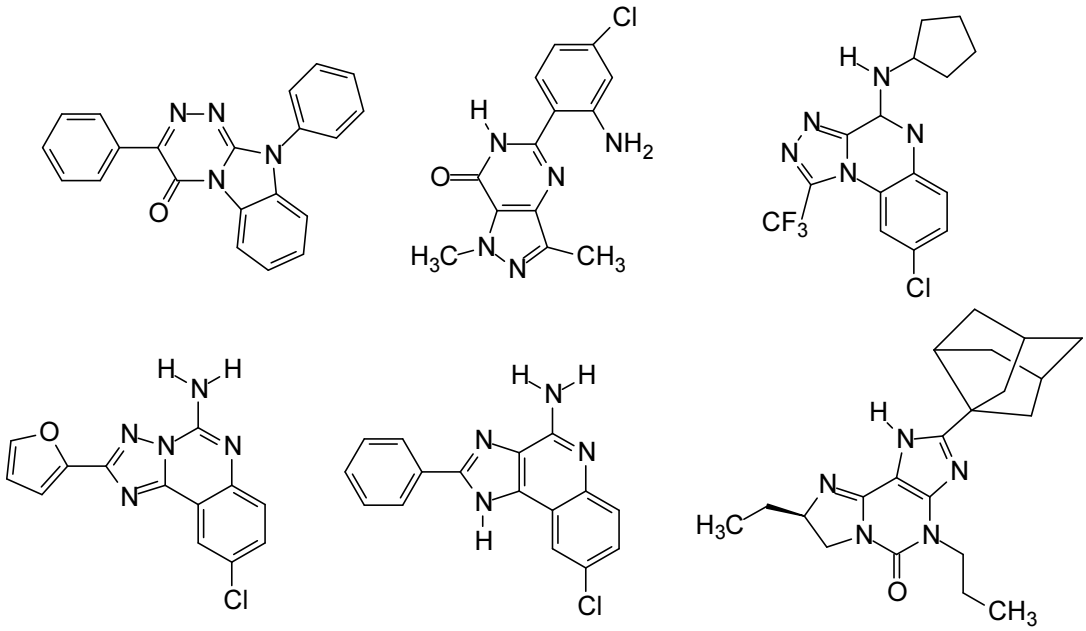


Figure 6-1: **Six A_1 AR antagonists used for the pharmacophore modeling (Novellino *et al.*, 2002)**

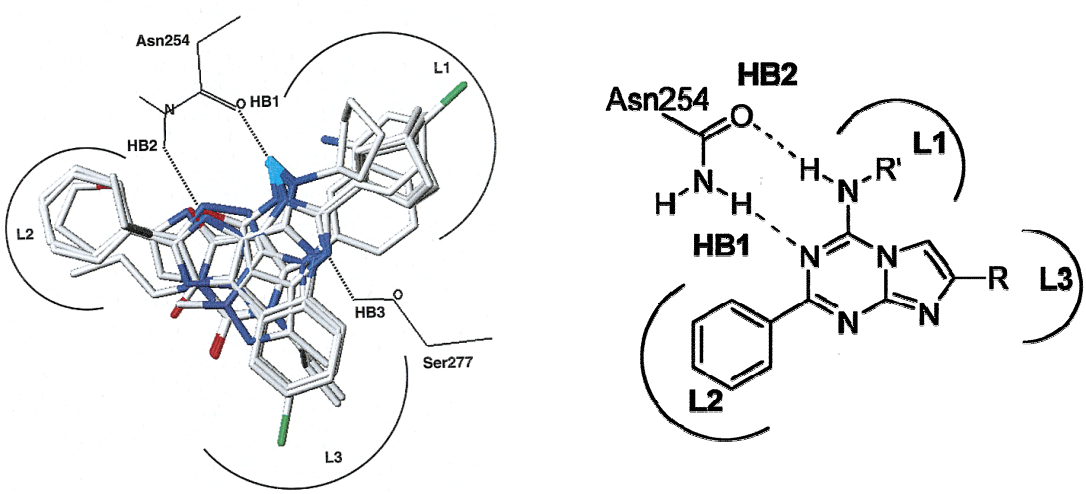


Figure 6-2: **Pharmacophoric modeling of six compounds in Figure 6-1 (left) showing the H-bonds, HB1, HB2 and HB3 corresponding to Asn254 and Ser277. On the right a pharmacophoric scheme shows the interaction of ITAs (Novellino *et al.*, 2002)**

The results of Novellino and co-workers (2002) support our findings by highlighting the importance of the CO linker. At first the introduction of an acetyl group at the N4 reduced A_1 affinity and diminished A_{2A} affinity. However, an enhancement in A_1 affinity was observed with CO-cyclohexyl and CO-phenyl, but A_{2A} affinity remained diminished. A_1 affinity was further enhanced by inserting a methylene spacer between the acetyl group and the N4. The introduction of a CO-cyclopentyl showed an increase in A_1 affinity and gained A_{2A} affinity. Replacement of the 7-CH_3 with a C_2H_5 and a phenyl maintained A_1 affinity and showed an increase in A_{2A} affinity (**Figure 6-3**). These results suggested that the insertion of the amidic CO group is indeed beneficial and that the favorable effect of the CO linker with respect to the CH_2 might be due to an improved H-bond donor ability of the NH group or a more precise orientation into the lipophilic pocket LP1 (**Figure 6-2**).

Revisiting the 2-acylaminobenzothiazinone scaffold of Gütschow and co-workers (2012), the chain elongation of Stöβel and co-workers (2013) and our synthesised 2-phenylalkylaminobenzothiazinones, it is evident that the CO group plays an important role in both A_1 and A_{2A} affinity (**Figure 6-4**). The same trend could be seen where the acyl group of 2-acylaminobenzothiazinones is replaced with a CH_2 group (2-phenylalkylaminobenzothiazinones), which lead to diminished A_1 and A_{2A} AR affinity. Unfortunately, chain elongation (compounds **41**, **42** and **43**) showed no improvement in A_1 and A_{2A} AR affinity (**Table 5-2**).

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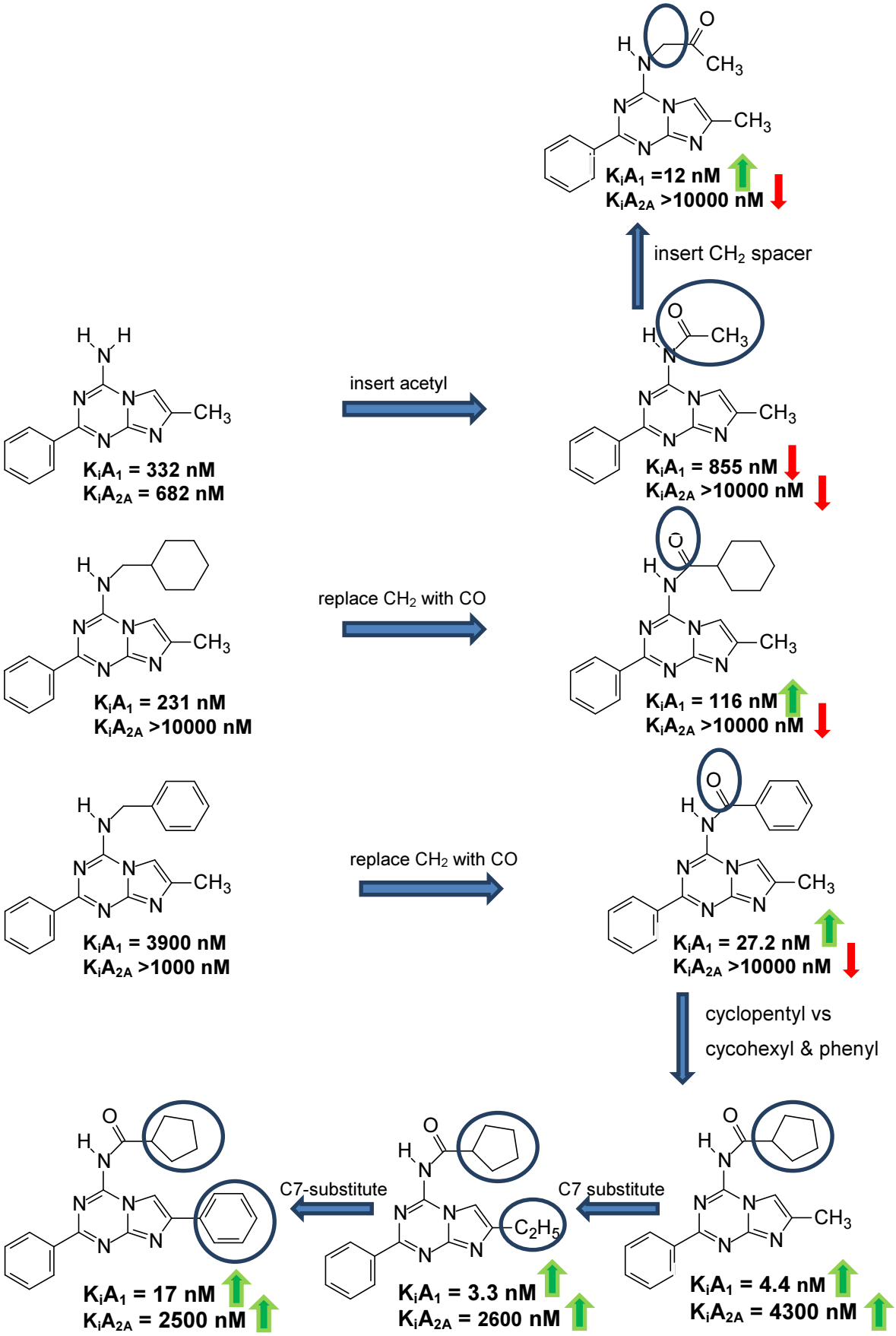

Figure 6-3: **Binding AR affinities of selected ITA derivatives (Novellino *et al.*, 2002)**

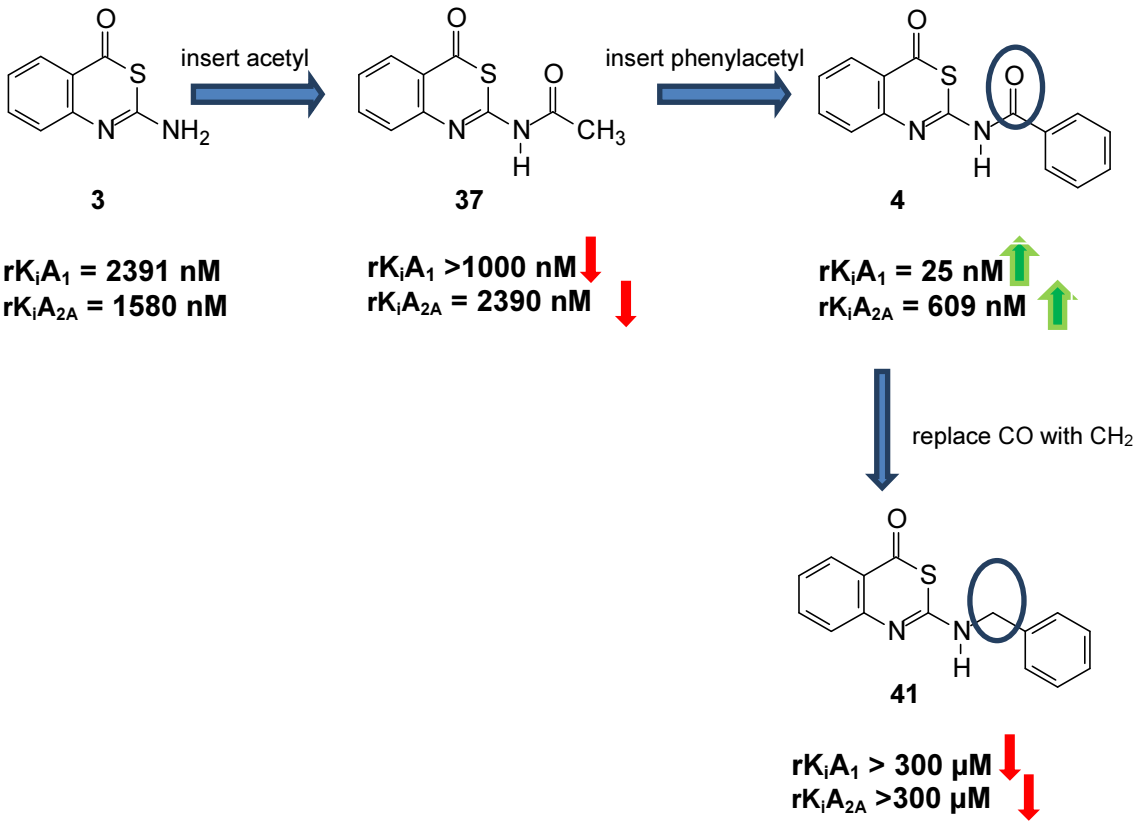

Figure 6-4: **Binding AR affinities of selected 2-acylaminobenzothiazinones (Gütschow *et al.*, 2012) and a 2-phenylalkylaminobenzothiazinone derivative of the current study**

In conclusion our results show the importance of the amidic CO moiety for both adenosine A_1 and A_{2A} affinity in the phenylacylaminobenzothiazinone scaffold at position C2 to improve H-bond donor ability of the NH group. Further research can investigate the insertion of a methylene linker between the NH and the acyl group to improve A_1 affinity and selectivity according to the results of Novellino and co-workers (2002) (**Figure 6-5**).

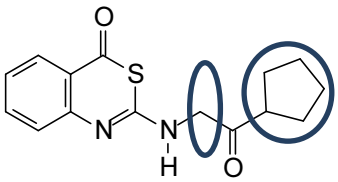

Figure 6-5: **Possible structural improvement to 2-acylaminobenzothiazinones to gain A_1 affinity (CH_2 linker) and selectivity and A_{2A} affinity (cyclopentane ring)**

Although the investigated 2-phenylalkylbenzothiazinones did not exhibit A_1 and A_{2A} AR affinity, the importance of the carbonyl group in the 2-acylaminobenzothiazianone scaffold for both A_1 and A_{2A} AR affinity was highlighted, which is of importance for the designing of new adenosine receptor antagonists for the treatment of PD in future.

6.1 References

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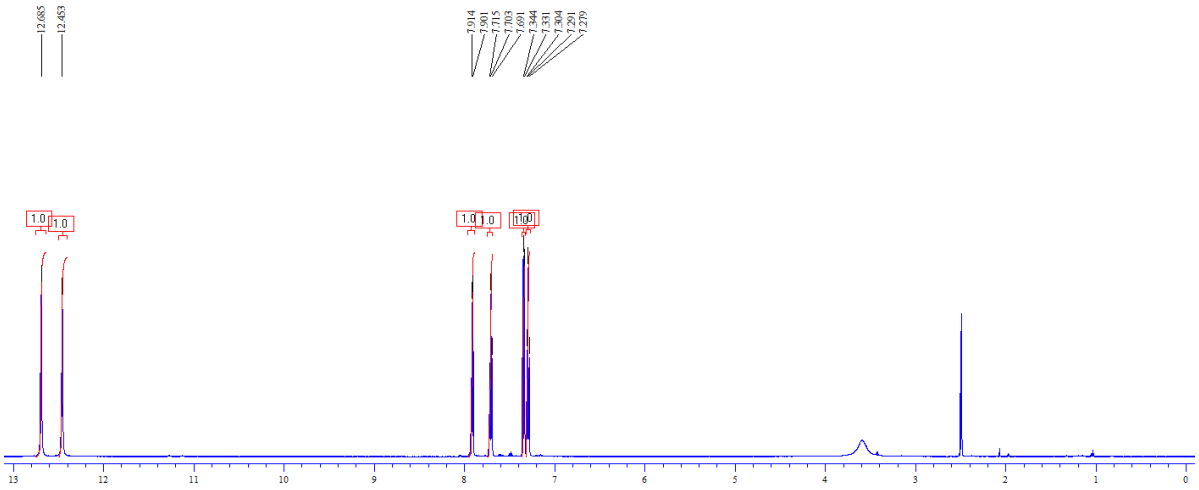
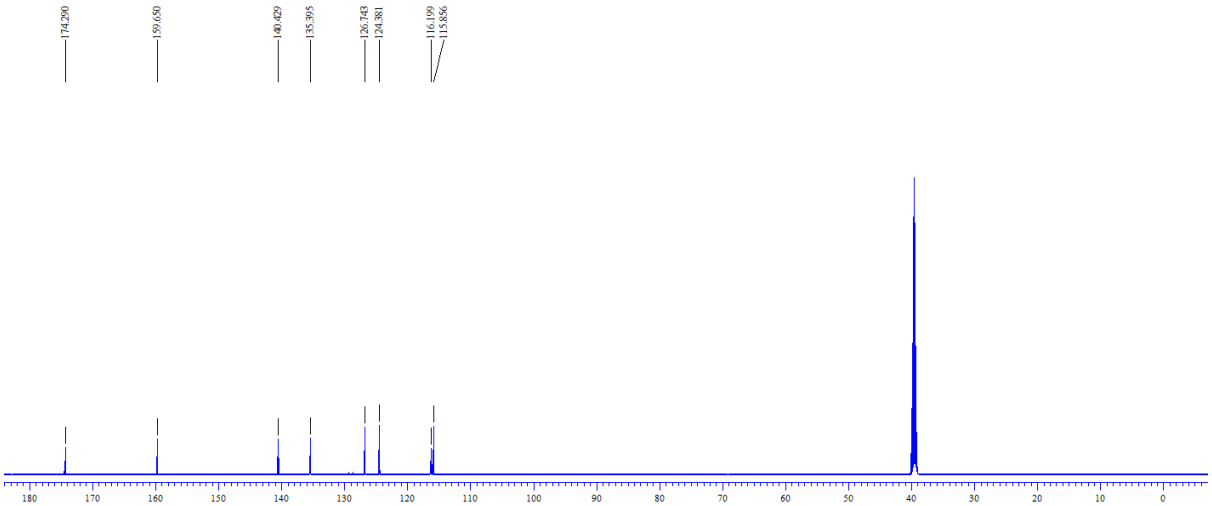
ANNEXURE A NMR DATA

