Affinity of dihydropyrimidone analogues for adenosine A_1 and A_{2A} receptors

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B.Pharm

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ABBREVIATIONS

[^{3}H]DPCPX	1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine
[^{3}H]NECA	[^{3}H]5'N-ethylcarboxamide-adenosine
[^{3}H]-R-PIA	[^{3}H]N^6-(R)-phenylisopropyladenosine
1,4-DHP	1,4-Dihydropyridines
δ	Delta scale indicating chemical shift
AD	Alzheimer's disease
ANR-94	8-ethoxy-9-ethyladenine
ATP	Adenosine- triphosphate
cAMP	Cyclic adenosine-monophosphate
BBB	Blood-brain barrier
br d	broad doublet
br s	broad singlet
br t	broad triplet
$CDCl_3$-*d*	Deuterochloroform
CNS	Central nervous system
COMT	Catechol-*O*-methyl-transferase
CPA	N^6-cyclopentyladenosine
CPM	Counts per minute
CSC	8-(3-chlorostyryl)-caffeine
d	doublet
DA	Dopamine
dd	doublet of doublets
DMF	Dimethylformamide
DMPX	3,7-Dimethyl-1-propargylxanthine
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterodimethyl sulfoxide
DYN	Dynorphin

EI	Electron ionization
EIMS	Electron impact ionization
ENK	Enkephalin
GABA	γ-Aminobutyric acid
G_i	Inhibitory G-protein
Glu	Glutamic acid
GPe	Globus pallidus externa
G_s	Stimulatory G-protein
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectra
IC_{50}	Half maximal inhibitory concentration
IR	Infrared spectroscopy
J	coupling constant
K_d	Dissociation constant
K_i	Inhibition constant
KW6002	Istradefylline
LBs	Lewy bodies
L-dopa	Levodopa
m	multiplet
mGlu5	Metabotropic glutamate
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase isoform B
mp	Melting point
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridinium ion
MPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPP^+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
MS	Mass spectrometry
MSX-2	3-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-propargylxanthine

NMDA	*N*-methyl-*D*-aspartate
NMR	Nuclear magnetic resonance
PD	Parkinson's disease
Phe	Phenylalanine
ppm	Parts per million
q	Quartet
qn	Quintet
ROS	Reactive oxygen species
s	singlet
SD	Standard deviation
SEM	Standard error of the mean
SNc	Substantia nigra pars compacta
SNr/GPi	Substantia nigra pars reticulate/globus pars interna
STN	Subthalamic nucleus
t	triplet
TBAB	Terta butyl ammonium bromide
TLC	Thin layer chromatography

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder that is characterised by a reduction of dopamine concentration in the striatum due to degeneration of dopaminergic neurons in the substantia nigra. Currently, first line treatment of PD includes the use of dopamine precursors, dopamine agonists and inhibitors of enzymatic degradation of dopamine, in an effort to restore dopamine levels and/or its effects. However, all these therapeutic strategies are only symptomatic and unfortunately do not slow, stop or reverse the progression of PD.

From the discovery of adenosine A_{2A} receptor-dopamine D_2 receptor heteromers and the antagonistic interaction between these receptors, the basis of a new therapeutic approach towards the treatment of PD emerged. Adenosine A_{2A} receptor antagonists have been shown to decrease the motor symptoms associated with PD, and are also potentially neuroprotective. The possibility thus exists that the administration of an adenosine A_{2A} antagonist may prevent further neurodegeneration. Furthermore, the antagonism of adenosine A_1 receptors has the potential of treating cognitive deficits such as those associated with Alzheimer's disease and PD. Therefore, dual antagonism of adenosine A_1 and A_{2A} receptors would be of great benefit since this would potentially treat both the motor as well as the cognitive impairment associated with PD.

The affinities (K_i-values between 0.6 mM and 38 mM) of a series of 1,4-dihydropyridine derivatives were previously illustrated for the adenosine A_1, A_{2A} and A_3 receptor subtypes by Van Rhee and co-workers (1996). These results prompted this pilot study, which aimed to investigate the potential of the structurally related 3,4-dihydropyrimidin-2(1H)-ones (dihydropyrimidones) and 2-amino-1,4-dihydropyrimidines as adenosine A_1 and A_{2A} antagonists.

In this pilot study, a series of 3,4-dihydropyrimidones and 2-amino-1,4-dihydropyrimidines were synthesised and evaluated as adenosine A_1 and A_{2A} antagonists. Since several adenosine A_{2A} antagonists also exhibit MAO inhibitory activity, the MAO-inhibitory activity of selected derivatives was also assessed. A modified Biginelli one pot synthesis was used for the preparation of both series of compounds under solvent free conditions. A mixture of a β-diketone, aldehyde and urea/guanidine hydrochloride was heated for an appropriate time to afford the desired compounds in good yields. MAO-B inhibition studies comprised of a fluorometric assay where kynuramine was used as substrate. A radioligand binding protocol described in literature was employed to investigate the binding of the compounds to the

adenosine A_{2A} and A_1 receptors. The displacement of N-[^{3}H]ethyladenosin-5'-uronamide ([^{3}H]NECA) from rat striatal membranes and 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) from rat whole brain membranes, was used in the determination of A_{2A} and A_1 affinity, respectively.

The results showed that both 3,4-dihydropyrimidones and 2-amino-1,4-dihydropyrimidines had weak adenosine A_{2A} affinity, with the *p*-fluorophenyl substituted dihydropyrimidone derivative (**1h**) in series 1, exhibiting the highest affinity for the adenosine A_{2A} receptor (28.7 µM), followed by the *p*-chlorophenyl dihydropyrimidine derivative (**2c**) in series 2 (38.59 µM). Both series showed more promising adenosine A_1 receptor affinity in the low micromolar range. The *p*-bromophenyl substituted derivatives in both series showed the best affinity for the adenosine A_1 receptor with K_i-values of 7.39 µM (**1b**) and 7.9 µM (**2b**). The *p*-methoxyphenyl dihydropyrimidone (**1d**) and *p*-methylpneyl dihydropyrimidine (**2e**) derivatives also exhibited reasonable affinity for the adenosine A_1 receptor with K_i-values of 8.53 µM and 9.67 µM, respectively. Neither the 3,4-dihydropyrimidones nor the 2-amino-1,4-dihydropyrimidines showed MAO-B inhibitory activity.

Comparison of the adenosine A_{2A} affinity of the most potent derivative (**1h**, K_i = 28.7 µM) from this study with that of the previously synthesised dihydropyridine derivatives (Van Rhee *et al.,* 1996, most potent compound had a K_i = 2.74 mM) reveals that an approximate 100-fold increase in binding affinity for A_{2A} receptors occurred. However, KW6002, a known A_{2A} antagonist, that has already reached clinical trials, has a K_i-value of 7.49 nM. The same trend was observed for adenosine A_1 affinity, where the most potent compound (**1b**) of this study exhibited a K_i-value of 7.39 µM compared to 2.75 mM determined for the most potent dihydropyridine derivatives (Van Rhee *et al.*, 1996). N^6-cyclopentyladenosine (CPA), a known adenosine A_1 agonist that was used as a reference compound, however had a K_i-value of 10.4 nM. The increase in both adenosine A_1 and A_{2A} affinity can most likely be ascribed to the increase in nitrogens in the heterocyclic ring (from a dihydropyridine to a dihydropyrimidine) since similar results were obtained by Gillespie and co-workers in 2009 for a series of pyridine and pyrimidine derivatives. In their case it was found that increasing the number of nitrogens in the heterocyclic ring (from one to two nitrogen atoms for the pyridine and pyrimidine derivatives respectively) increased affinity for the adenosine A_{2A} and adenosine A_1 receptor subtypes, while three nitrogen atoms in the ring (triazine derivatives) were associated with decreased affinity. It thus appears that two nitrogen atoms in the ring (pyrimidine) are required for optimum adenosine A_1 and A_{2A} receptor affinity.

The poor adenosine A_{2A} affinity exhibited by the compounds of this study can probably be attributed to the absence of an aromatic heterocyclic ring. The amino acid, Phe-168 plays a very important role in the binding site of the A_{2A} receptor, where it forms aromatic π-π-stacking interactions with the heterocyclic aromatic ring systems of known agonists and antagonists. Since the dihydropyrimidine ring in both series of this pilot study was not aromatic, the formation of aromatic π-π-stacking interactions with Phe-168 is unlikely.