2-aminobenzothiazinone (3)

^{1}H-NMR

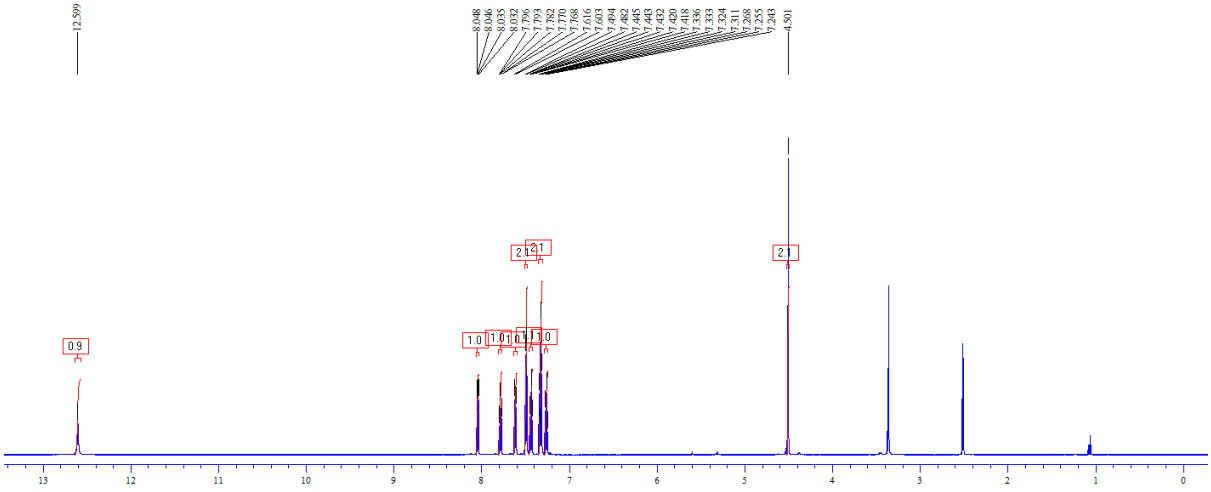


^{13}C NMR

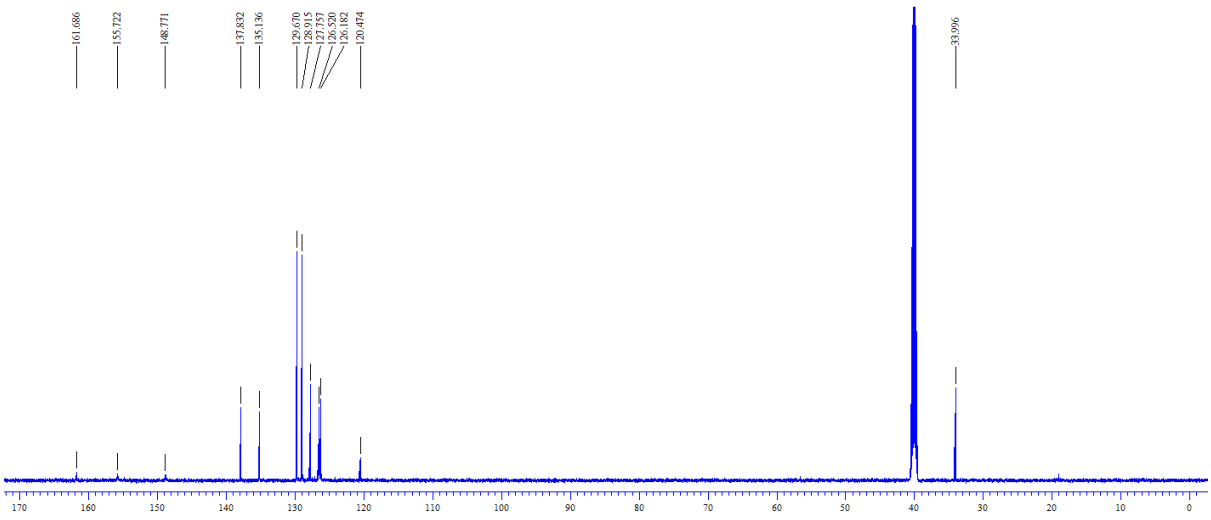


2-(benzylamino)-4H-3,1-benzothiazin-4-one (41)

^{1}H NMR

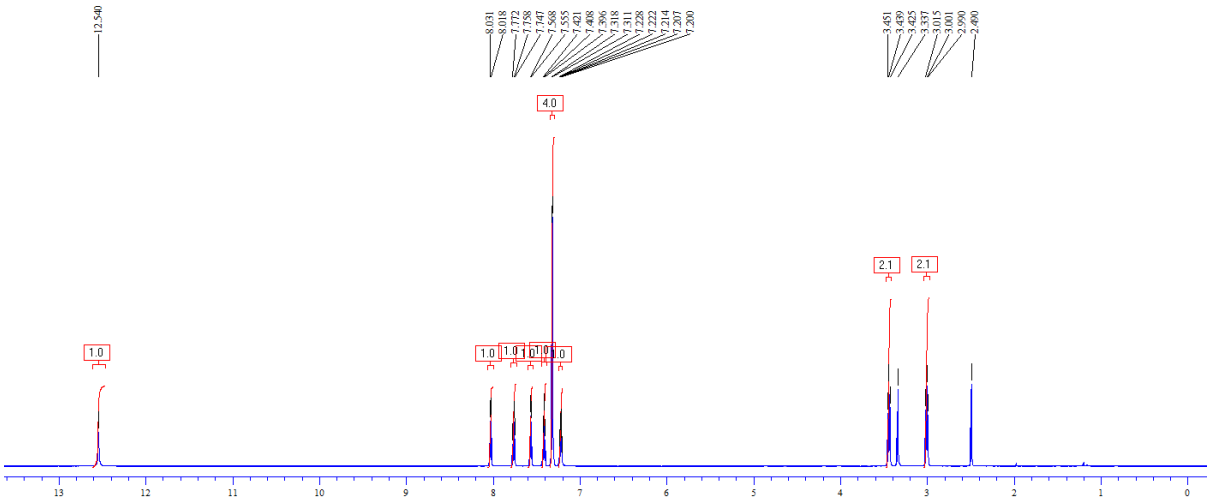


^{13}C NMR

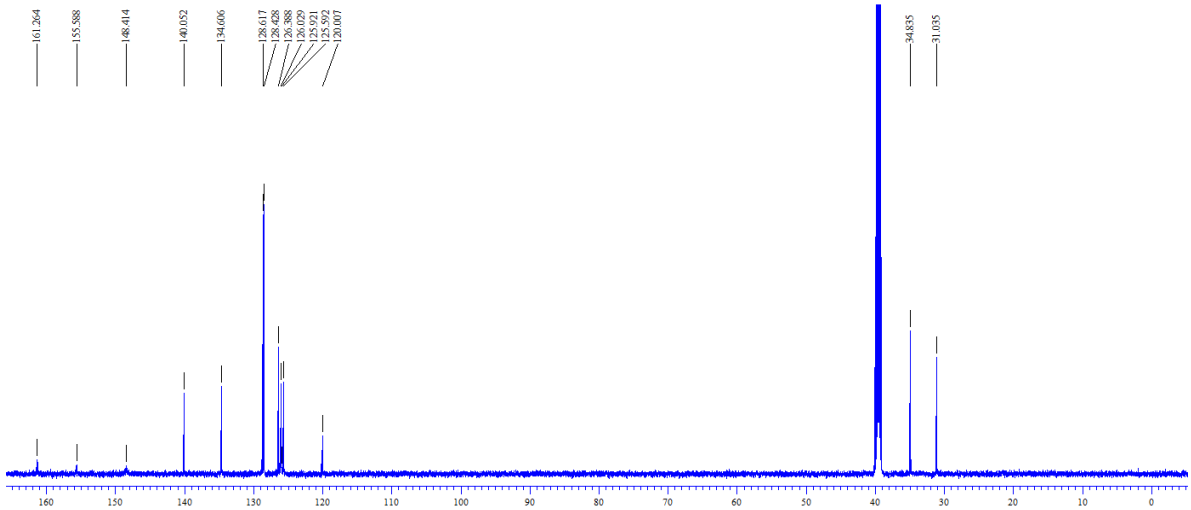


2-[(2-phenylethyl)amino]-4H-3,1-benzothiazin-4-one (42)

^{1}H NMR

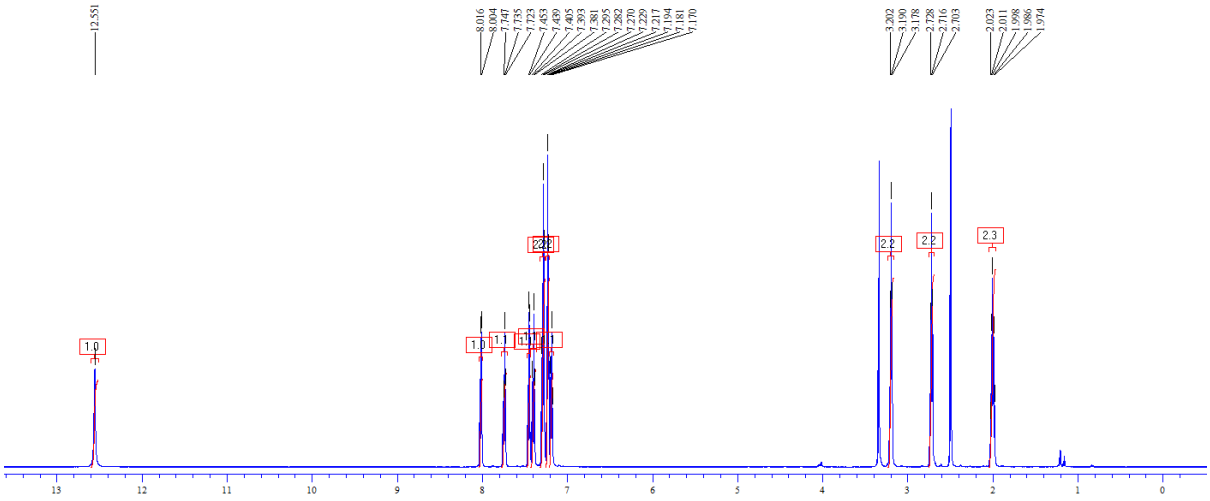


^{13}C NMR

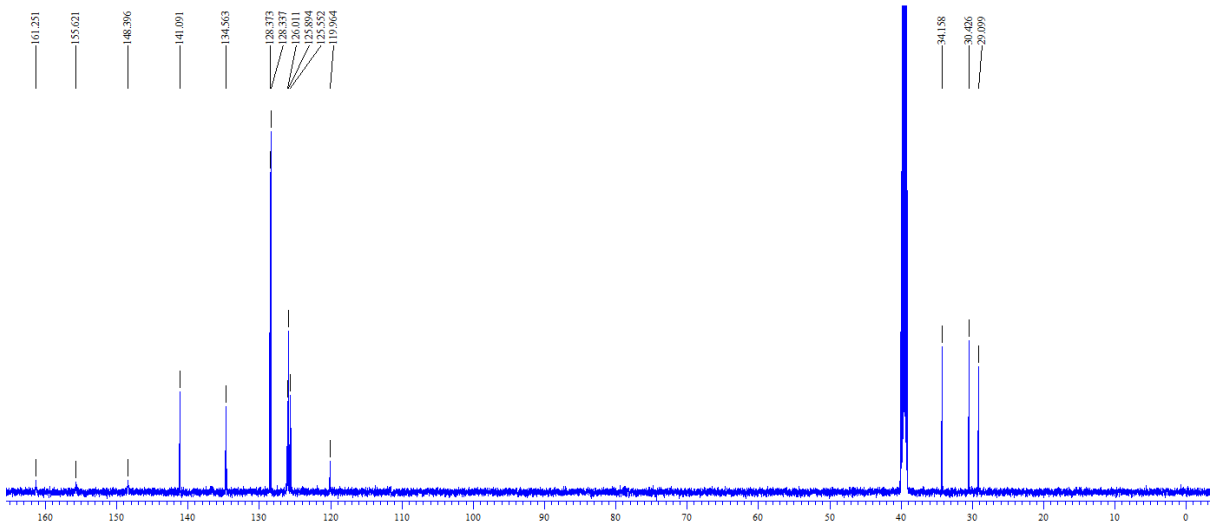


2-[(3-phenylpropyl)amino]-4H-3,1-benzothiazin-4-one (43)

^{1}H NMR

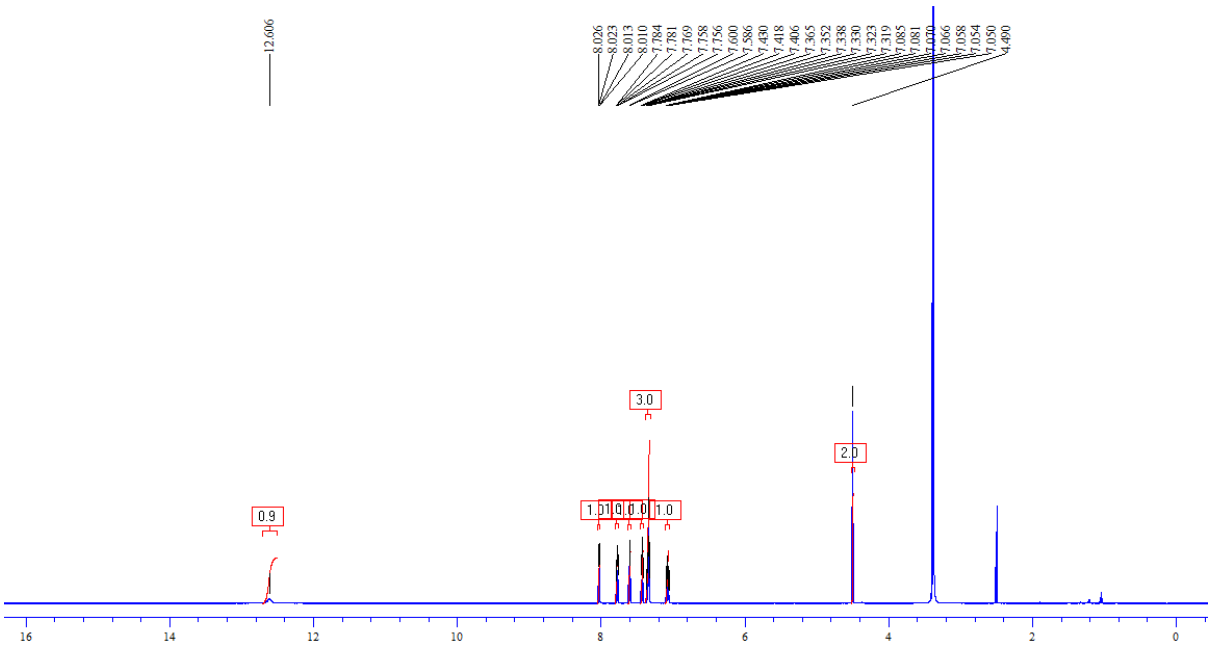


^{13}C NMR

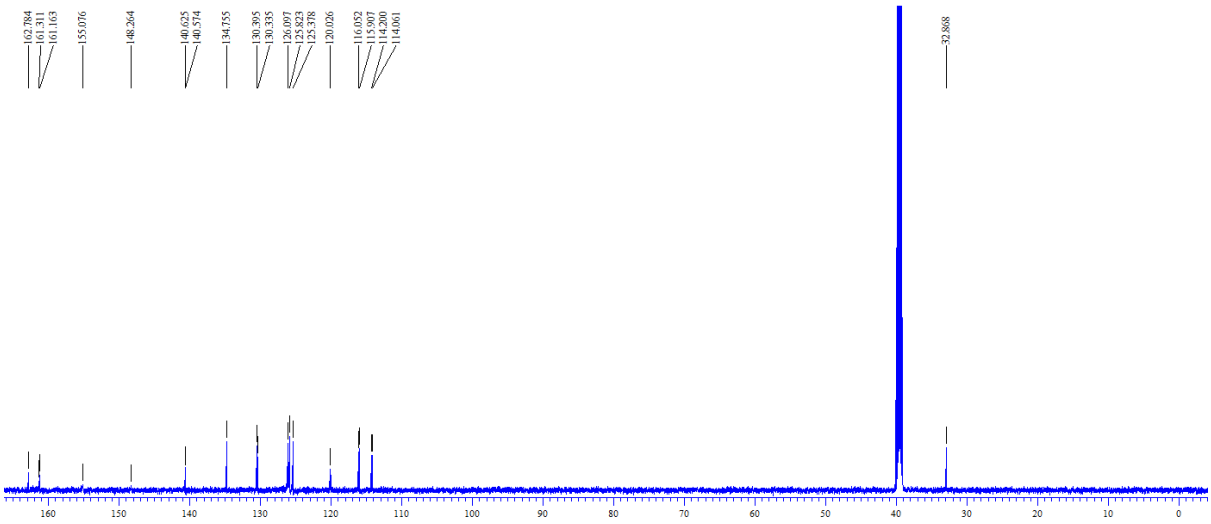


2-{[(3-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44a)

^{1}H NMR

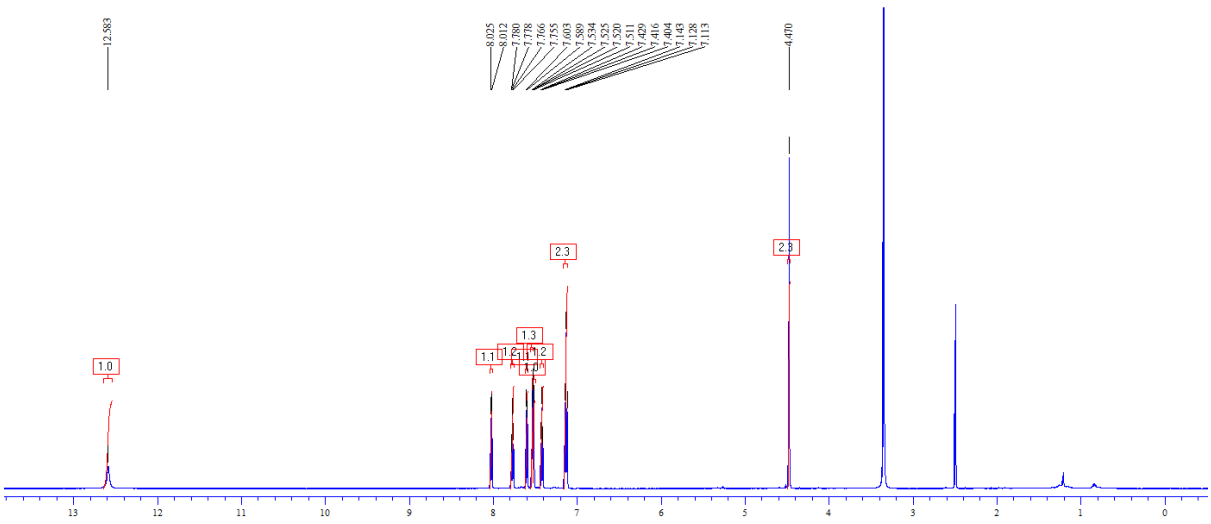


^{13}C NMR

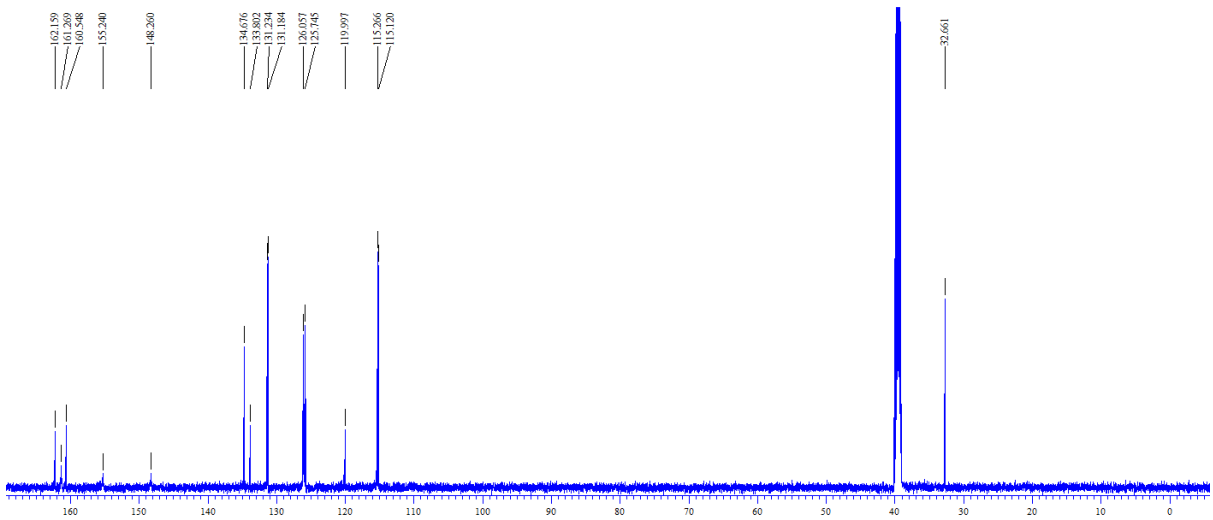


2-{[(4-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44b)

^{1}H NMR

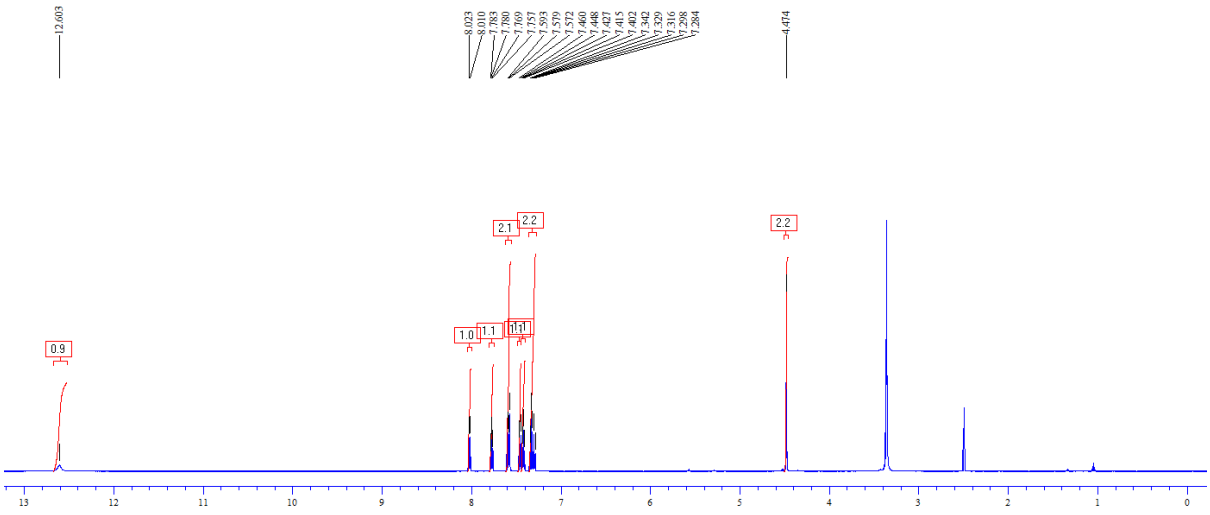


^{13}C NMR

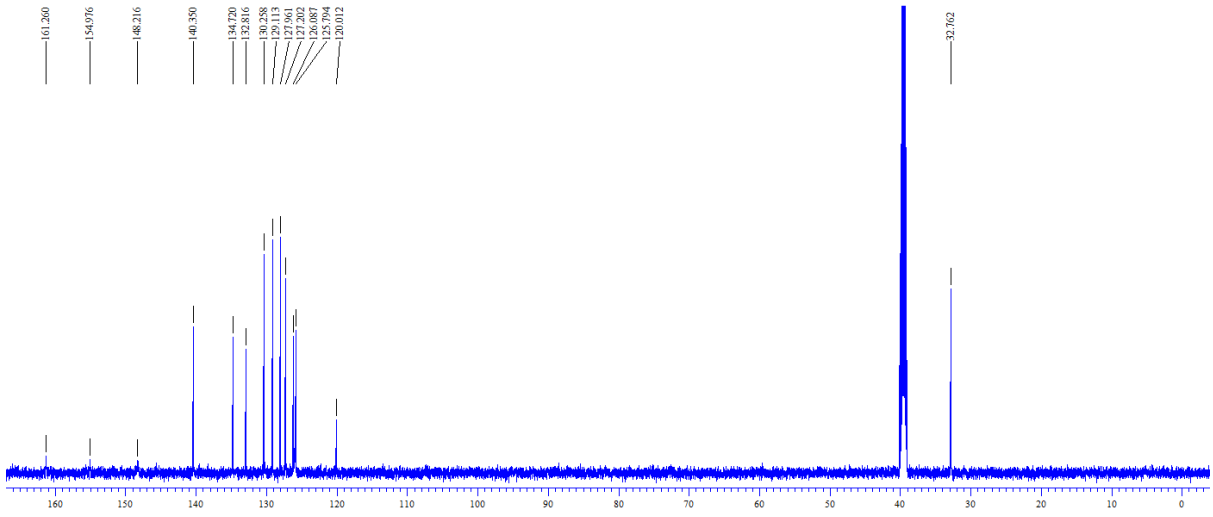


2-{[(3-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45a)

^{1}H NMR

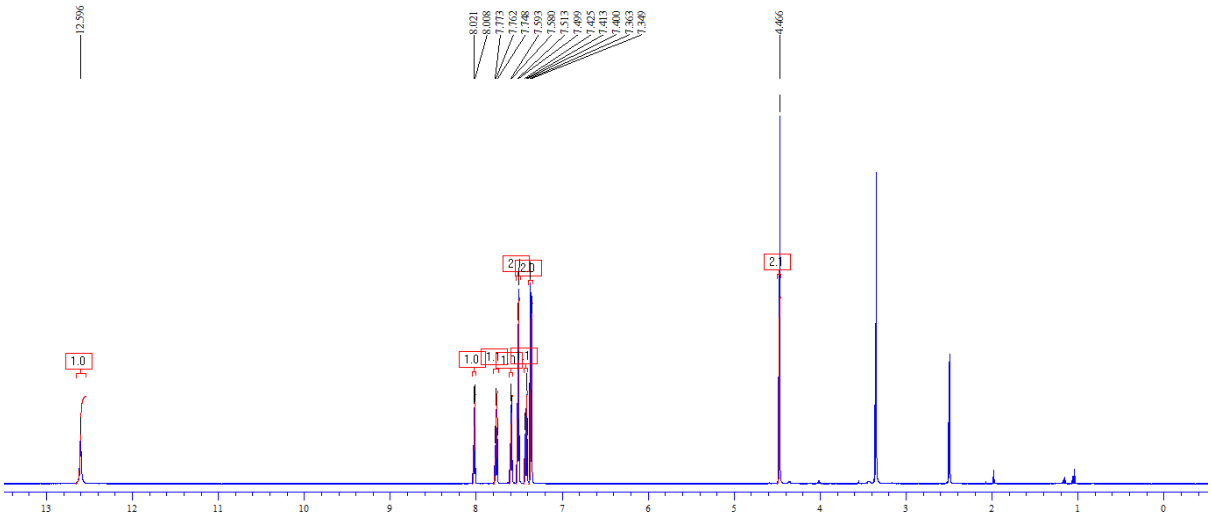


^{13}C NMR

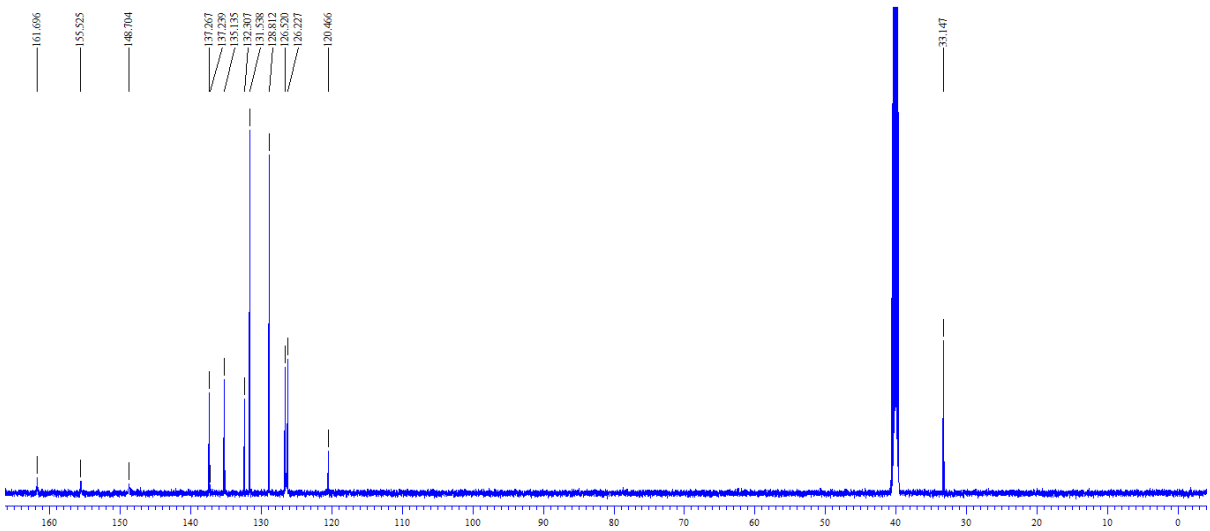


2-{[(4-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45b)

^{1}H NMR

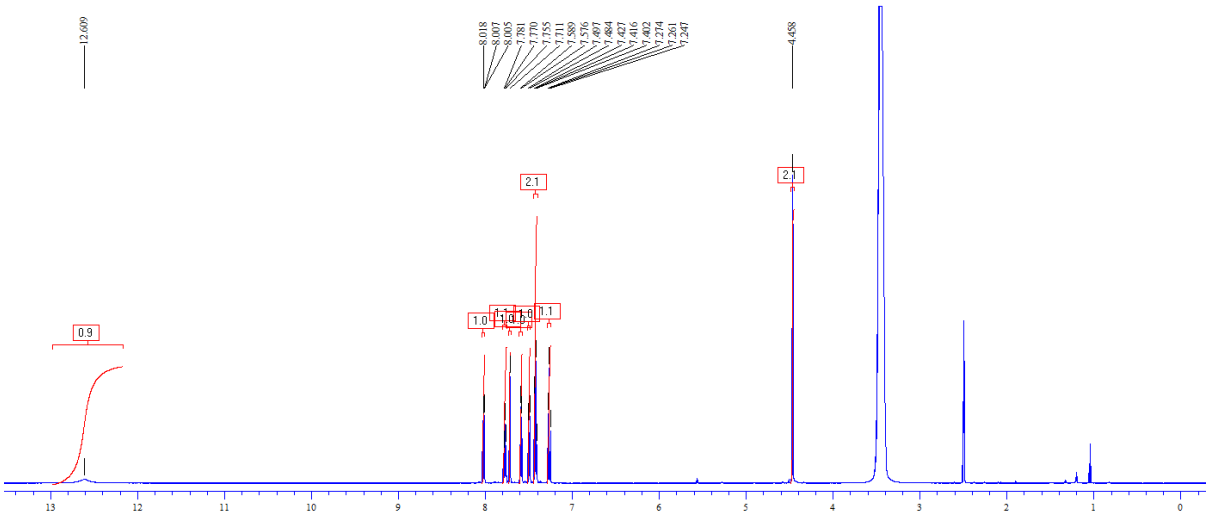


^{13}C NMR

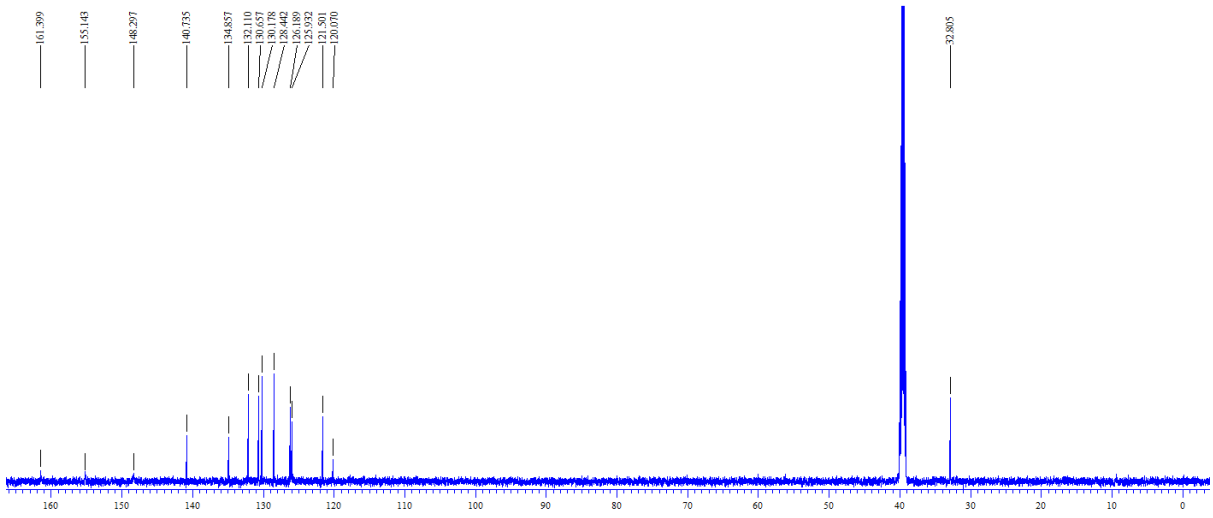


2-{[(3-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46a)