In conclusion, the 3,4-dihydropyrimidone and 2-amino-1,4-dihydropyrimidine scaffolds can be used as a lead for the design of novel adenosine A_1 and A_{2A} antagonists, although further structural modifications are required before a clinically viable candidate will be available as potential treatment of PD.

Keywords: Parkinson's disease, 3,4-dihydropyrimidin-2(1H)-ones, 2-amino-1,4-dihydropyrimidines, adenosine A_1 antagonist, adenosine A_{2A} antagonist, monoamine oxidase B.

OPSOMMING

Parkinson se siekte (PS) is ‘n neurodegeneratiewe siektetoestand wat gekenmerk word deur ‘n verlaagde dopamienkonsentrasie in die striatum as gevolg van die degenerasie van dopaminergiese neurone in die substantia nigra. Huidige behandeling van PS is gemik op die herstel van dopamienvlakke en/of -effekte en sluit die gebruik van dopamienvoorlopers, dopamienagoniste en inhibeerders van die ensiematiese afbraak van dopamien as eerstelinie behandeling in. Hierdie behandelingstrategieë verskaf egter slegs simptomatiese verligting, maar vertraag of stop nie die siekteverloop nie.

Die ontdekking dat adenosien A_{2A}- en dopamien D_2-reseptore heteromere vorm en verder dat daar ’n antagonistiese interaksie tussen die twee reseptore is, het die grondslag gelê vir ‘n nuwe benadering tot die behandeling van PS. Bo- en behalwe die gevolg dat adenosien A_{2A}-reseptorantagoniste die motoriese simptome wat met PS geassosieer word verminder, is hulle ook potensieel neurobeskermend, en die moontlikheid bestaan dus dat verdere neurodegenerasie voorkom kan word deur die toediening van ‘n adenosien A_{2A}-antagonis. Verder kan die antagonisme van adenosien A_1-reseptore moontlik die kognitiewe defekte wat geassosieer word met Alzheimer- en Parkinson se siekte verlig. Dualistiese antagonisme van adenosien A_1- en A_{2A}-reseptore kan dus van groot waarde wees, aangesien dit die moontlikheid bied om beide die motoriese- sowel as die kognitiewe verswakking, wat geassosieer word met PS, te behandel.

Die affiniteit (K_i waardes tussen 0.6 mM en 38 mM) van ‘n reeks 1,4-dihidropiridienderivate vir die A_1-, A_{2A}- en A_3-adenosienreseptorsubtipes is in 1996 deur Van Rhee en medewerkers geïllustreer. Bogenoemde resultate het tot dié loodsstudie gelei waar daar gepoog is om die potensiaal van die struktuurverwante 3,4-dihidropirimidien-2(1H)-one (dihidropirimidone) en 2-amino-1,4-dihidropirimidiene as moontlike adenosien A_1- en A_{2A}-antagoniste te ondersoek.

In hierdie loodsstudie is ‘n reeks 3,4-dihidropirimidone en 2-amino-1,4-dihidropirimidiene gesintetiseer en as adenosien A_1- and A_{2A}-antagoniste geëvalueer. Aangesien MAO-inhiberende effekte ook voorheen vir verskeie adenosien A_{2A}-antagoniste aangetoon is, is geselekteerde verbindings ook as MAO-inhibeerders geëvalueer. ‘n Gemodifiseerde Biginelli-eenpotsintese, in ‘n oplosmiddelvrye omgewing, is gebruik om albei reekse te sintetiseer. In hierdie reaksie is ‘n β-diketoon, aldehied en ureum/tioureum hidrochloried gemeng en vir ‘n geskikte tyd verhit om die gewenste verbindings met goeie opbrengste te lewer. MAO-B remming-studies behels ‘n fluorometriese toets waar kinuramien as substraat gebruik word om die ensiemaktiwiet te bepaal. ‘n Radioligandbindingsprotokol, soos in die

literatuur beskryf, is gebruik om die binding van die toetsverbindings aan die adenosien A_1- en A_{2A}-reseptore te ondersoek. Verplasing van N-[^{3}H]etieladenosien-5'-uronamied ([^{3}H]NECA) uit rot striatale membrane en 1,3-[^{3}H]-dipropiel-8-siklopentielxantien ([^{3}H]DPCPX) uit rot volbreinmembrane is onderskeidelik gebruik om adenosien A_{2A}- en A_1-binding te bepaal.

Die resultate het getoon dat beide die 3,4-dihidropirimidone en 2-amino-1,4-dihidropirimidine swak affinteit vir die adenosien A_{2A}-reseptor het. Die *p*-fluoorfeniel-gesubstitueerde dihidropirimidoon (**1h**) in reeks 1 het die beste affiniteit vir die adenosien A_{2A}-reseptor (28.7 µM) getoon, terwyl die *p*-chloorfeniel-dihidropirimidien (**2c**) in reeks 2 (38.59 µM) die tweede beste affiniteit gehad het. Albei reekse het beter adenosien A_1-affiniteit getoon met K_i-waardes in die lae mikromolaar gebied. Redelike adenosien A_1-affiniteitswaardes van 7.39 µM (**1b**) en 7.9 µM (**2b**) is byvoorbeeld vir die *p*-broomfeniel-gesubstitueerde derivate van albei reeks bepaal. Die *p*-metoksiefeniel-dihidropirimidoon (**1d**) en die *p*-metielfeniel-dihidropirimidien (**2e**) het ook matige adenosien A_1-bindingsaffiniteit getoon met waardes van 8.53 µM en 9.67 µM onderskeidelik. Nie die 3,4-dihidropirimidone of die 2-amino-1,4-dihidropirimidiene het MAO-B inhibisie getoon nie.

Wanneer die adenosien A_{2A}-affiniteit van die mees potente verbinding, (**1h**, K_i = 28.7 µM), vergelyk word met dié van die dihidropiridienderivate wat voorheen bestudeer is (Van Rhee *et al.*, 1996, K_i van mees potente verbinding = 2.74 mM) blyk dit dat die affiniteit ongeveer 100 maal verbeter het. KW6002, ‘n bekende adenosien A_{2A}-antagonis, wat reeds kliniese toetsing bereik het, het egter ‘n K_i-waarde van 7.49 nM. Dieselfde tendens is waargeneem vir adenosien A_1-affiniteit, waar ‘n K_i van 7.39 µM. bepaal is vir die die mees potente verbinding (**1b**) van hierdie studie teenoor dié van Van Rhee en medewerkers (1996) wat ‘n K_i-waarde van 2.75 mM getoon het. CPA, ‘n bekende adenosien A_1-agonis wat as verwysing gebruik is, het egter ‘n K_i-waarde van 10.4 nM. Die verhoging in beide adenosien A_1- and A_{2A}-affiniteit kan waarskynlik aan die vermeerdering van stikstofatome in die heterosikliese ring (van dihidropiridien na dihidropirimidien)toegeskryf word, aangesien soortgelyke resultate verkry is deur Gillespie en medewerkers (2009) vir ‘n reeks piridien- en pirimidienderivate. In dié geval het die vermeerdering van die aantal stikstowwe in die heterosikliese ring (vanaf een na twee vir die piridien- en pirimidienderivate, onderskeidelik) ook tot verhoogde adenosien A_1- en A_{2A}-affiniteit gelei, terwyl drie stikstofatome in die ring (triasienderivate) weer ‘n verlies aan affiniteit tot gevolg gehad het. Dit blyk dus dat die teenwoordigheid van twee stikstofatome in die ring (pirimidienderivate) optimale adenosien A_1- en A_{2A}-affiniteit veroorsaak.

Die swak adenosien A_{2A}-affiniteit van die verbindings in hierdie studie kan waarskynlik toegeskryf word aan die afwesigheid van ‘n aromatiese heterosikliese ring. Die aminosuur, Phe 168, speel ‘n belangrike rol in die A_{2A}-reseptorbindingsetel, waar dit gewoonlik aromatiese π-π-stapelingsinteraksies met die aromatiese heterosikliese ringstelsels van bekende agoniste en antagoniste vorm. Aangesien die dihidropirimidienring in albei die reekse van hierdie loodsstudie nie aromaties is nie, is die vorming van aromatiese π-π-stapelingsinteraksies met Phe-168 onwaarskynlik.