^{1}H NMR

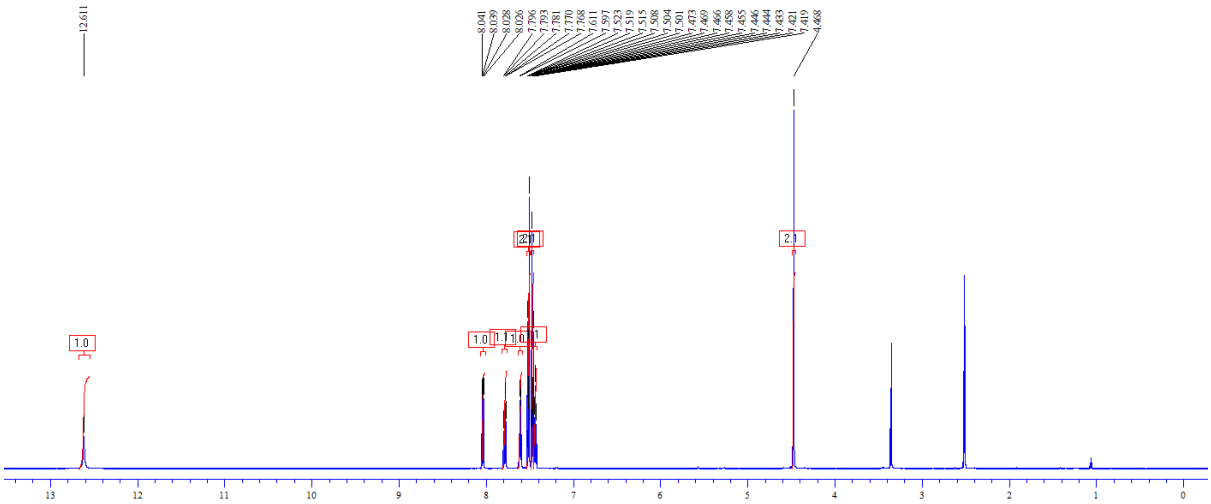


^{13}C NMR

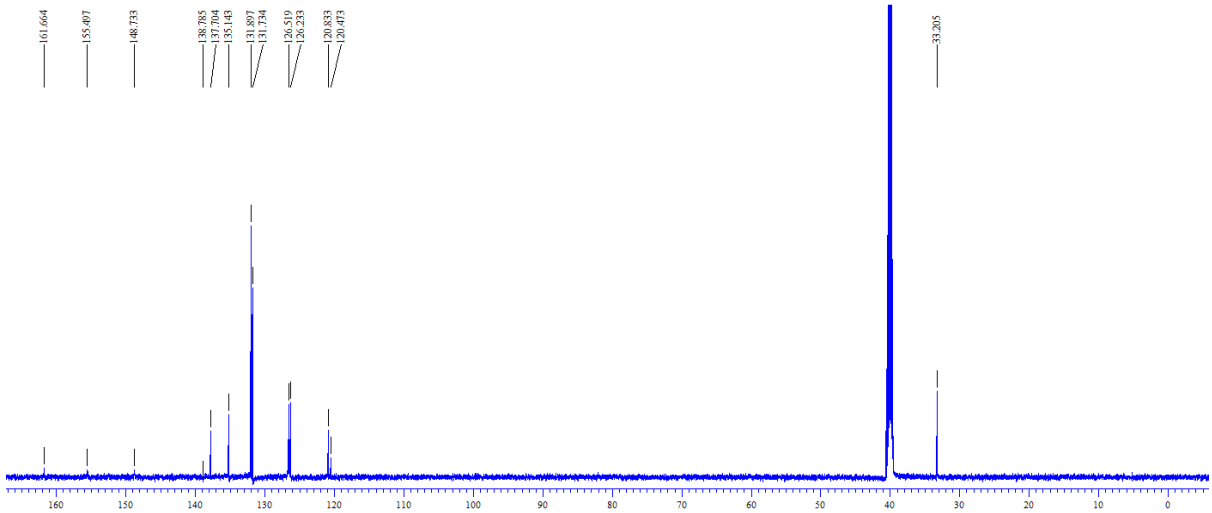


2-{[(4-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46b)

^{1}H NMR

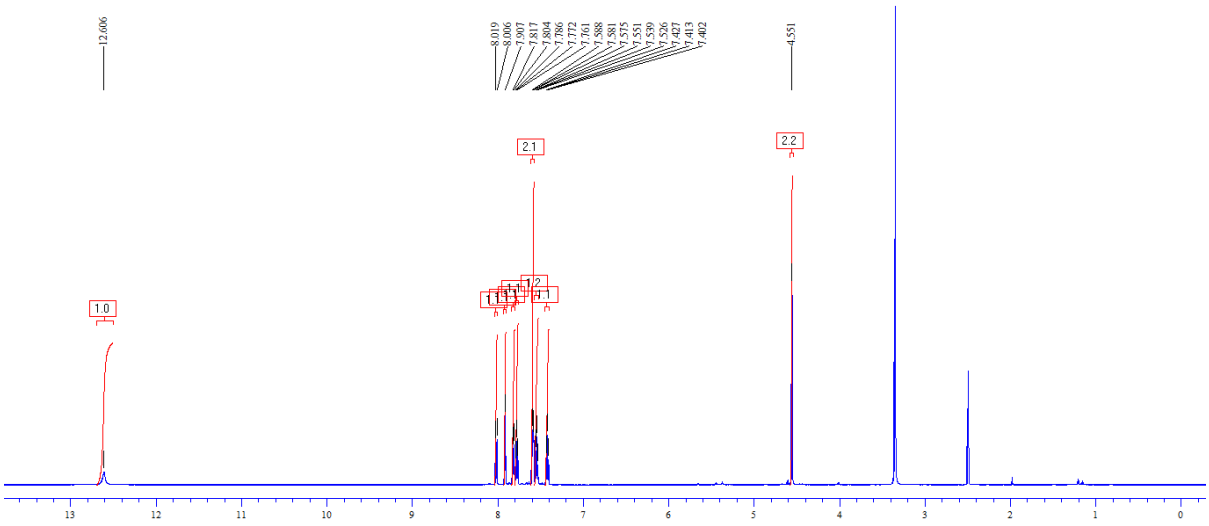


^{13}C NMR

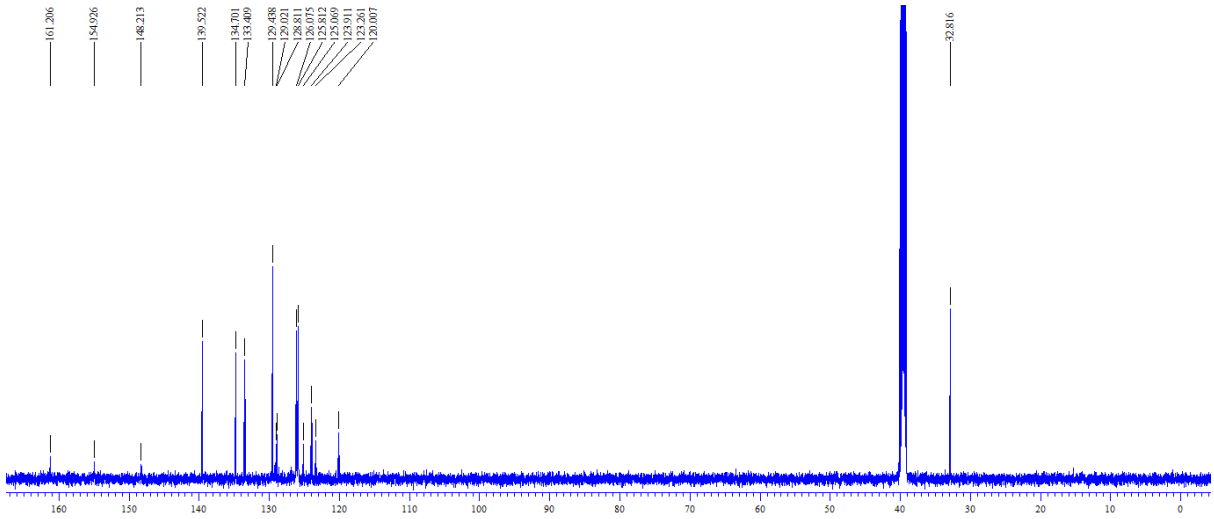


2-({[3-(trifluoromethyl)phenyl]methyl}amino)-4H-3,1-benzothiazin-4-one (47)

^{1}H NMR

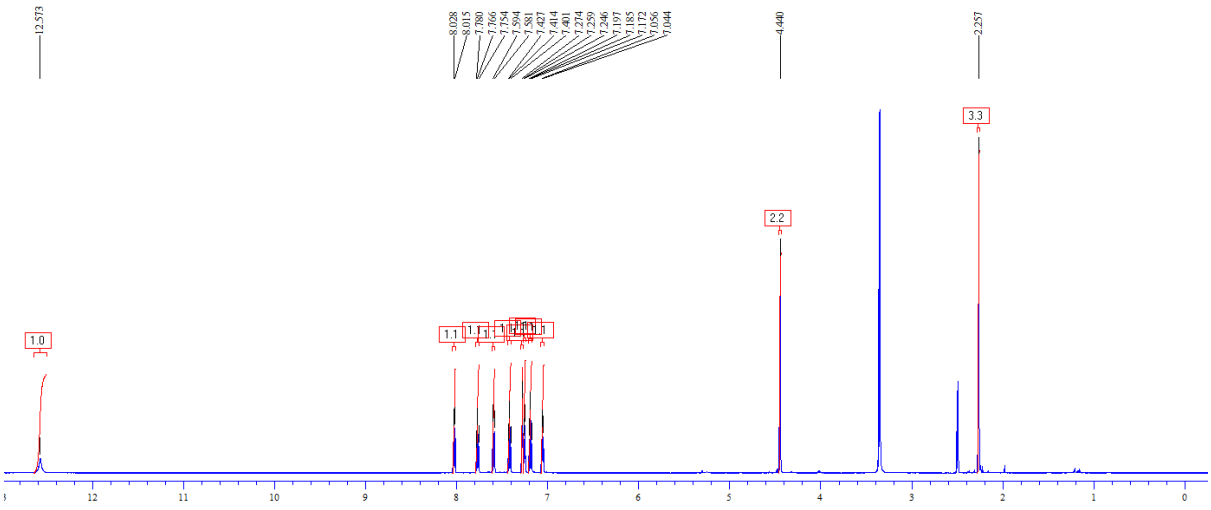


^{13}C NMR

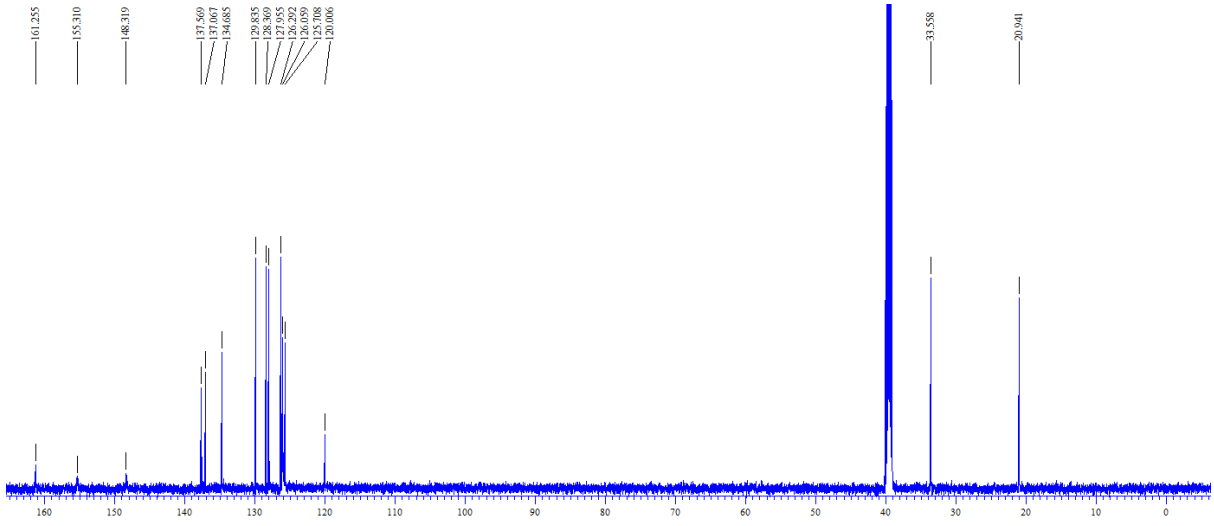


2-{[(3-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48a)

^{1}H NMR

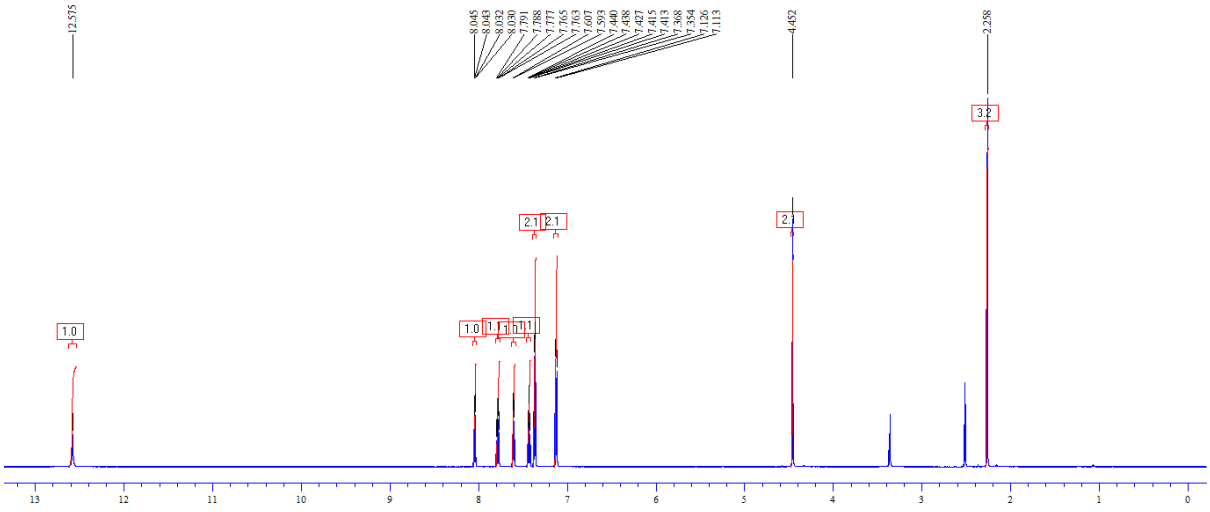


^{13}C NMR

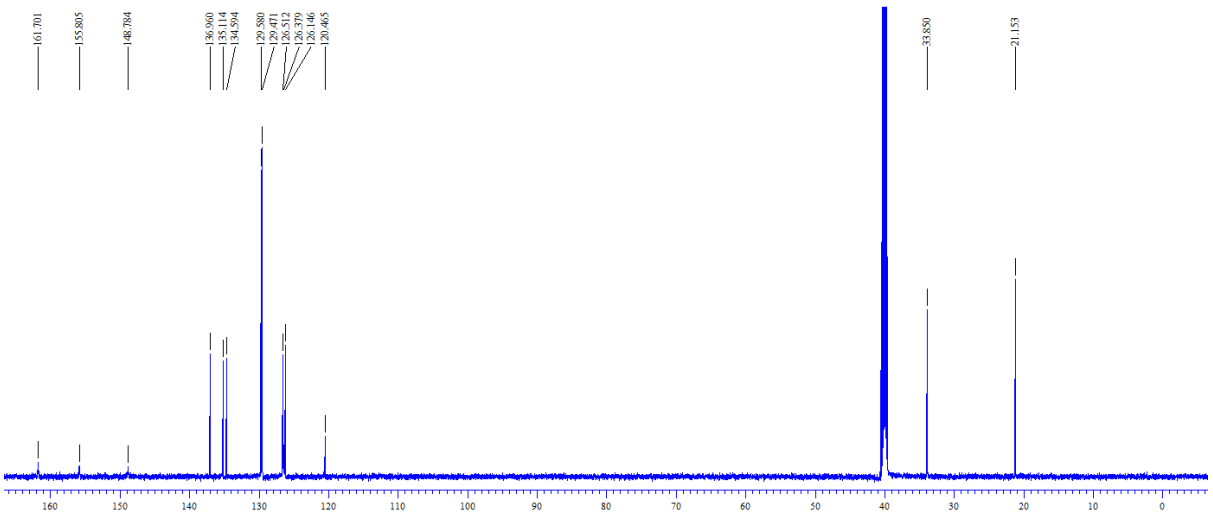


2-{[(4-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48b)