Die gevolgtrekking kan dus gemaak word dat die 3,4-dihidropirimidoon- en 2-amino-1,4-dihidropirimidien-kernstrukture as leidraadverbindings vir die ontwikkeling van nuwe adenosien A_1- en A_{2A}-antagoniste gebruik kan word, alhoewel verdere strukturele veranderinge nodig is om die affiniteit te verbeter ten einde ‘n klinies lewensvatbare kandidaat vir die behandeling van PS te verkry.

Sleutelwoorde: Parkinson se siekte, 3,4-dihidropirimidien-2(1H)-one, 2-amino-1,4-dihidropirimidiene, adenosien A_1-antagonis, adenosien A_{2A}-antagonis, monoamienoksidase B.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Currently, the treatment of Parkinson's disease (PD) is focused on dopamine replacement strategies with levodopa (L-dopa), a precursor of dopamine, and dopamine agonist drugs. Although these strategies are highly effective in controlling the early stages of the disease, long-term treatment is associated with drug-related complications such as a loss of drug efficacy, the onset of dyskinesias and the occurrence of psychosis and depression. The inadequacies of dopamine replacement therapy have prompted the search for alternative drug targets. Signalling at adenosine receptors plays a role in several diseases (Moro *et al.*, 2006) and antagonism of the adenosine A_{2A} receptor holds promise as symptomatic treatment of PD. Evidence also suggests that adenosine A_{2A} antagonists may slow the course of PD by protecting against the underlying neurodegenerative processes and may further prevent the development of dyskinesias that are normally associated with long term L-dopa use. Since adenosine A_{2A} antagonists produce additive anti-PD when administered in combination with L-dopa, it allows for a reduction in L-dopa usage and a decrease in the occurrence of side effects. Adenosine A_{2A} antagonists are therefore a promising adjunctive to dopamine replacement therapy.

There are four different subtypes of adenosine receptors, A_1, A_{2A}, A_{2B} and A_3, of which the A_{2A} receptors are specifically localized in the striatum (Svenningsson *et al.*, 1999), where they are co-expressed with dopamine D_2 receptors. Adenosine A_1 receptors are found throughout the brain, including the hippocampus and prefrontal cortex, which are important areas for cognition. 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 antagonises 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} antagonists effectively reduce catalepsy and reverse locomotor activity suppressed by D_2 antagonists (Salamone *et al.*, 2008; Antoniou *et al.*, 2005; Correa *et al.*, 2004; Moo-Puc *et al.*, 2003; Malec, 1997). The rationale for adenosine A_{2A} receptor antagonists in the therapy of PD is thus based upon the co-localisation of adenosine A_{2A} receptors with D_2 receptors on the striatopallidal neurons, which is an instrumental tool to restoring motor behaviour (Ferre *et al.*, 1993).

Evidence from epidemiological studies indicates a strong inverse relationship between coffee drinking and a reduced risk of PD within many populations (Gale & Martyn, 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 other adenosine receptor antagonists have furthermore been shown to decrease the symptoms of PD (Schwarzchild *et al.,* 2002).

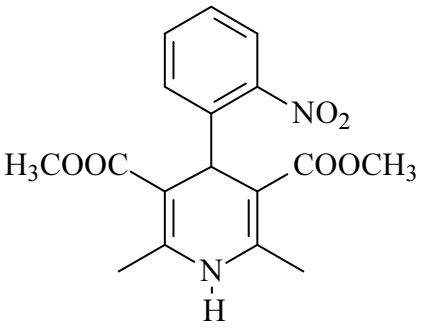
The effects evoked by caffeine include stimulatory actions on motor activity, alertness, attention, cognitive performance and reduced sleep. The cognitive effects of caffeine are mostly due to its ability to antagonise adenosine A_1 receptors in the hippocampus and prefrontal cortex (Ribeiro & Sebastião, 2010). Adenosine A_1 antagonists depolarize postsynaptic neurons and presynaptically enhance the release of a number of neurotransmitters, e.g. acetylcholine, glutamate, serotonin and norepinephrine. This release of neurotransmitters could find application in the treatment of cognitive deficits such as those associated with Alzheimer's disease (AD) and PD.

Jacobson and co-workers (1993) also found evidence of the existence of synergism between the motor-activating effects of adenosine A_1 and A_{2A} antagonists. Their results indicated that A_{2A} receptors are necessary, but not sufficient, for caffeine to produce motor activation, and that the role of adenosine A_1 receptors should thus not be discarded (Jacobson *et al.,* 1993; Nikodijevic *et al.*, 1991).

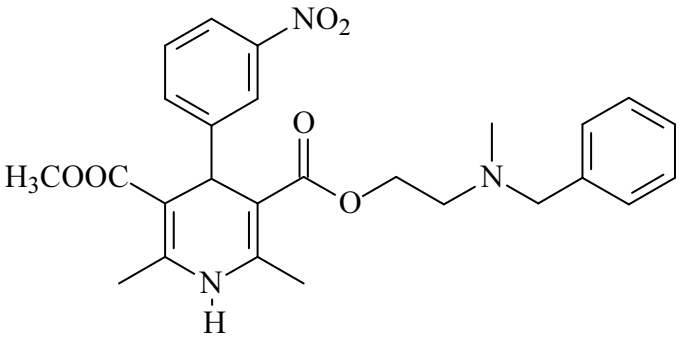
The dual antagonism of both adenosine A_1 and A_{2A} receptors therefore may be a solution for improving motor impairments, enhancing cognitive function via adenosine A_1 antagonism and also offer neuroprotection through adenosine A_{2A} antagonism.

1.2 **RATIONALE**

1,4-Dihydropyridines (1,4-DHP) have been developed as potent blockers and activators of L-type calcium channels and a number of these channel blockers, such as nifedipine and nicardipine, are used in the treatment of hypertension and coronary heart diseases (Borchard, 1994). By structural modification, it has been possible to synthesise 1,4-DHP which have affinity for other sites than Ca^{2+} channels, for example binding to α_{1a}-adrenergic receptors and to platelet activating factor receptors (Wetzel *et al.,* 1995; Sunkel *et al.,* 1990).

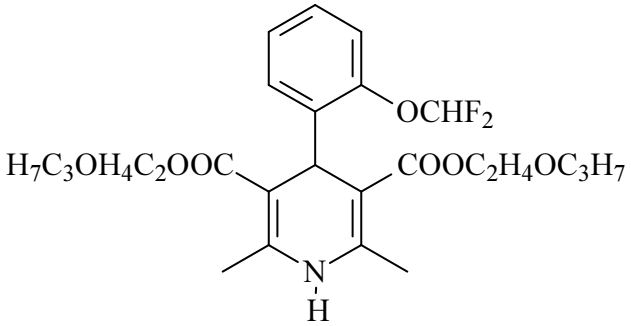


Nifedipine



Nicardipine

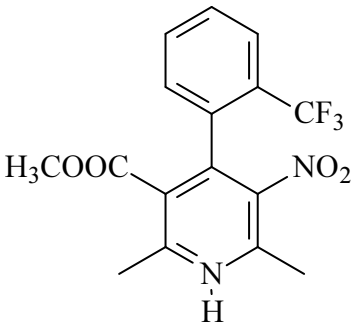
The neuroprotective properties of cerebrocrast, a novel atypical neuronal non-calcium antagonistic 1,4-DHP derivative, have been illustrated by its ability to protect cerebellar granule cells from 1-methyl-4-phenylpyridinium (MPP^+) -induced neuronal death (Klimaviciusa *et al.,* 2007). Other studies in animal models of PD have also identified other dihydropyridine calcium channel blockers (amlodipine, nifedipine, felodipine, isradipine, nimodipine) as having potential neuroprotective effects that may be relevant to reducing continued neurodegeneration (Surmeier, 2007; Kupsch *et al*., 1996; Kupsch *et al*., 1995).



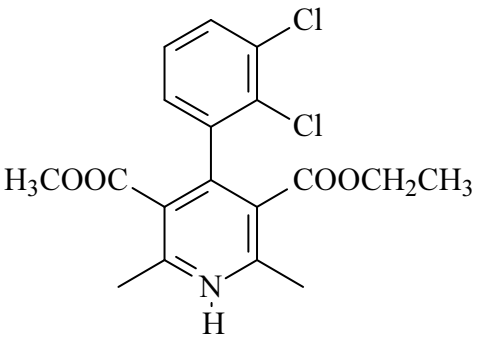
Cerebrocrast

Several dihydropyridines were also found to have affinity for adenosine A_1 receptors in the rat brain. Hu and co-workers (1987) confirmed the finding that the dihydropyridine calcium channel agonist BayK 8644 is able to displace the radioligand [^{3}H]-R-PIA ([^{3}H]N^6-(R)-phenylisopropyladenosine) from the adenosine A_1 receptor (Fredholm *et al.,* 1986) with a K_i-

value of 5.2 µM. Two dihydropyridine calcium channel antagonists nifedipine and felodipine had K_i-values of 4.2 µM and 8.7 µM, respectively for the adenosine A_1 receptor.

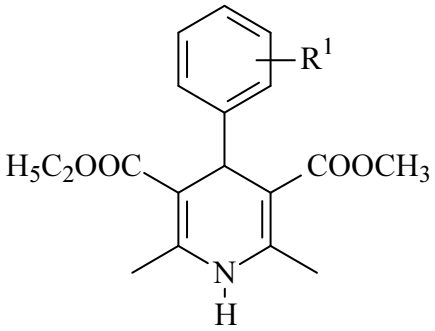


BayK 8644

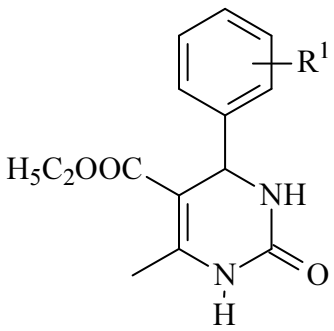


Felodipine

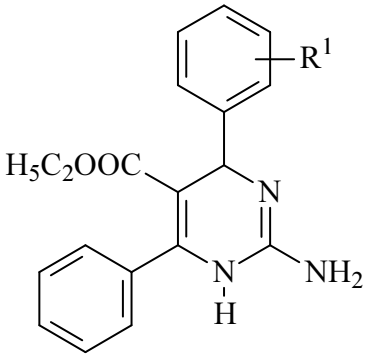
Van Rhee and co-workers (1996) synthesised 1,4-DHP derivatives and reported that these derivatives had affinity for three adenosine receptor subtypes (A_1, A_{2A} and A_3) in the millimolar range. These results prompted the current pilot study which aimed to investigate the potential of the structurally related 3,4-dihydropyrimidin-2(1H)-ones (3,4-dihydropyrimidones) and 2-amino-1,4-dihydropyrimidines as adenosine A_1 and A_{2A} antagonists.



1,4-DHP



3,4-dihydropyrimidones



2-amino-1,4- dihydropyrimidines

The classical adenosine A_1 and A_{2A} receptor antagonists are xanthine structures, currently there are already a large number of non-xanthine antagonists known for their adenosine A_1 and A_{2A} receptor activity.

1.3 **AIMS AND OBJECTIVES**

The objectives of this pilot study are summarised below:

- 3,4-Dihydropyrimidone analogues (table 1) will be synthesised: The Biginelli one pot synthesis will be used to obtain the desired dihydropyrimidones. The three-component coupling of a substituted arylaldehyde, β-ketoester and urea will be carried out under refluxing, solvent free conditions in the presence of a catalytic amount of tetra-butyl ammonium bromide (TBAB).

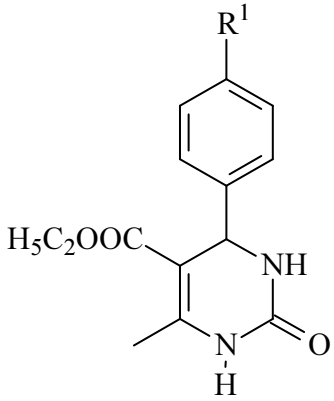
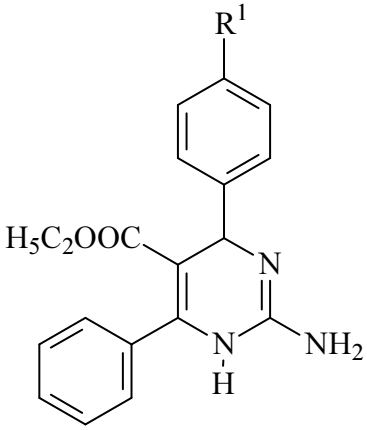
- 2-Amino-1,4-dihydropyrimidines (table 1) will be synthesised using a similar modified Biginelli one pot synthesis where a mixture of an aldehyde, ethyl benzoylacetate, guanidine hydrochloride and sodium hydrogen carbonate will be heated in dimethylformamide (DMF).

- The synthesised compounds will be characterised with nuclear magnetic resonance (NMR) and infrared spectroscopy (IR) as well as mass spectrometry (MS).

- Selected compounds will be evaluated as MAO-B inhibitors. Inhibition potencies will be expressed as IC_{50} values, which indicate the concentration of inhibitor that produces 50% enzyme inhibition. A fluorometric assay will be used to determine the inhibitory activity, where the enzyme activity will be based on the amount of kynuramine that is enzymatically converted to 4-hydroxyquinoline.

- Affinities of the synthesised compounds for adenosine A_1 and A_{2A} receptors will be determined *in vitro* using a radioligand binding study. A protocol described in literature (Van der Walt *et al.,* 2013) will be employed, where the displacement of N-[^{3}H]ethyladenosin-5'-uronamide ([^{3}H]NECA) from rat striatal membranes and 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) from rat whole brain membranes, will be used to indicate binding of compounds to the adenosine A_{2A} and A_1 receptor subtypes, respectively.

Table 1: Structures of dihydropyrimidones and dihydropyrimidines that will be synthesised and analysed during this pilot study.

	Series 1	**Series 2**
R^1	**3,4-Dihydropyrimidinones**	**2-Amino-1,4-dihydropyrimidines**
H		
4-Br		
4-Cl		
4-F		
4-OCH_3		
4-NO_2		
4-CH_3		
4-OH		
4-CF_3		

The remainder of the dissertation is set out as follows: Chapters 2 and 3 will provide an overview of literature pertaining to PD, the treatment of PD and the role of adenosine antagonists in the treatment of PD. Chapter 4 contains the synthetic procedures and structural characterisation of the 1,4-dihydropyrimidone and 2-amino-1,4-dihydropyrimidine derivatives synthesised in this pilot study. In chapters 5 and 6 all the experimental procedures employed in the investigation of the adenosine A_1 and A_{2A} affinities and MAO-B inhibitory effects of the synthesised compounds will be described. These chapters also include the results obtained, a short discussion and suggestions for future studies. In Chapter 7 final conclusions, as drawn from the results are presented.

CHAPTER 2

PARKINSON'S DISEASE

2.1 GENERAL BACKGROUND

In 1817, James Parkin was the first to describe an unrecognized condition in his monograph "Essay on shaking Palsy" (Dauer & Przedborski, 2003). It was not until 1861 when Jean Martin Charcot added more symptoms that it was officially named "Parkinson's disease (PD)" (Lees *et al.,* 2009). PD remains the most common cause of Parkinsonism; a condition characterized by tremor, rigidity, bradykinesia, postural instability and freezing as a result of striatal dopamine (DA) deficiency or direct striatal damage (Dauer & Przedborski, 2003). Lees and co-workers (2009) defined PD as a progressive bradykinetic disorder which is characterized by severe pars compacta nigral cell loss and accumulation of aggregated α-synuclein in the brain stem, spinal cord and cortical regions. In other words, PD is a degenerative condition affecting the brain (Bove *et al*., 2005) leading to various neurological symptoms.

Ageing is a major risk factor of PD (Lees *et al.,* 2009), about 75% of all cases usually begin from the age of 60, rapidly rises to about 80 years old and then gradually decrease. Men have been found to be 1.5 times more likely to develop this disease than women (Wooten *et al*., 2004). The three major motor symptoms are tremor, bradykinesia and rigidity which may be accompanied by akinesia, speech disturbances, gait and balance problems (Lees *et al.,* 2009; Dauer & Przedborski, 2003). These symptoms usually begin on one side of the body and remain worse on that side compared to the other side. The non motor symptoms associated with PD are dementia, psychoses, sleep disorders, loss of smell, constipation, mood disorder, orthostatic hypotension, drooling and depression (Lees *et al.,* 2009; Dauer & Przedborski, 2003).

There is no cure for PD and all current treatments are symptomatic. DA replacement therapy through oral administration of L-dopa remains the most effective available treatment (Fernandez & Chen, 2007; Koller & Cersosimo, 2004; Dauer & Przedborski, 2003). However each symptomatic treatment has its own inadequacies and unfavourable side effects resulting in an ongoing search focused on prevention of dopaminergic neuron degeneration as well as search for alternative drug targets (Dauer & Przedborski, 2003).