^{1}H NMR

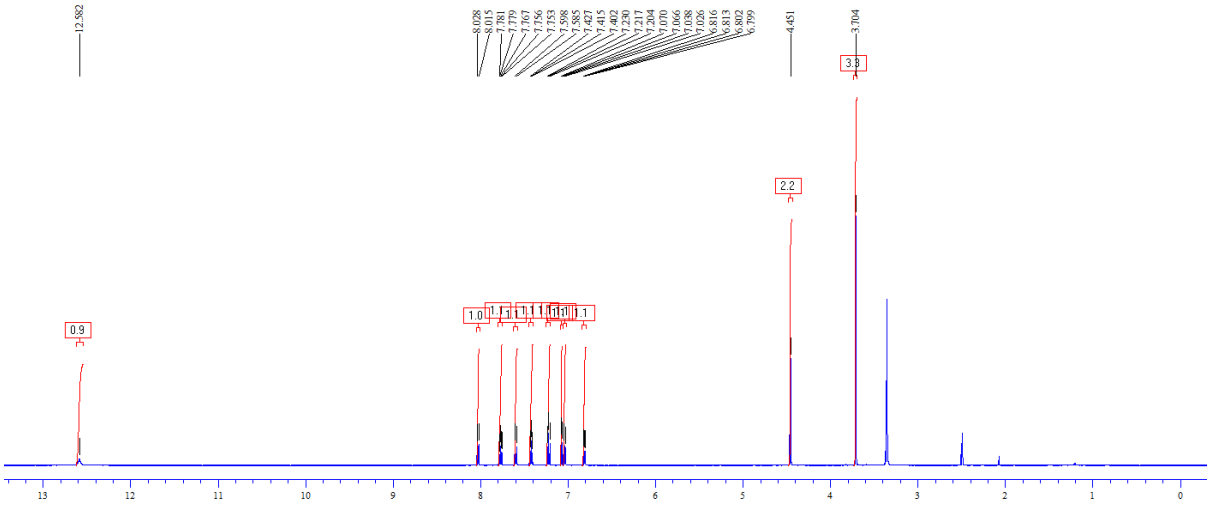


^{13}C NMR

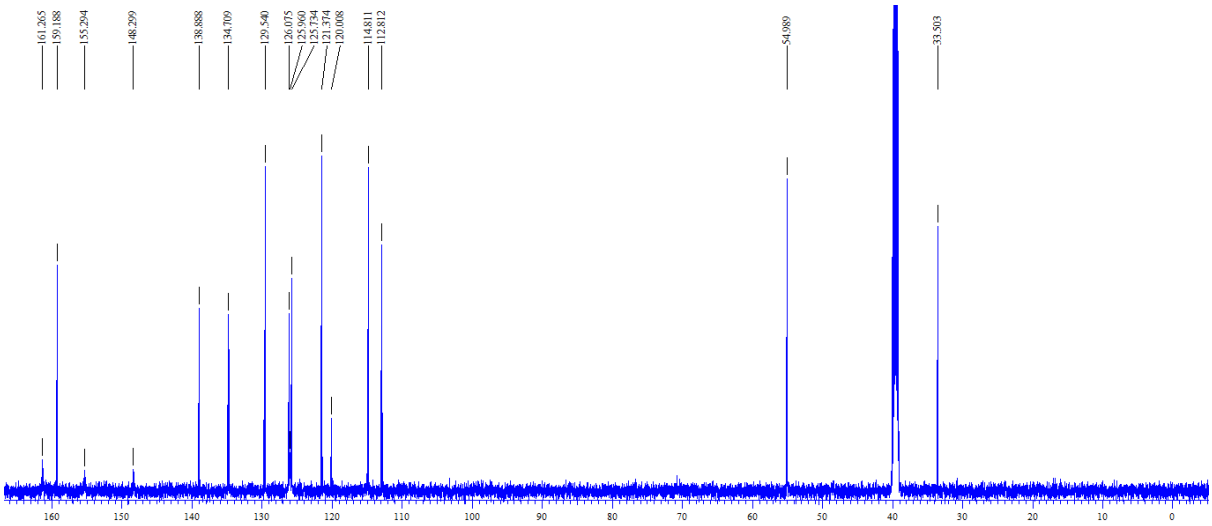


2-{[(3-methoxyphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (49)

^{1}H NMR

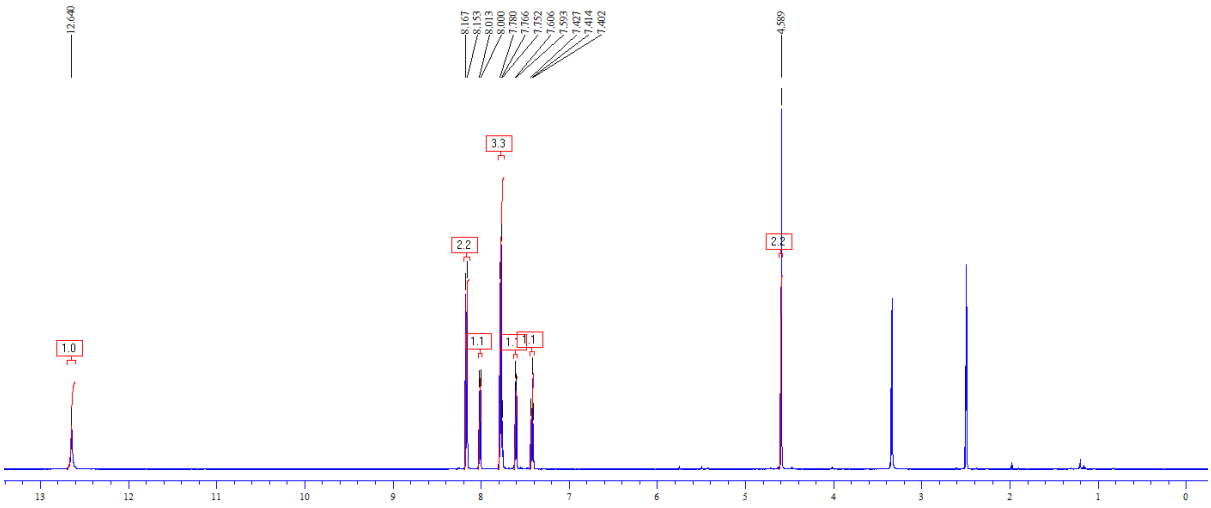


^{13}C NMR

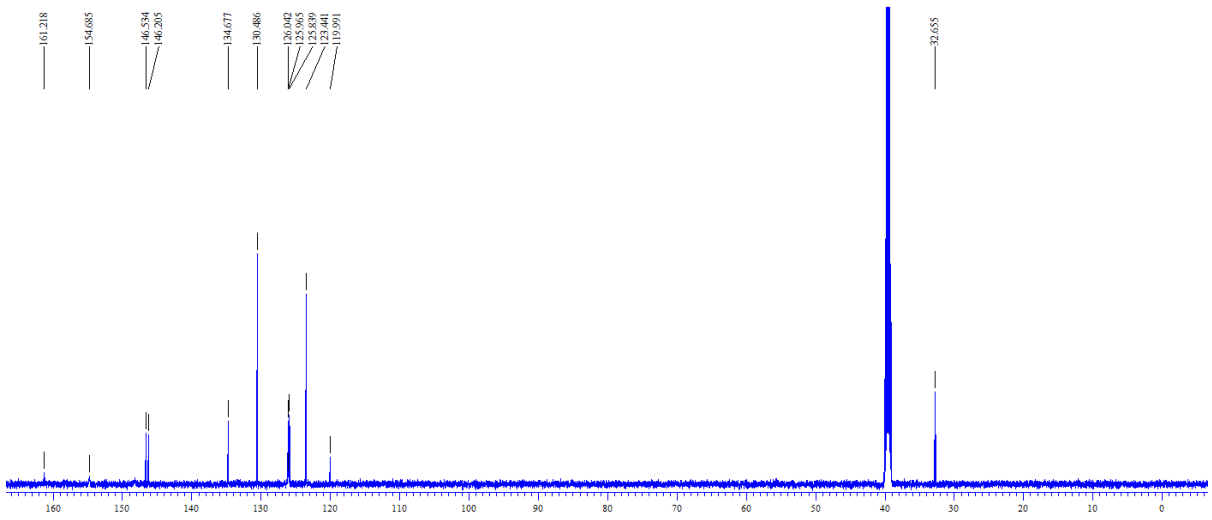


2-{[(4-nitrophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (50)

^{1}H NMR

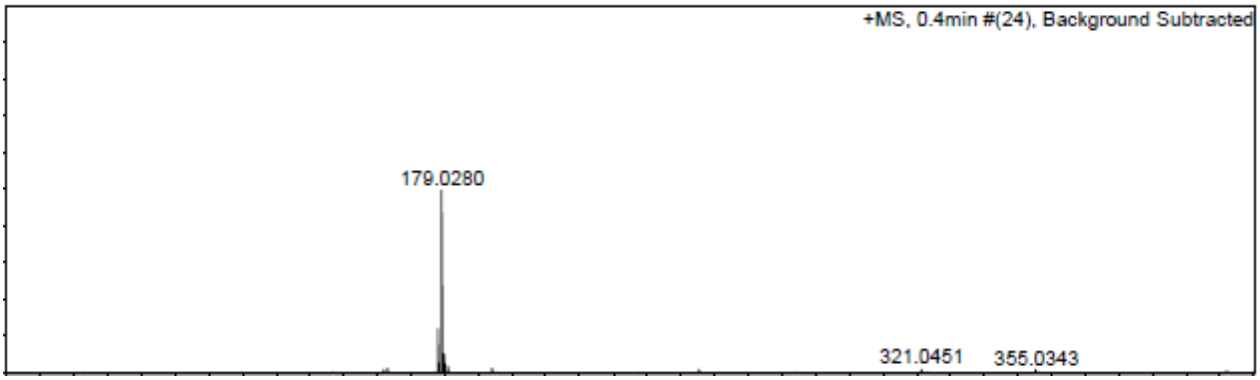


^{13}C NMR



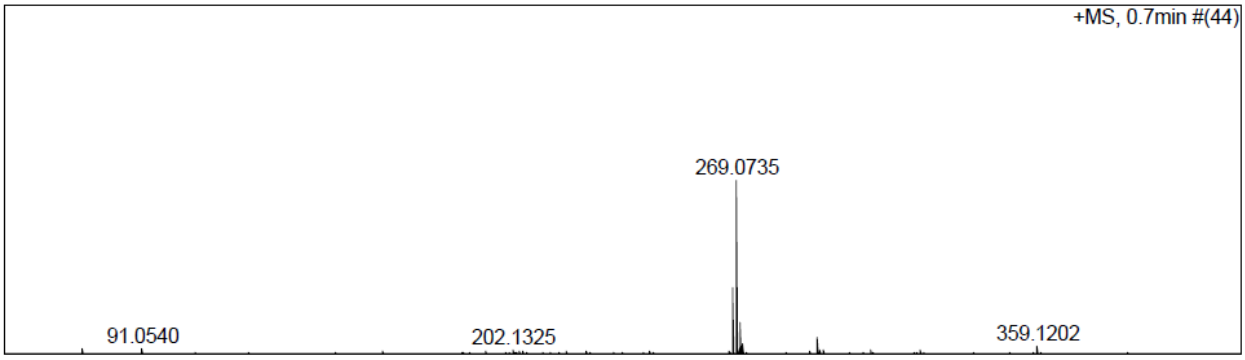
ANNEXURE B MS DATA

2-aminobenzothiazinone (3)



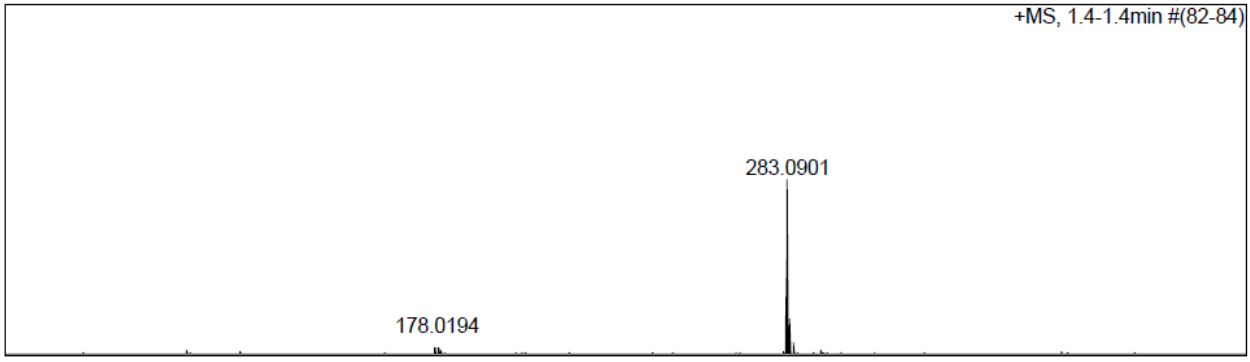
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
179.0280	1	C 8 H 7 N 2 O S	100.00	179.0274	-0.6	-3.5	5.1	6.5	ok	even

2-(benzylamino)-4H-3,1-benzothiazin-4-one (41)



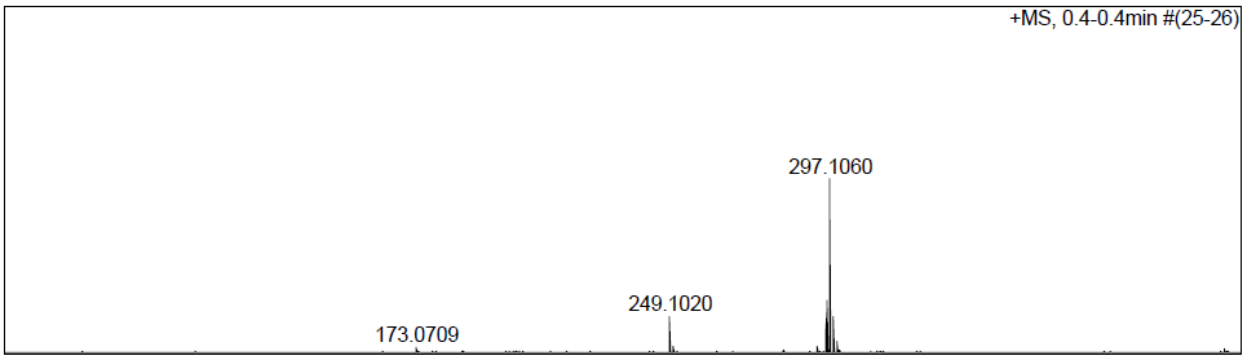
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
269.0735	1	C 15 H 13 N 2 O S	100.00	269.0743	0.8	3.0	4.0	10.5	ok	even

2-[(2-phenylethyl)amino]-4H-3,1-benzothiazin-4-one (42)



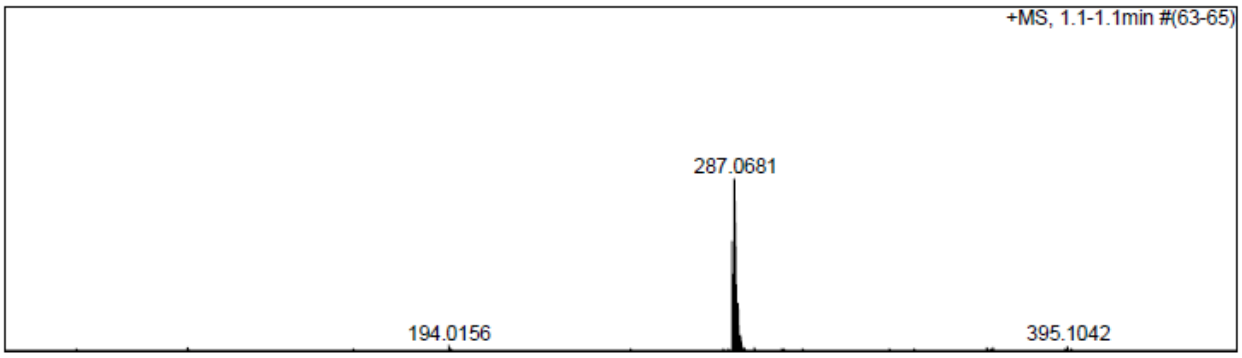
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
283.0901	1	C 16 H 15 N 2 O S	100.00	283.0900	-0.2	-0.6	5.0	10.5	ok	even

2-[(3-phenylpropyl)amino]-4H-3,1-benzothiazin-4-one (43)