2.2 NEUROCHEMICAL AND NEUROPATHOLOGICAL FEATURES

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 of the substantia nigra pars compacta (SNc) as can be seen in the diseased nigrostriatal pathway, shown in figure 1 (Dauer & Przedborski, 2003; Marsden,1983).

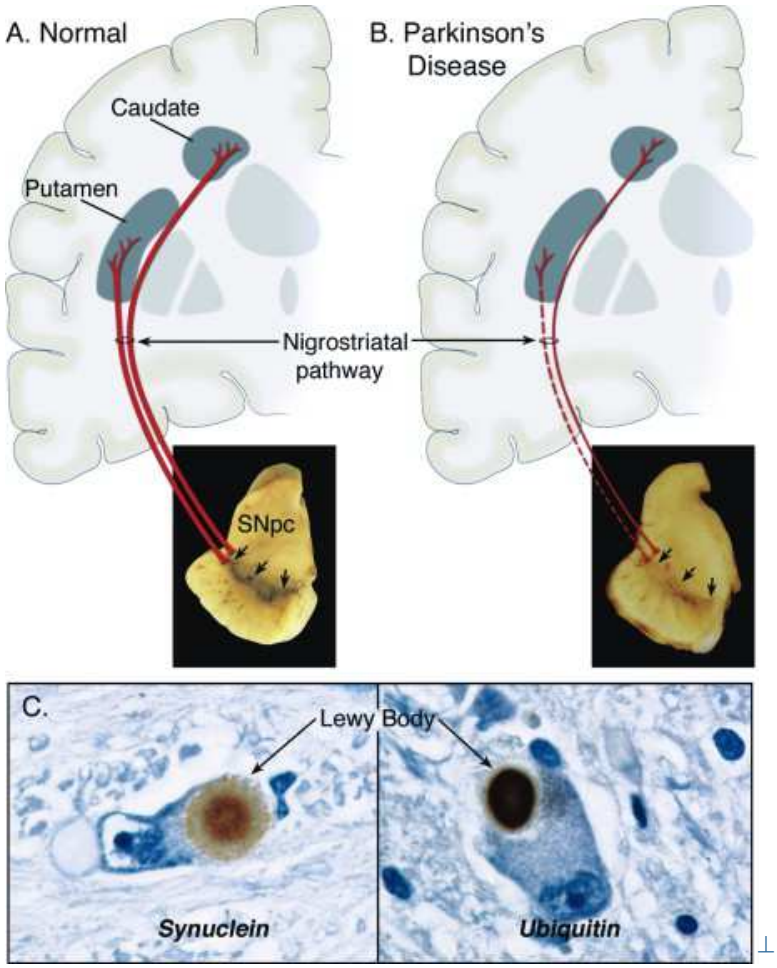


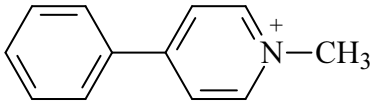
Figure 1: Neuropathology of PD. (**A**) Schematic representation of the normal nigrostriatal pathway (red) compared to (**B**) the diseased nigrostriatal pathway (red) and (**C**) immunohistochemical labelling of Lewy bodies (Dauer & Przedborski, 2003).

By definition, LBs are spherical eosinophilic cytoplasmic protein aggregates found in all affected brain regions and compose of numerous proteins including α-synuclein, parkin, ubiquitin and neurofilaments (Spillantini *et al.,* 1998; Forno, 1996). However, their role in neuronal cell death remains controversial (Dauer & Przedborski, 2003). In 1987, Hornykieicz and Kish reviewed that there are other factors contributing to neurodegeneration other than

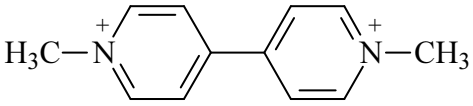
the loss of dopaminergic neurons. These factors relate to altered behaviour and cognition such as: i) abnormal α-synuclein at the synapses rather than LBs and neuritis; ii) impaired dopaminergic, noradrenergic, cholinergic and serotoninergic cortical innervations and iii) altered neuronal function resulting from reduced alteration (Ferrer, 2011). It has also been shown that neurodegeneration and LB formation extends beyond dopaminergic neurons i.e. they are also found in noradrenergic, serotogenic, cholinergic systems, cerebral cortex, olfactory bulb and autonomic nervous system (Dauer & Przedborski, 2003; Hornykiewicz & Kish, 1987). This may explain why some PD patients suffer from other conditions like depression as well as dementia (Dauer & Przedborski, 2003).

2.3 **ETIOLOGY**

The specific cause of PD is not known. Dauer and Przedborski (2003) pointed out that environmental toxins, genetic factors and endogenous toxin may play a role in the development of PD. The environmental hypothesis indicate that neurodegeneration results from dopaminergic neurotoxin exposure as explained by the findings that people intoxicated with MPTP (1-methyl-4-pheny-1, 2, 3, 6-tetrahydropyridine) develop Parkinsonism (Langston *et al.,* 1983).



MPP^+



Paraquat

The environmental hypothesis is further supported by the observation that rural environment residences exposed to herbicides and pesticides have shown high risk of PD (Tanner, 1992). One example is the herbicide paraquat which is structurally similar to MPP^+ (1-methly-4-phenylpyridium ion), the active metabolite of MPTP (Dauer & Przedborski, 2003). The mechanism of action is via the toxic MPP^+ metabolite which interferes with complex I of the electron transport chain, leading to depletion of cells produced during oxidative phosphorylation. This exposes the dopaminergic neurons to irreversible damage from energy depletion and oxidative stress induced by free radicals (Nussbaum & Polymeropounles, 1997).

Genetic factors which play a role in the etiology of sporadic PD include (but are not limited to) polymorphism in the gene encoding enzyme, cytochrome P-450 (Sandy *et al.,* 1996).

Additionally, the inherited differences in metabolic pathways lead to distortions of normal metabolism thus creating toxic substances (Dauer & Przedborski, 2003). The neurodegeneration process may also be triggered by endogenous toxins such as reactive oxygen species (ROS) generated by DA metabolism as well as the isoquinoline derivatives as found in PD brains (Nagatsu, 1997; Cohen, 1984).

2.4 **PATHOGENESIS**

The two major hypotheses regarding pathogenesis of PD are: i) altered protein metabolism i.e. misfolding and aggregation of proteins and ii) mitochondrial dysfunction oxidative stress including toxic oxidized DA species or glutamatergic excitotoxicity (Ebrahimi-Fakari *et al*., 2011; Dauer & Przedborski, 2003). Neuroinflammation, excitotoxicity, apoptosis and loss of trophic factors also take part in the pathogenesis of PD and they act synergistically to promote neurodegeneration (Yacoubain & Standaert, 2009). However, it is uncertain whether cell death pathways activated during PD neurodegeneration, participate or not in the common downstream machinery e.g. apoptosis (Dauer & Przedborski, 2003).

2.5 **MECHANISM OF NEURODEGENERATION**

The molecular events responsible for neurodegeneration are not well understood (Yacoubian & Standaert, 2009). Nevertheless, the responsible mechanisms include oxidative stress, mitochondrial dysfunction, protein aggregation and misfolding, inflammation, excitotoxicity, apoptosis, other cell death pathways and loss of trophic support. The primary event of these factors is unknown but it has been suggested that one process affects the other (Koller & Cersosimo, 2004) i.e. all these mechanisms are thought to act synergistically through a complex interactions which lead to a final common pathway involving protein aggregation and apoptosis (Yacoubain & Standaert, 2009; Koller & Cersosimo, 2004). Genetic mutations (e.g. α-synuclein), oxidative damage, mitochondrial dysfunction and abnormal DA metabolism all promote accumulation of misfolded proteins, a major cause of PD neurodegeneration (Dauer & Przedborski, 2003). Furthermore, pathogenic mutations may also damage the ability of the cellular machinery to detect and degrade misfolded proteins (Parkin, UCH-L1, DJ-1). However, it is not clear whether misfolded proteins directly cause toxicity or damage cells via the formation of protein aggregates (LBs), since controversy exists to whether LBs are neuroprotective or neurotoxic (figure 2) (Dauer & Przedborski, 2003). Moreover, adenosine triphosphate (ATP) depletion also plays a role in promoting neurodegeneration (Giasson *et al.,* 2000).

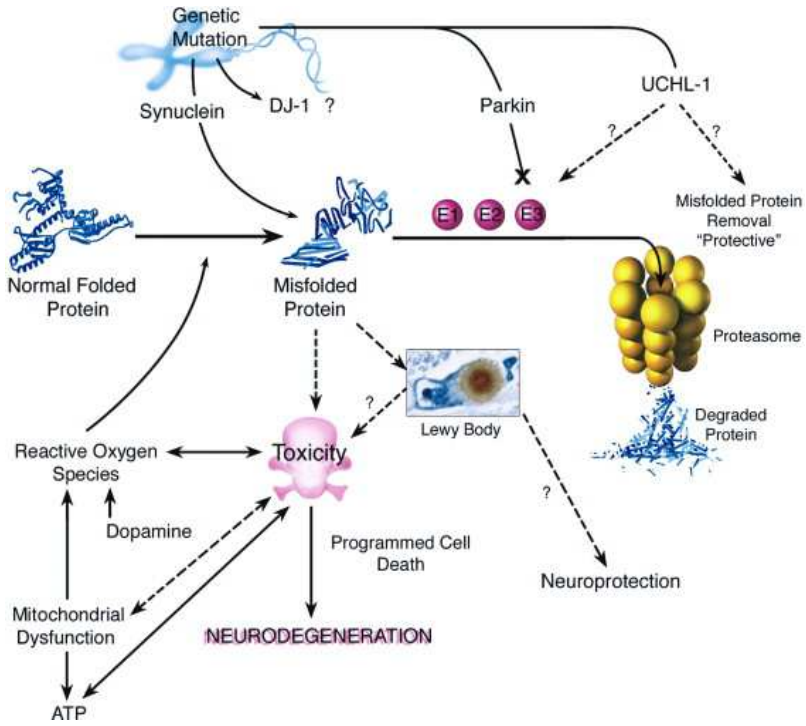


Figure 2: Pathogenic pathways in Parkinson's disease showing the mechanism of neurodegeneration (Dauer & Przedborski, 2003).

2.6 **TREATMENT OF PARKINSON'S DISEASE**

Current treatment for PD involves symptomatic management with dopaminergic replacement therapy. L-dopa remains the most effective oral treatment, although long-term use is associated with complications such as dyskinesias and on-off fluctuations. Non-dopaminergic medications that improve PD symptoms and motor fluctuations and also offer neuroprotection are in demand.

2.7 **DRUGS FOR NEUROPROTECTION**

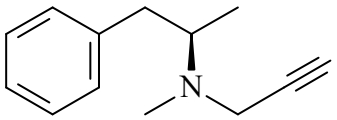
Schapira (2010) defined neuroprotection as the ability to prevent neuronal cell death by intervening in and inhibiting the pathogenetic cascade that results in cell dysfunction and death. The aim of neuroprotection is to prevent further dopaminergic cell death by halting or slowing disease progression (Clarke, 2004). This can be achieved by preventing molecular mechanisms that are responsible for neurodegeneration. The drugs used include antioxidants, antiapoptosis, glutamate antagonists, MAO-B inhibitors, adenosine antagonists, anti-inflammatory and mitochondrial stabilizing agents.

2.7.1 **MAO-B inhibitors**

Catabolism of dopamine by MAO-B produces the hydroxyl radical and other ROS that lead to oxidative stress (Spencer *et al.,* 1996). MAO-B inhibitors therefore block this reaction

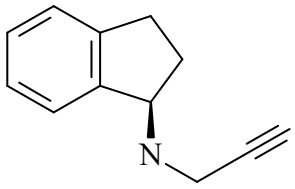
hence increasing the amount of DA available and thus slowing the progression of dopaminergic neurons (LeWitt, 2006). The MAO-B inhibitors are suggested to have a dual action i.e. i) improvement of symptoms related to dopamine deficiency and ii) potential antioxidants. They also increase the half life of DA in the synaptic cleft and enhance receptor simulation and reuptake into the synaptic bulb (Schapira, 2010). The prevention of MPTP conversion to MPP+ also suggests that MAO-B inhibitors may be neuroprotective (Koller & Cersosimo, 2004).

Selegiline (R-deprenyl) is a propargylamine and selective MAO-B inhibitor. It reduces oxidative stress associated with MAO-B mediated DA metabolism and glutamate induced toxicity (Fernandez & Chen, 2007). Selegiline has dopamine potentiating, antioxidant and antiapoptotic properties. It alters gene expression for pro and antiapoptotic proteins, resulting in mitochondrial integrity preservation during oxidative stress (Fernandez & Chen, 2007). Selegiline further inhibits the metabolism of MPTP to MPP^+ and was found to be neuroprotective in MPTP induced animal models (Clarke, 2004). It was also shown to have mild symptomatic effects that improve motor symptoms in PD (Olanow *et al.,* 2008).



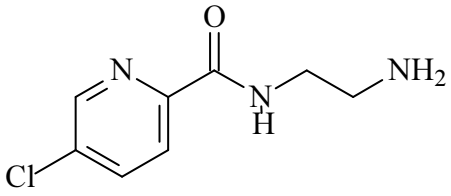
Selegiline

Rasagiline is a more potent irreversible MAO-B inhibitor than selegiline and its neuroprotective activity lies in its propargyl moiety and not its MAO inhibition activity. This is supported by the observation that it exerts neuroprotection at concentrations below the MAO-B inhibition threshold (Schapira, 2010; Olanow *et al.,* 2008). Rasagiline's neuroprotective action could also be related to antioxidant, antiapoptosis and growth factor induction properties.



Rasagiline

Lazabemide is a non propargylamine selective reversible inhibitor of MAO-B with higher selectivity towards MAO-B than selegiline (LeWitt & Taylor, 2008).

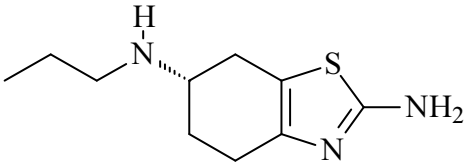


Lazabemide

2.7.2 **Dopaminergic drugs**

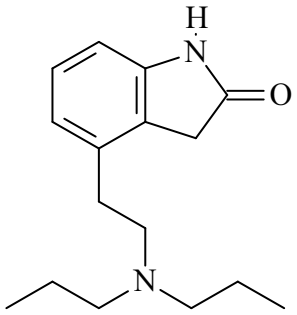
The neuroprotection mechanism of action of DA agonists is uncertain but might be based on dopaminergic stimulation that mediates recovery of dopaminergic nigrostriatal neurons from toxic effects (Lewitt & Taylor, 2008). They possess a hydroxylated benzene ring structure which implies antioxidant activity (Schapira, 2010). Dopaminergic drugs reduce DA turnover and production of ROS in the nigrostriatal neurons. These drugs act as radical scavengers at relatively high concentrations but it is unknown whether they reach such levels in the central nervous system (LeWitt & Taylor, 2008; Fernandez & Chen 2007).

Pramipexole reduces 6-hydroxydopamine and MPTP toxicity. Its neuroprotection activity may be due to inactivation of dopaminergic neurons or anti apoptotic actions (Schapira, 2010; LeWitt, 2006; Abramova *et al.,* 2002; Ling *et al*., 1999). It has been shown to exert free radical scavenging properties (Grunblatt *et al.,* 2001; Le & Jankovic, 2001).



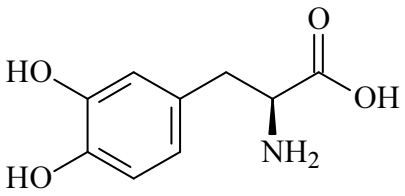
Pramipexole

Ropinirole, a dopamine D_2,/D_3 receptor agonist, has been shown to scavenge hydroxyl radicals; nitric oxide but not superoxide radicals at relatively high concentration. It exerts its protective effects via D_2 receptors (Schapira, 2010; LeWitt & Taylor, 2008). Ropinirole has shown protection against 6-hydroxydopamine induced toxicity in mice (Takata *et al.,* 2000).



Ropinirole

L-dopa is an amino acid precursor of DA and possesses antioxidant neuroprotective effects in the striatum (LeWitt & Taylor, 2008; LeWitt, 2006; Camp *et al.,* 2000). Its benefits include good control of motor symptoms and improvement in quality of life and life expectancy. It can either act as a pro-oxidant and antioxidant depending on the concentration. Low concentration of L-dopa enhances the production of protective molecules whilst high concentrations of L-dopa have shown toxicity in culture models (Schapira, 2010).