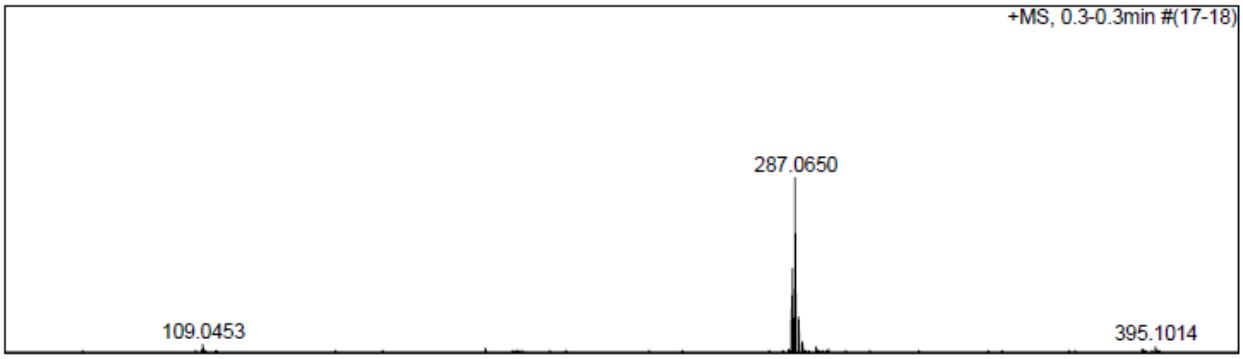
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
297.1060	1	C 17 H 17 N 2 O S	100.00	297.1056	-0.4	-1.3	3.7	10.5	ok	even

2-{[(3-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44a)



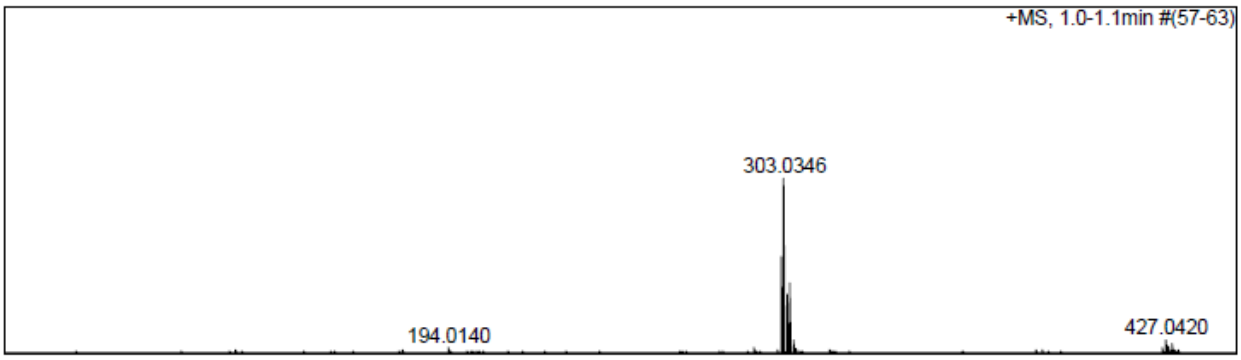
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
287.0681	1	C 15 H 12 F N 2 O S	100.00	287.0649	-3.3	-11.3	49.5	10.5	ok	even

2-{[(4-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44b)



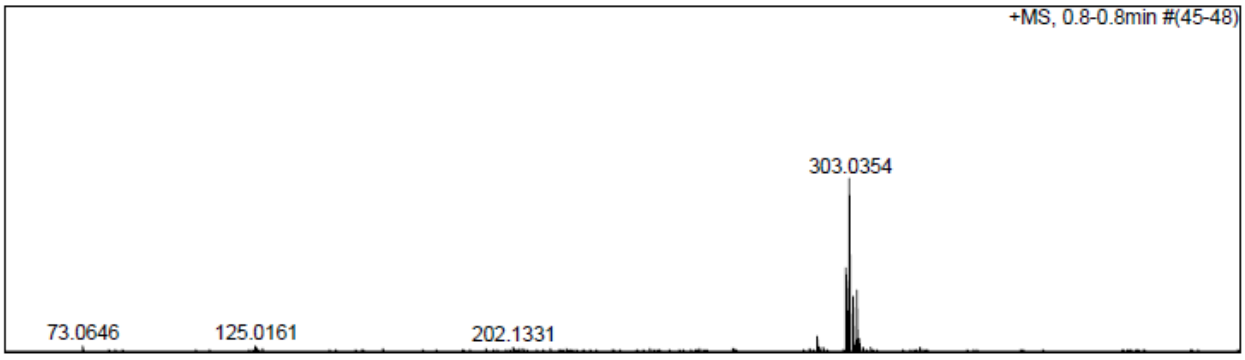
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
287.0650	2	C 15 H 12 F N 2 O S	100.00	287.0649	-0.1	-0.2	9.5	10.5	ok	even

2-{[(3-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45a)



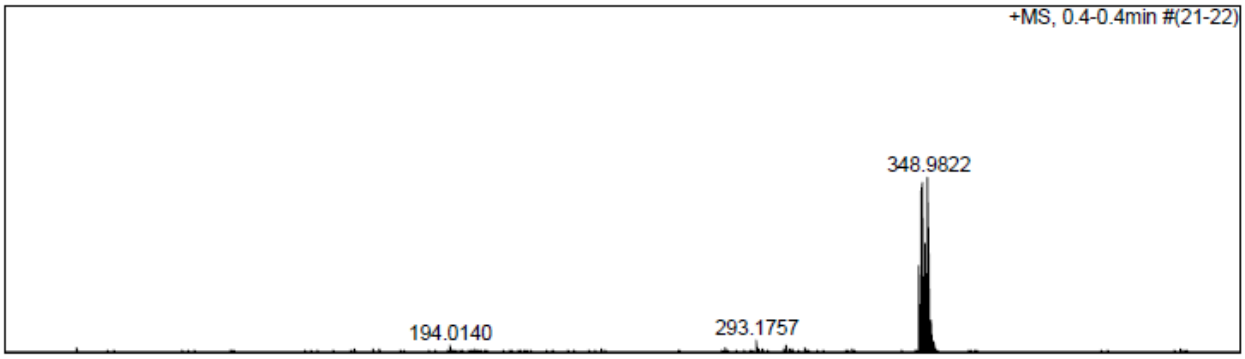
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
303.0346	2	C 15 H 12 Cl N 2 O S	100.00	303.0353	0.7	2.4	72.1	10.5	ok	even

2-{[(4-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45b)



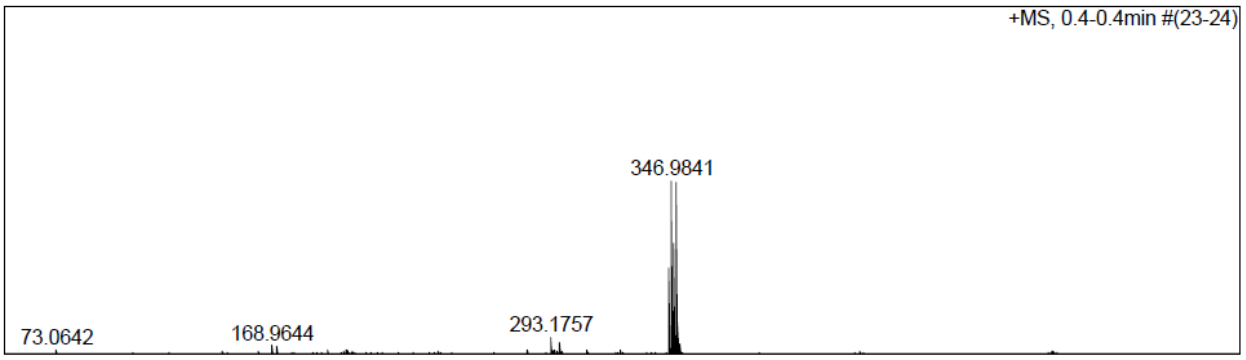
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
303.0354	2	C 15 H 12 Cl N 2 O S	100.00	303.0353	-0.0	-0.1	62.1	10.5	ok	even

2-{[(3-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46a)



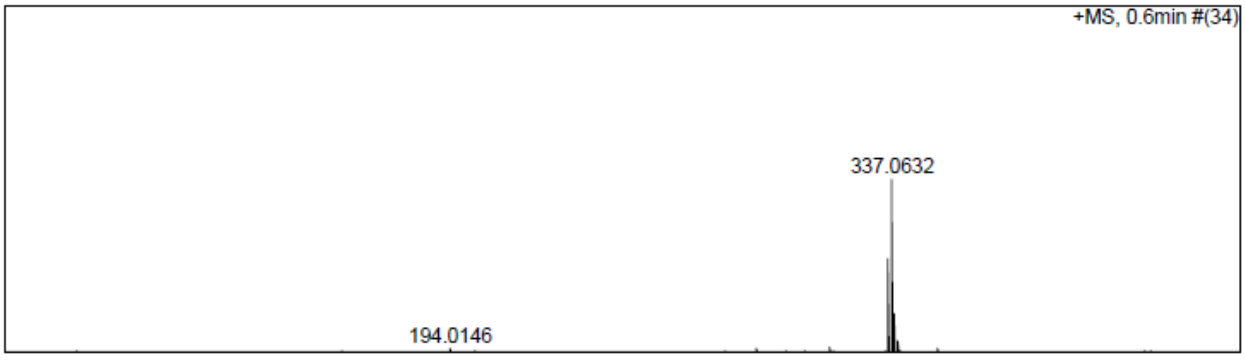
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
346.9839	1	C 15 H 12 Br N 2 O S	100.00	346.9848	0.9	2.7	185.0	10.5	ok	even

2-{[(4-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46b)



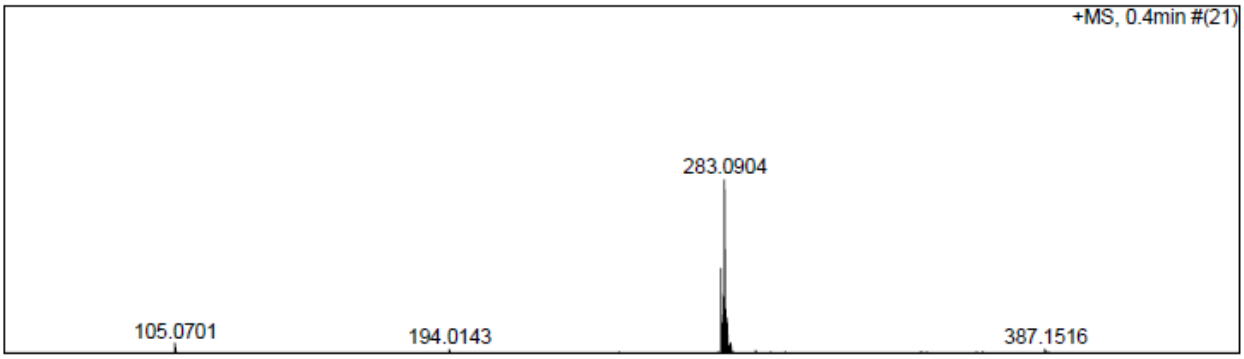
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
346.9841	1	C 15 H 12 Br N 2 O S	100.00	346.9848	0.7	2.0	192.1	10.5	ok	even

2-({[3-(trifluoromethyl)phenyl]methyl}amino)-4H-3,1-benzothiazin-4-one (47)



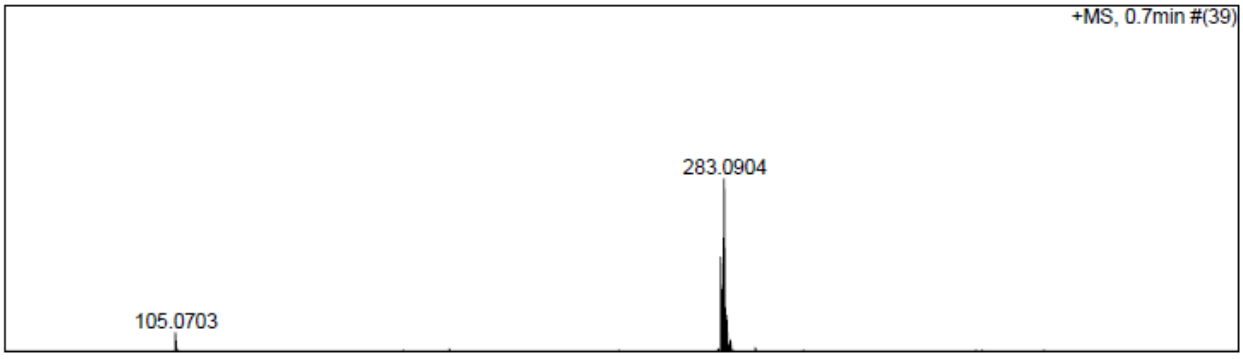
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
337.0632	1	C 16 H 12 F 3 N 2 O S	100.00	337.0617	-1.5	-4.5	19.1	10.5	ok	even

2-{[(3-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48a)



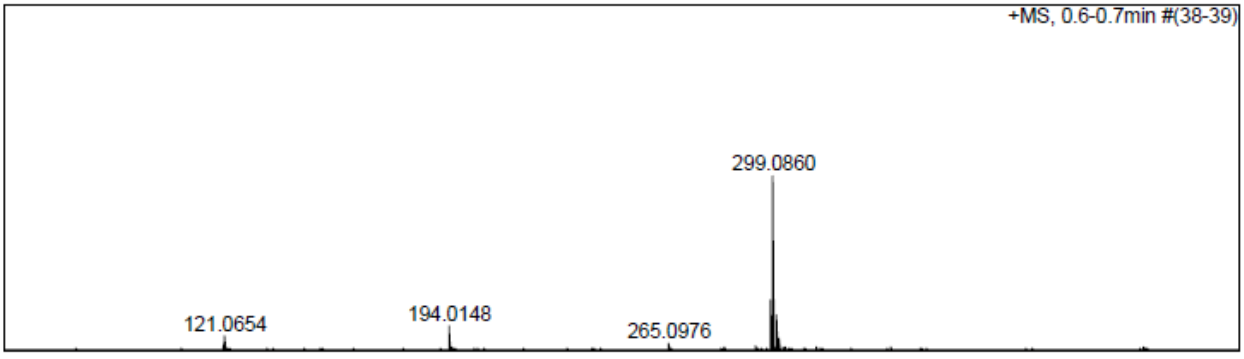
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
283.0904	1	C 16 H 15 N 2 O S	100.00	283.0900	-0.4	-1.5	6.9	10.5	ok	even

2-{[(4-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48b)



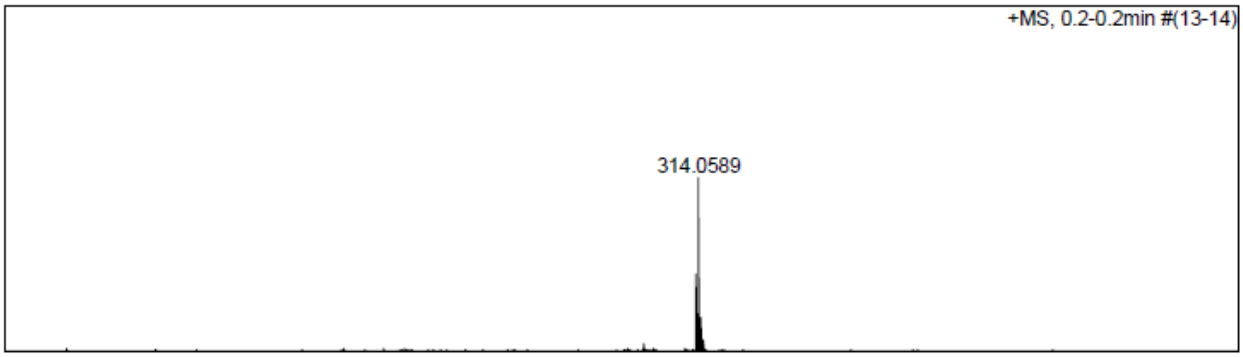
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
283.0904	1	C 16 H 15 N 2 O S	100.00	283.0900	-0.4	-1.4	12.0	10.5	ok	even

2-{[(3-methoxyphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (49)



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
299.0860	1	C 16 H 15 N 2 O 2 S	100.00	299.0849	-1.2	-3.9	4.7	10.5	ok	even

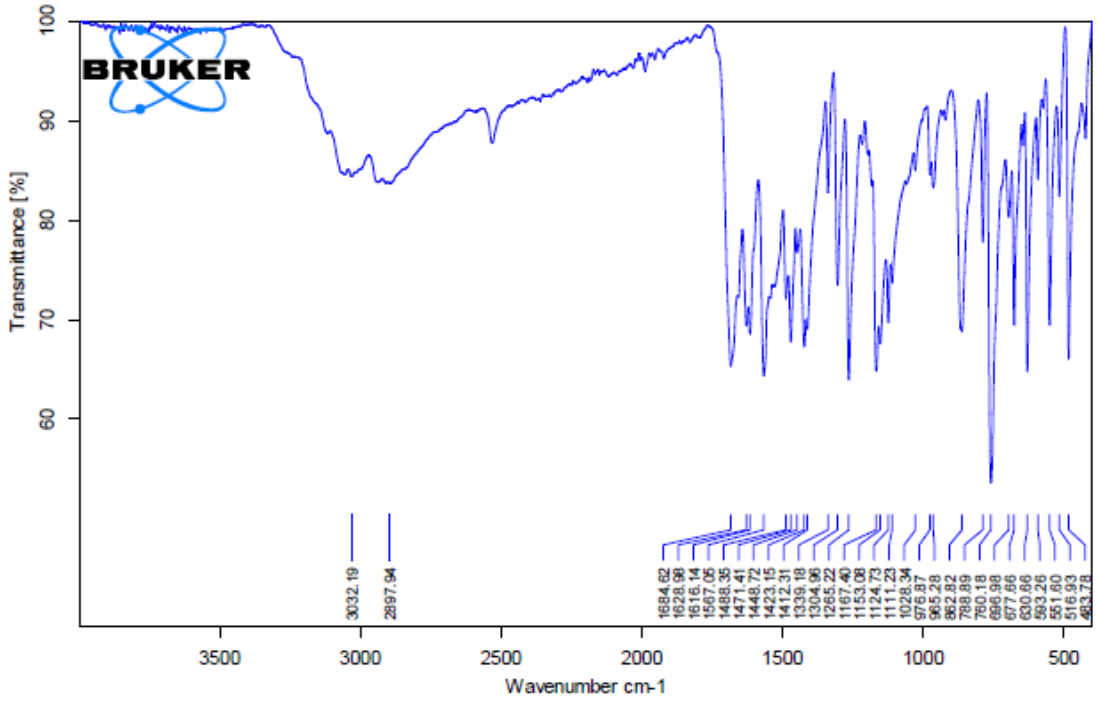
2-{[(4-nitrophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (50)