L-dopa

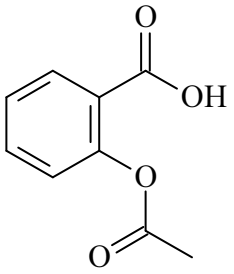
2.7.3 **Antioxidant therapy**

Drugs with antioxidant properties include α-tocopherol, rasagiline, selegiline, vitamin E, coenzyme Q_{10} and creatine. α-Tocopherol is an antioxidant that acts by quenching oxyradical species. Coenzyme Q_{10} is a cofactor in the mitochondrial electron transport chain whereas creatine promotes mitochondrial ATP production (Yacoubain & Standaert, 2009; LeWitt & Taylor, 2008). However, there is no evidence for α-tocopherol in PD and severe deficiency does not lead to parkinsonism. Also no difference has been observed between vitamin E treated patients and placebo treated patients thus there are no conclusions to the effectiveness of using antioxidant vitamins (vitamin E and vitamin C) (Yacoubain & Standaert, 2009; LeWitt & Taylor 2008).

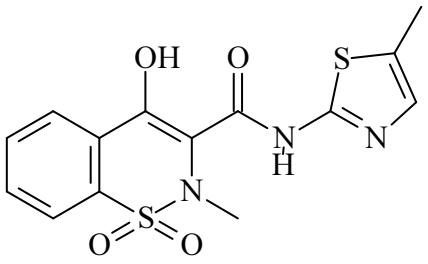
2.7.4 **Anti-inflammatory drugs**

The use of a nonsteroid anti-inflammatory drug, two or more times per week showed to produce a 45% lower risk for PD (Yacoubain & Standaert, 2009). When given before the

toxin, acetylsalicylic acid and meloxicam showed neuroprotective effects in an MPTP mouse model of PD (Schapira, 2010). Another promising agent is minocycline a second generation tetracycline and an anti-inflammatory agent with antiapoptotic action (Schapira, 2010; Yacoubain & Standaert, 2009). It acts by inhibiting microglial activation, a prominent feature suggesting an inflammatory component to the pathogenesis of PD neurodegeneration (LeWitt, 2006).

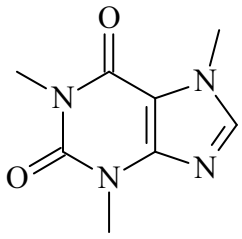


Acetylsalicylic acid



Meloxicam

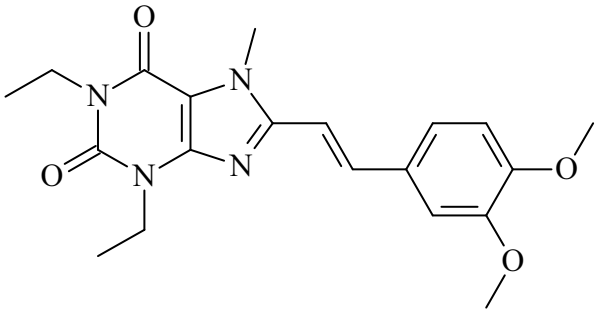
2.7.5 **Adenosine A_{2A} receptor antagonists**



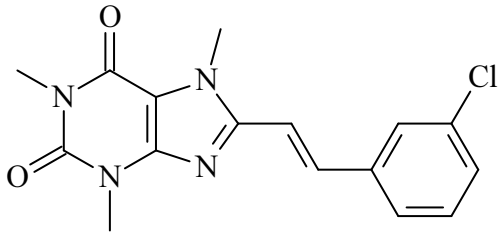
Caffeine

The incidence of PD was found to be significant lower in people with a higher coffee intake (Ross *et al.*, 2000). Since caffeine, present in coffee, mediates its action by antagonizing adenosine receptors, it has led to the evaluation of adenosine receptor antagonists as potential neuroprotective agents (Schwarzchild *et al.,* 2006). In the striatum, adenosine stimulation of the A_{2A} receptor leads to inhibition of dopamine signalling via the heterodimerization of the A_{2A} receptor with the D_2 receptor. In the presence of caffeine, the adenosine A_{2A} receptors are inhibited thereby promoting DA function leading to a reduced incidence of PD (Yacoubain & Standaert, 2009). Caffeine has been shown to have neuroprotective effects on the dopaminegic nigrostriatal system in MPTP induced mouse model (Xu *et al.*, 2002; Chen *et al.*, 2001). It protects against both neurotoxicity and degeneration of MPTP induced dopaminergic system. Several caffeine derivatives have been tested for their ability to mimic caffeine's attenuation of MPTP toxicity. These include the xanthine caffeine derivatives

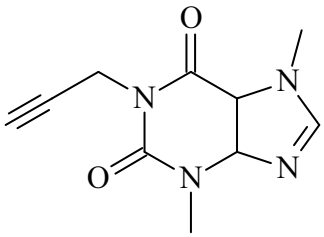
Istradefylline (KW6002), 8-(3-chlorostyryl)-caffeine (CSC) and 3,7-dimethyl-1-propargylxanthine (DMPX) (Kalda *et al.*, 2006).



KW6002



CSC



DMPX

It is also suggested that adenosine A_{2A} receptors antagonist reduce glutamate release and neuroinflammation leading to anti-excitotoxicity properties, although the anti-inflammatory effects of adenosine are still debatable (Kalda *et al.,* 2006).

2.8 **SYMPTOMATIC TREATMENT**

PD remains incurable but treatment improves quality of life and functional capacity. Lees and co-workers (2009) stated that the main goal of PD treatment is the restoration of striatal DA function. Symptomatic treatment for this disease is relatively successful, and a number of effective agents are available (Standaert & Roberson, 2011).

2.8.1 **L-dopa**

According to Fernandez and Chen (2007), L-dopa (L-3,4-dihydroxyphenylalanine) remains the most effective agent for the symptomatic treatment for PD (see 2.7.2 for structure). It is primarily converted by decarboxylation to dopamine in the striatum (Standaert & Roberson, 2011). L-dopa is effective in relieving bradykinesia and any resulting disabilities and can ameliorate all of the clinical features of Parkinsonism (Aminoff, 2007). However, L-dopa does not ameliorate nonmotor symptoms such as dementia and it is associated with long term development of motor complications such as dyskinesia and motor fluctuations (Fernandez

& Chen, 2007). Moreover, L-dopa induced dyskinesias typically develop with motor fluctuations and become more severe as the disease progresses and with increases in L-dopa dosages. To extend its efficacy and decrease motor complications, L-dopa narrows as its efficacy shortens and its adverse effects, including dyskinesias, become less tolerable (Fernandez & Chen, 2007).

Although L-dopa can cross the blood-brain barrier (BBB), it is administered in combination with peripheral dopa decarboxylase inhibitors (benserazide or carbidopa) to increase its availability (Youdim *et al.,* 2006). If administered alone, L-dopa is peripherally decarboxylated and less than 1% penetrates the central nervous system (CNS). Carbidopa or benserazide do not penetrate the CNS and blocks the premature decarboxylation of L-dopa by aromatic L-amino acid decarboxylase in the peripheral circulation. This leads to an increase in the fraction of administered L-dopa that remains unmetabolised and available to cross the BBB thus increasing brain concentration of L-dopa and reducing side effects like nausea and vomiting that are associated with DA production in the peripheral (Standaert & Roberson , 2011; Lees, 2005; Clark, 2004).

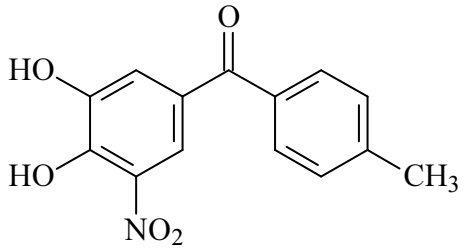
2.8.2 **Dopamine agonists**

Dopamine agonists provide effective relief of Parkinsonian symptoms either as first line therapy in early Parkinson's disease or as an adjunct to L-dopa (Fernandez & Chen, 2007). They are direct agonists of the striatal dopamine receptors and are responsible for delaying L-dopa-induced motor complications (Standaert & Roberson, 2011; Fernandez & Chen, 2007). Their use is associated with a lower incidence of response fluctuations and dyskinesias that occur with long term L-dopa therapy (Aminoff, 2007). The non ergoline dopamine agonists (pramipexole, ropinirole, rotigotine and piribedil) are efficacious drugs that in contrast to L-dopa, when used as a monotherapy, do not provoke dyskinesia (see 2.7.2 for structures) (Lees *et al.*, 2009).