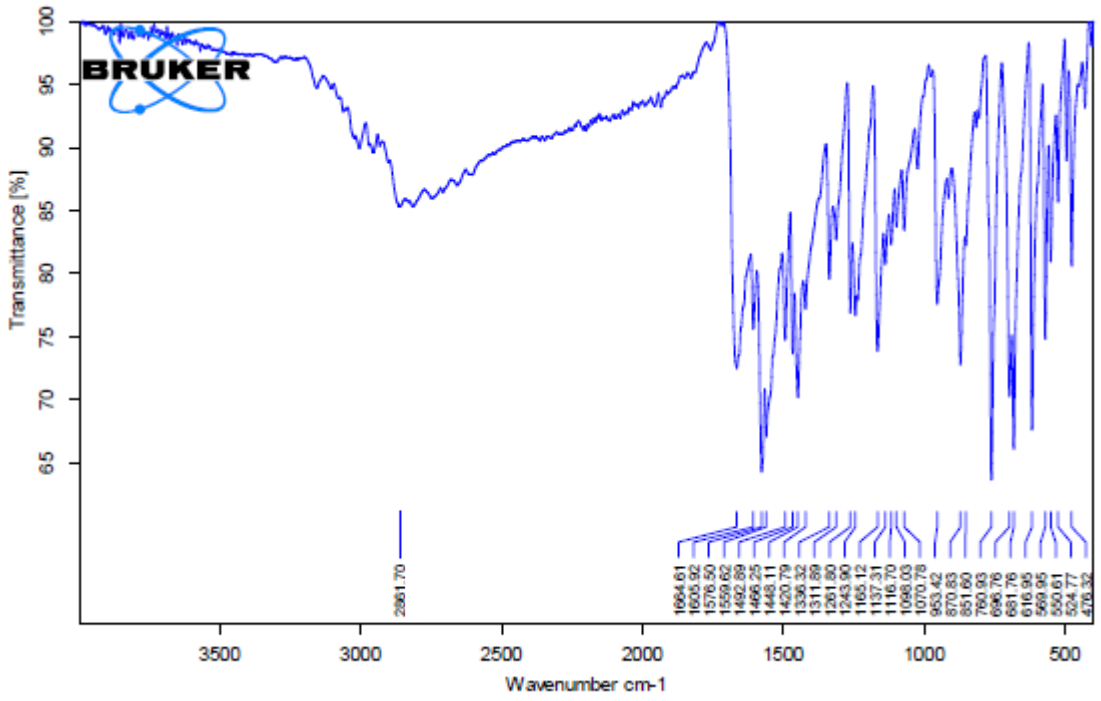
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
314.0589	1	C 15 H 12 N 3 O 3 S	100.00	314.0594	0.5	1.5	6.3	11.5	ok	even

ANNEXURE C IR DATA

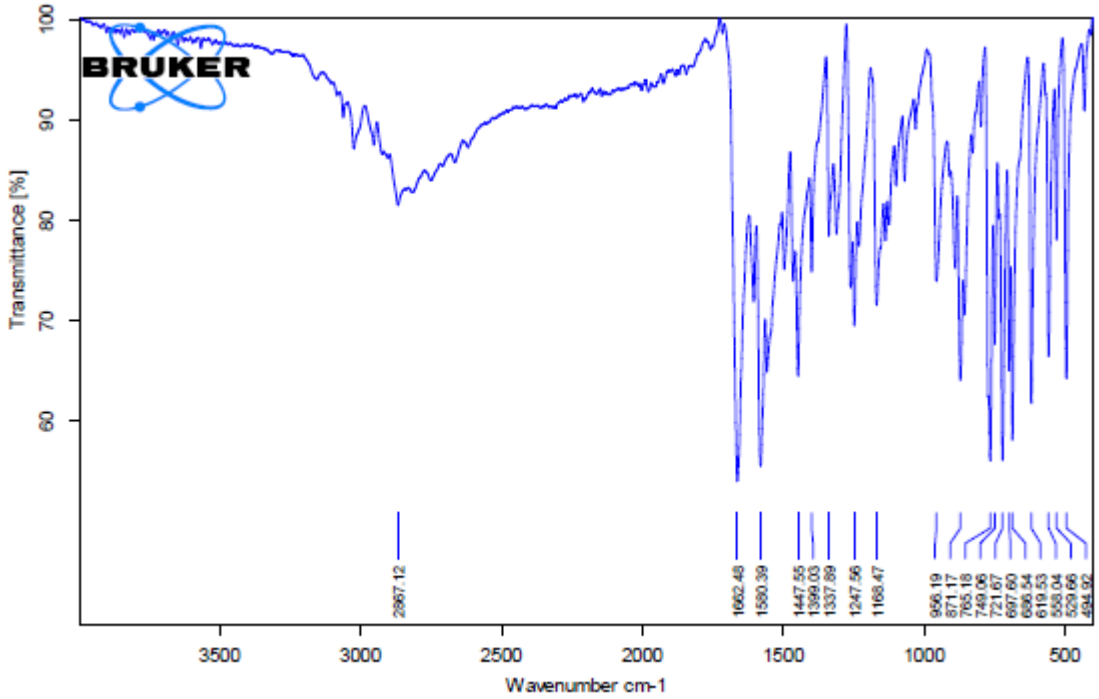
2-aminobenzothiazinone (3)



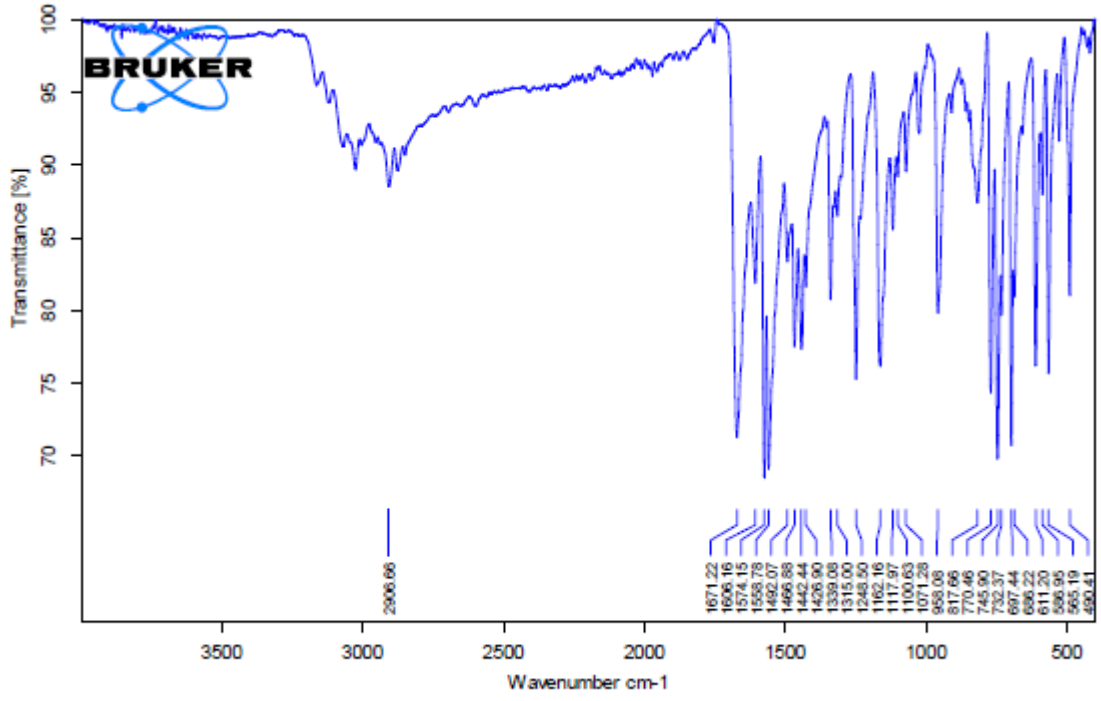
2-(benzylamino)-4H-3,1-benzothiazin-4-one (41)



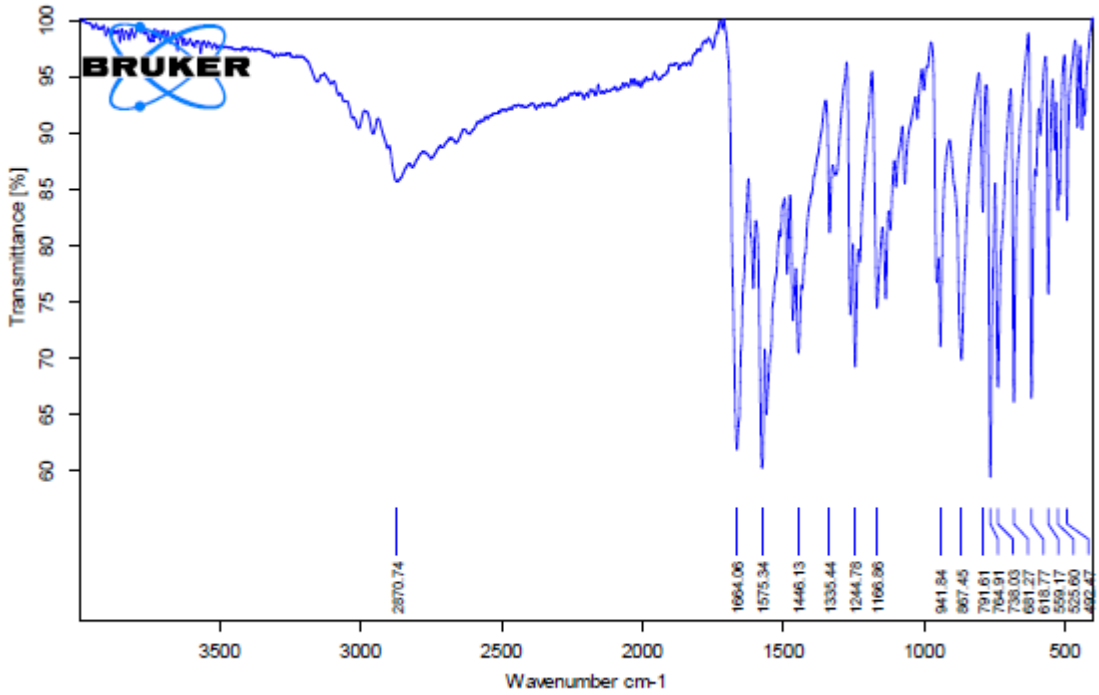
2-[(2-phenylethyl)amino]-4H-3,1-benzothiazin-4-one (42)



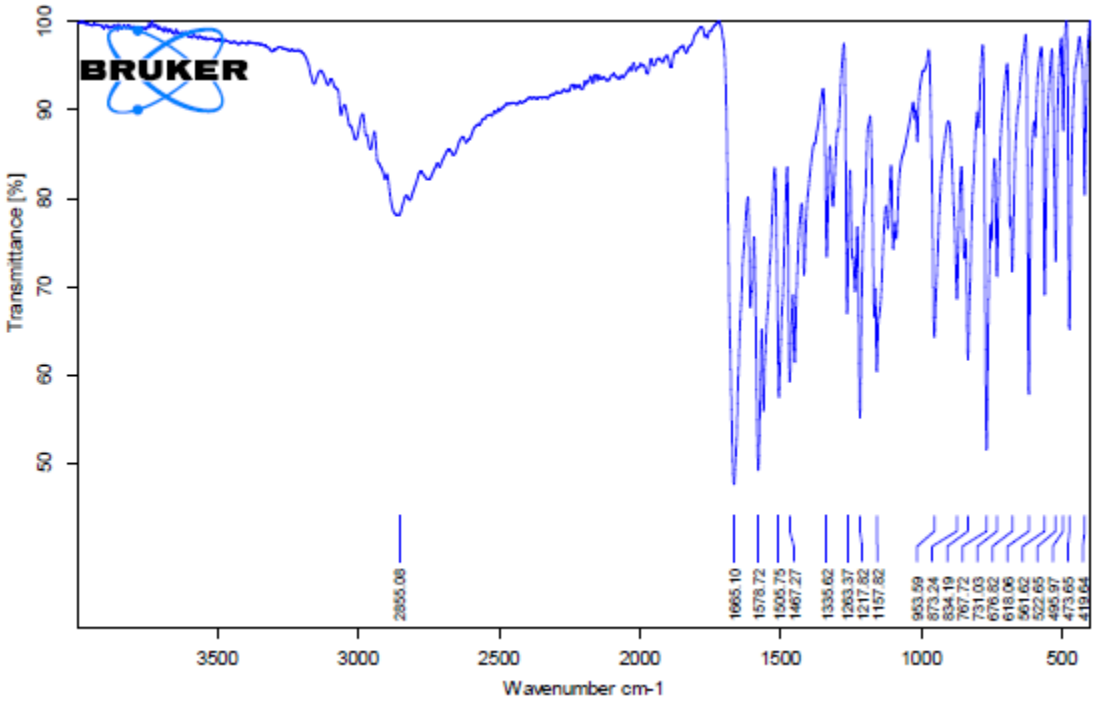
2-[(3-phenylpropyl)amino]-4H-3,1-benzothiazin-4-one (43)



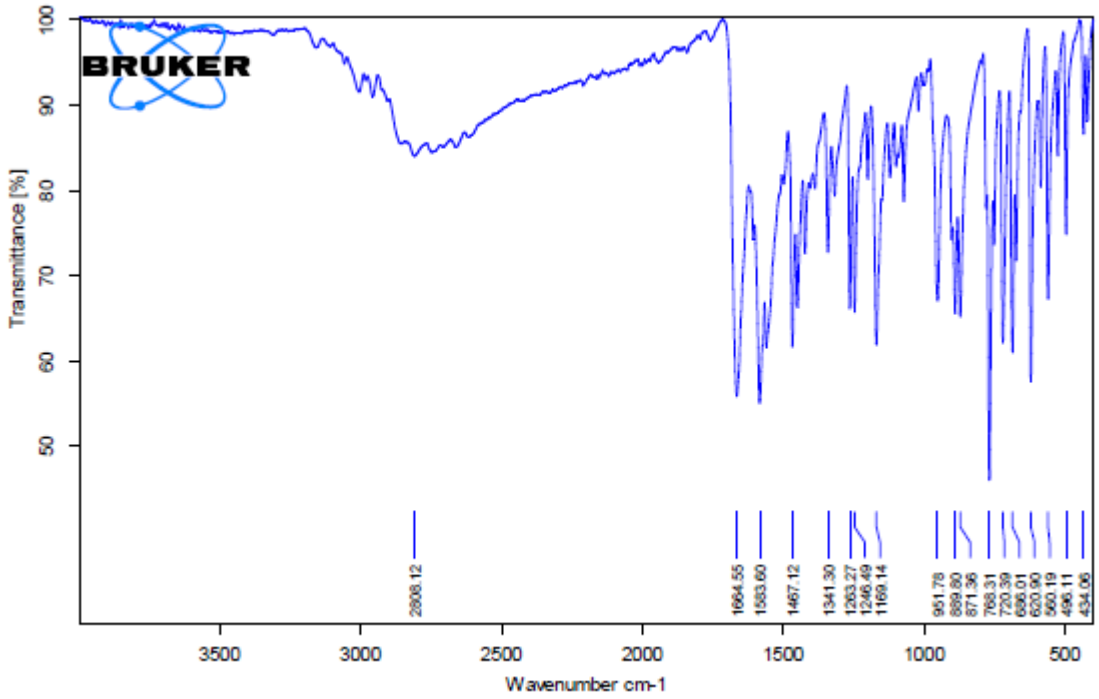
2-{[(3-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44a)



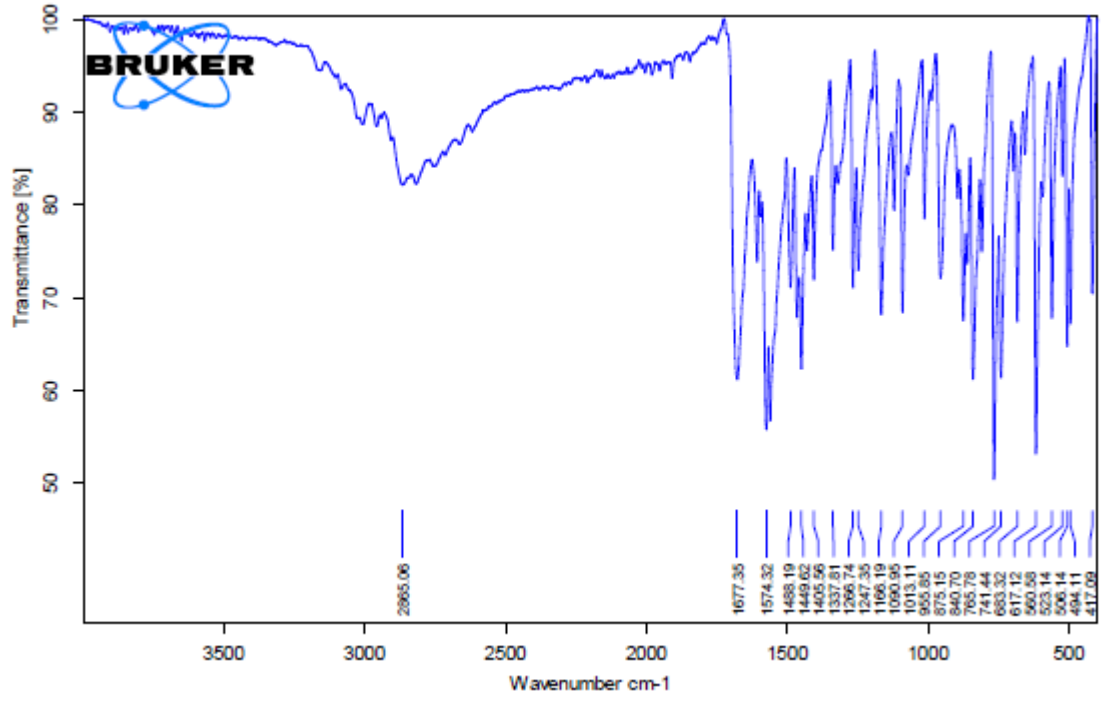
2-{[(4-fluorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (44b)



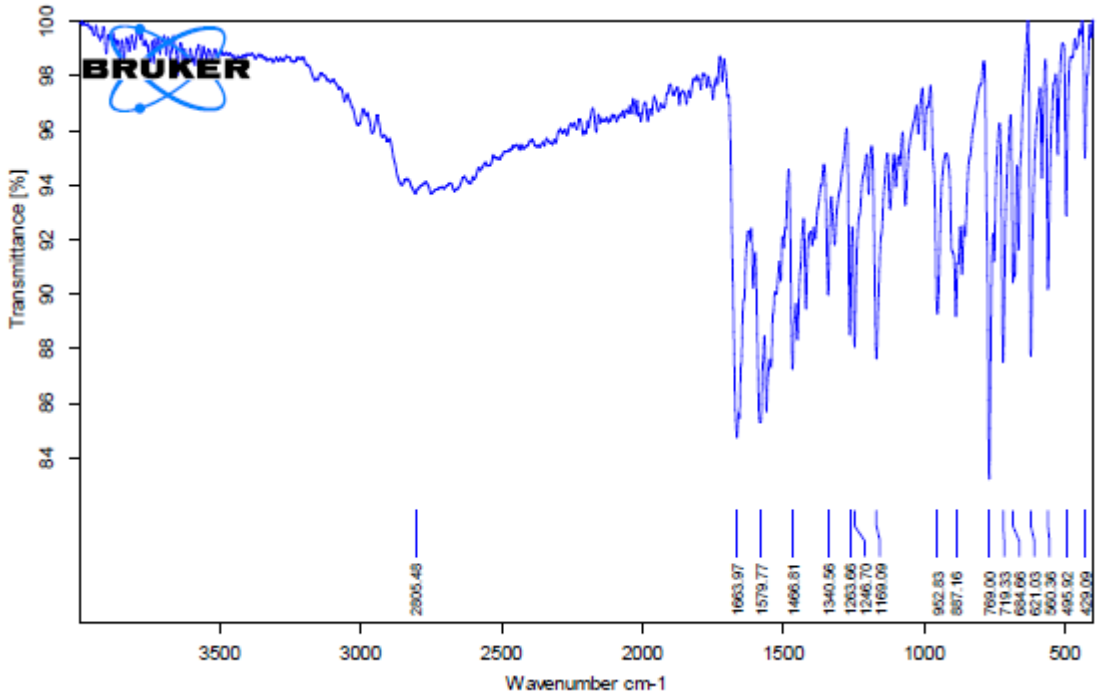
2-{[(3-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45a)



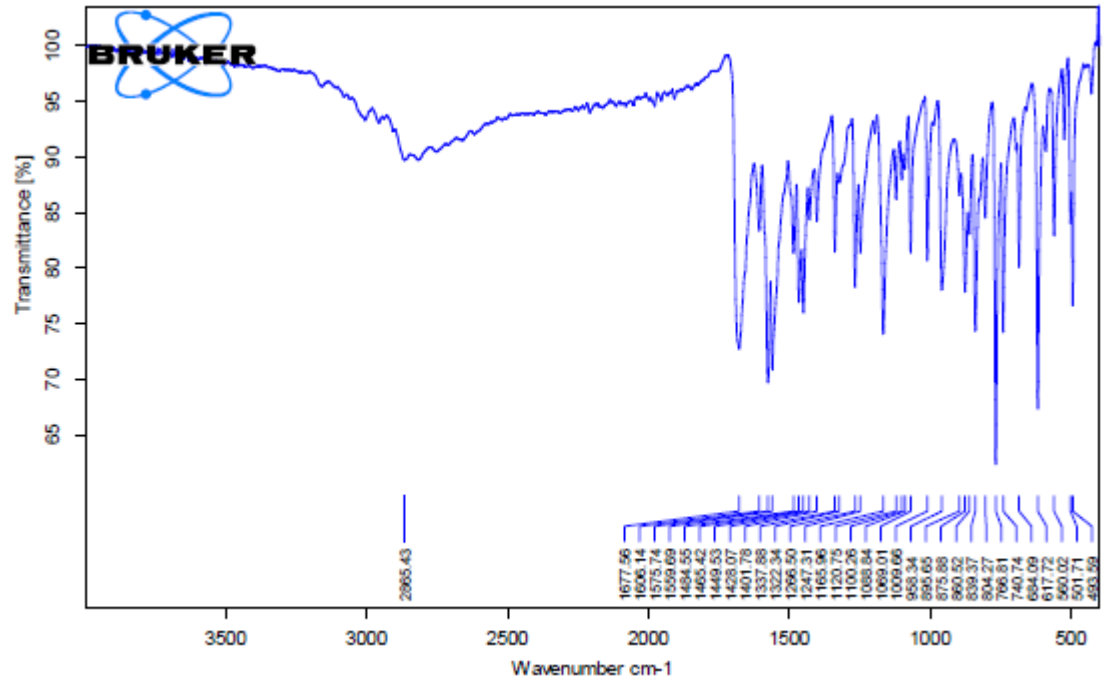
2-{[(4-chlorophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (45b)



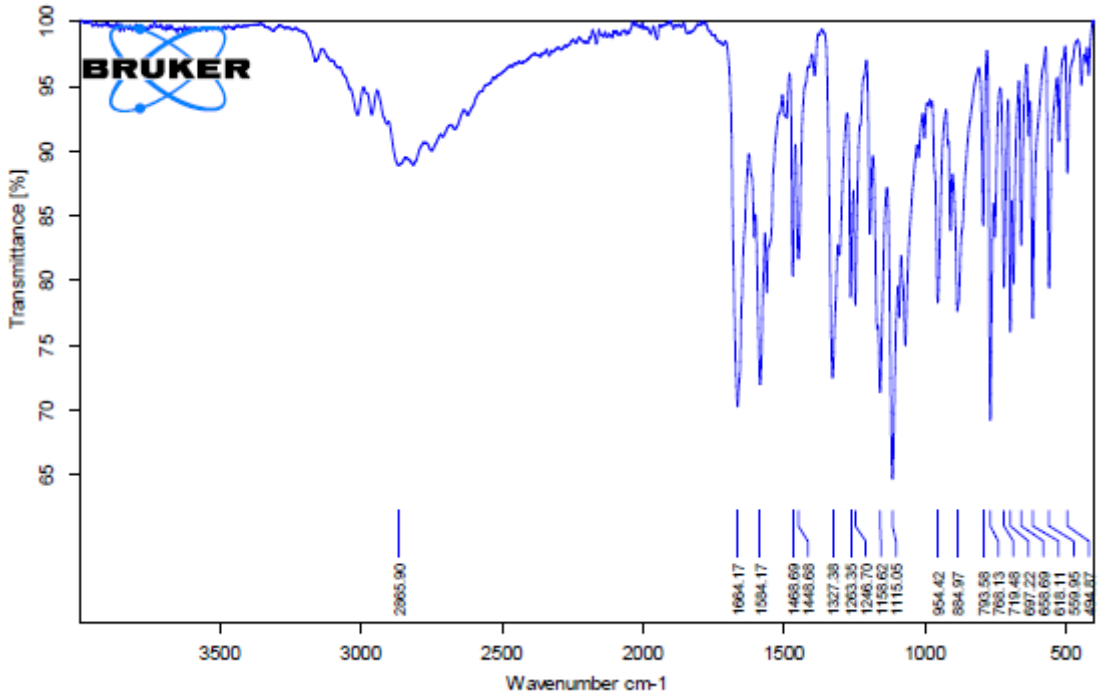
2-{[(3-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46a)



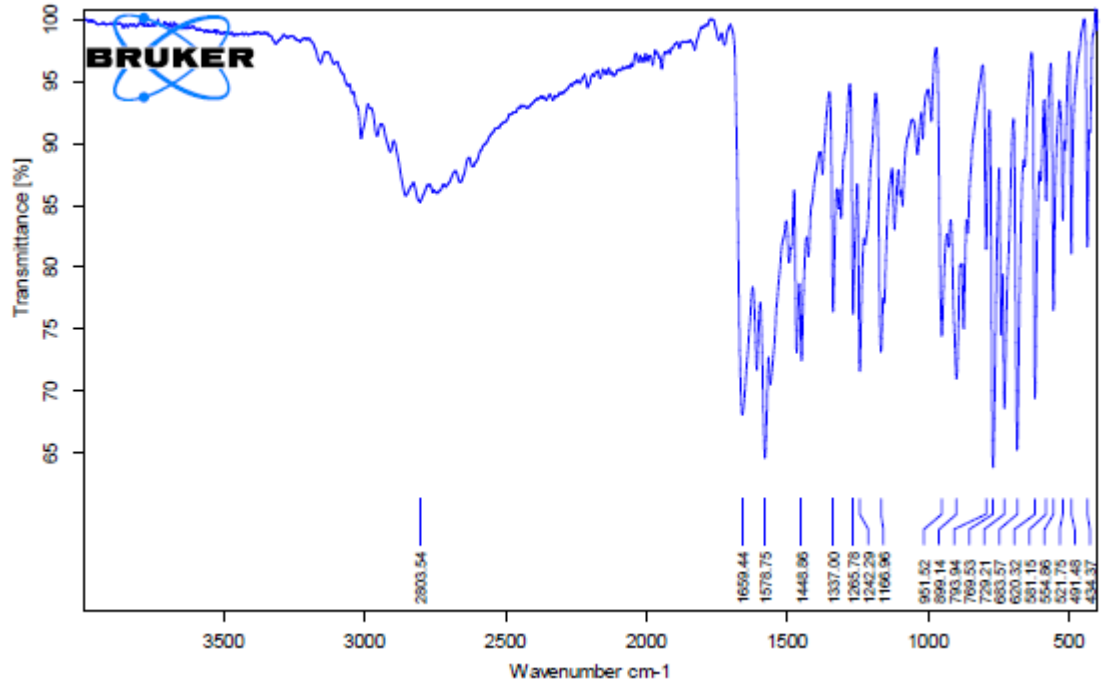
2-{[(4-bromophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (46b)



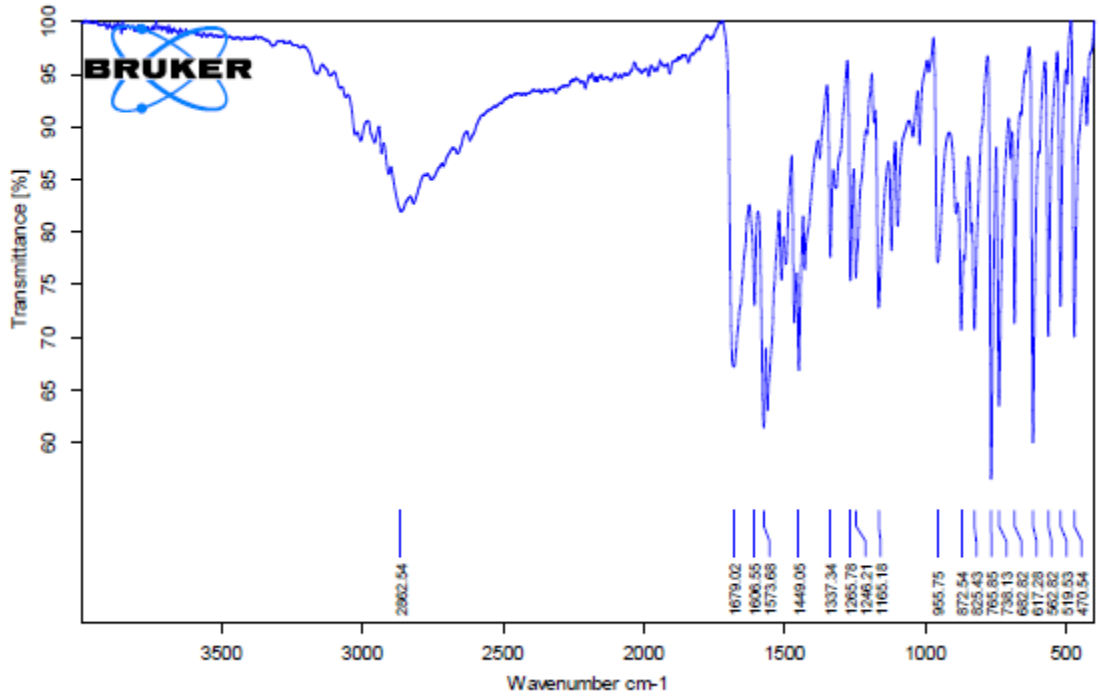
2-({[3-(trifluoromethyl)phenyl]methyl}amino)-4H-3,1-benzothiazin-4-one (47)



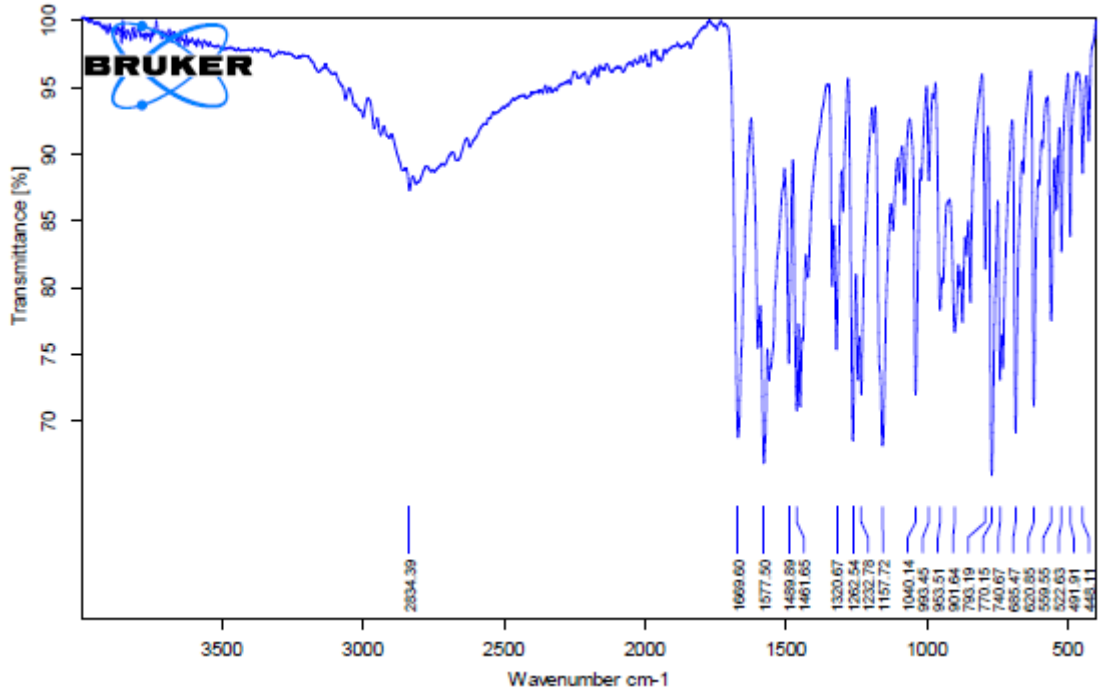
2-{[(3-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48a)



2-{[(4-methylphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (48b)



2-{[(3-methoxyphenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (49)



2-{[(4-nitrophenyl)methyl]amino}-4H-3,1-benzothiazin-4-one (50)