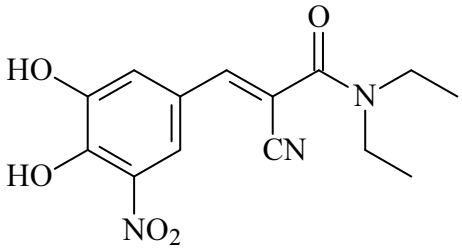
2.8.3 **COMT inhibitors**

Inhibitors of catechol-*O*-methyltransferase (COMT) also enhance L-dopa availability and prevent the inactivation of DA by COMT. The principal therapeutic action of COMT inhibitors is to block this peripheral conversion of L-dopa to 3-O-methyldopa, increasing both the plasma half life of L-dopa as well as the fraction that crosses the BBB and reaches the CNS (Standaert & Roberson, 2011; Clarke, 2004). Two examples of COMT inhibitors available, are entacapone and tolcapone. Both agents significantly reduce the wearing of symptoms in patients treated with L-dopa/carbidopa, whilst entacapone further improves motor

impairments and disability (Clarke, 2004). Side effects include nausea, vomiting, diarrhoea, confusion, and vivid dreams. However, due to hepatotoxicity, tolcapone should only be used in patients who have not responded to other therapies and with appropriate monitoring for hepatic injury (Standaert & Roberson, 2011).



Tolcapone



Entacapone

2.8.4 **MAO inhibitors**

MAO-B is also responsible for the biotransformation of MPTP into MPP^+, a potent parkinsonism-inducing neurotoxin, thus the inhibition of MAO-B may modify the underlying processes of PD. Fernandez and Chen (2007) showed that MAO-B inhibition improves striatal dopaminergic activity by inhibiting the metabolism of DA, thereby improving PD motor symptoms. Besides the metabolism of DA into 3,4-dihydroxyphenylacetic acid, MAO-B also deaminates β-phenylethylamine, an endogenous amine that stimulates DA release and inhibits neuronal DA uptake (Fernandez & Chen, 2007). Selegiline and rasagiline (see 2.7.1 for structures) are examples of two irreversible selective MAO-B inhibitors which are currently recommended for adjunctive therapy in patients with PD to reduce "off" time associated with motor fluctuations (Fernandez & Chen, 2007). These agents also do not exhibit the cheese effect and may modify disease activity or be neuroprotective (Youdim & Bakhle, 2006; Lees, 2005; Przedborski, 2005). An example of a reversible inhibitor of MAO-B is lazabemide (see 2.7.1 for structures).

2.8.5 **Anticholinergic drugs**

Anticholinergics may also reduce painful dystonic phenomena in young onset cases (Lees *et al.*, 2009). These drugs improve tremor and rigidity but have little effect on bradykinesia (Aminoff, 2007). Examples are benztropine mesylate, biperidine, orpnenadrine, trihexyphenidyl, procycline and diphenhydramine hydrochloride. All these drugs have relatively modest antiparkinsonian activity and are only used in treatment of early PD or as an adjunct to dopamimetic therapy. Anticholinergics are effective in rest tremor and they are used in young patients with severe high amplitude tremor. However, several adverse effects

result from anticholinergic properties e.g. sedation, mental confusion etc., and thus limit the use of anticholinergics in the modern management of PD (Standaert & Roberson, 2011; Clarke, 2004).

2.8.6 **Amantadine**

Amantadine, an antiviral agent, can be used as initial therapy and it is an effective anti-dyskinetic agent in some patients (Lees *et al.,* 2009). The mechanism of action is unclear but amantadine appears to alter DA release in the striatum, has anticholinergic properties and blocks N-methyl-D-aspartate (NMDA) glutamate receptors (Standaert & Roberson, 2011; Clarke, 2004). It may potentiate dopaminergic function by influencing the synthesis, release, or reuptake of DA and catecholamines (Aminoff, 2007).

2.8.7 **Adenosine A_{2A} receptor antagonists**

DA via dopamine D_2 receptors antagonises adenosine A_{2A} receptor mediated signalling (Vortherms & Watts, 2004). Loss of DA will therefore lead to unopposed adenosine A_{2A} signalling resulting in over activity of the striatopallidal pathway hence excess inhibition of movement. Adenosine A_{2A} antagonists may therefore lead to reversibility of movement dysfunction. It may also improve mobility during both monotherapy as well as with co-administration with DA antagonist and L-dopa, leading to reduced L-dopa usage and side effects (Ciéslak *et al.,* 2008; Pretorius *et al.,* 2008). Adenosine A_{2A} antagonists may also prevent L-dopa induced dyskinesia. Evidence include KW6002 (see 2.7.5 for structure) which has been shown to exhibit antiparkinsonian activity without provoking both hypersensitivity and dyskinesia (Ikeda *et al.,* 2002). A clinical study also showed that KW6002 reduced “off” time and increased “on” time that is associated with motor fluctuations (Kalda *et al.,* 2006), suggesting that adenosine A_{2A} antagonists are promising agents for symptomatic treatment of PD (Pretorius *et al*., 2008).

2.9 **CONCLUSION**

The symptoms of PD can be divided into two domains: motor and non-motor. Motor symptoms are the clinical hallmarks of a diagnosis of PD. More recently, there has been increased emphasis on the disabling burden and management challenges of the non-motor symptoms. These symptoms include sleep disturbance, pain, autonomic dysfunction, and cognitive, behavioural and psychological problems, e.g. dementia. When the significance between higher caffeine intake and a lower incidence of PD was realised some years ago, adenosine A_{2A} receptors became a promising target to treat PD.

CHAPTER 3

ADENOSINE RECEPTORS

3.1 GENERAL BACKGROUND AND TISSUE DISTRIBUTION

The adenosine receptors are bound to the cell membranes of neurons, glia cells and endothelial cells of the brain blood vessels. The receptor sub-types are G-protein-coupled glycoproteins A_1, A_{2A}, A_{2B} and A_3 with A_1 as the most abundant type (Ciéslak *et al*., 2008; Jaakola *et al*., 2008). Adenosine A_1 receptors are located in the neuron synaptic membranes of the brain core, hippocampus, cerebellum, spinal cord, thalamus and striatum. In the brain the adenosine A_3 subtype are located in hippocampus, thalamus, and hypothalamus. The adenosine A_{2B} receptors are found in brain core, hippocampus, cerebellum, thalamus, hypothalamus and striatum. The A_{2A} receptors are found only in the dopaminergic regions of the brain whilst the A_{2B} receptor is wide spread throughout the CNS (Ciéslak *et al*., 2008).

3.2 ADENOSINE A_{2A} RECEPTORS

The adenosine A_{2A} receptors activate adenylate cyclase and stimulate neuronal activity (Schwarzichild *et al*., 2006) while their antagonists are promising symptomatic therapy for PD. Adenosine A_{2A} antagonist retard PD progression by neuroprotection and may also prevent long term L-dopa associated dyskinesia (Pretorius *et al.,* 2008; Schwarzschild *et al*., 2006).

High levels of adenosine A_{2A} receptors are found on the external neuronal surfaces in the basal ganglia and are located in the striatum, globus pallidus and substantia nigra. Regionally, adenosine A_{2A} receptors are highly concentrated in the striatum, presynaptically (23%), postsynaptically (70%), neuron body (3%) and on glia cells (3%) with very low levels in the cortex and other brain regions (Ciéslak *et al*., 2008; Müller & Ferré, 2007; Schwarzschild *et al*., 2006). They modulate the neurotransmission of γ–aminobutyric acid (GABA), acetylcholine and glutamate transmission (Schapira, 2010). The adenosine A_{2A} receptors are co-localized with the dopaminergic D_2 receptors only on the indirect pathways between striatum, globus pallidus, and substantia nigra (Ciéslak *et al*., 2008). They form heteromeric complexes postsynaptically with metabotropic glutamate (mGlu5) and dopamine D_2 receptors whilst presynapticaly mainly with adenosine A_1, but also mGlu5 and dopamine D_2 receptors (figure 3) (Müller & Ferré, 2007). The heterodimerization of adenosine A_{2A}

receptor with dopamine D_2 receptor inhibits DA signalling and therefore inhibition will promote DA function (Yacoubain & Standaert, 2009).

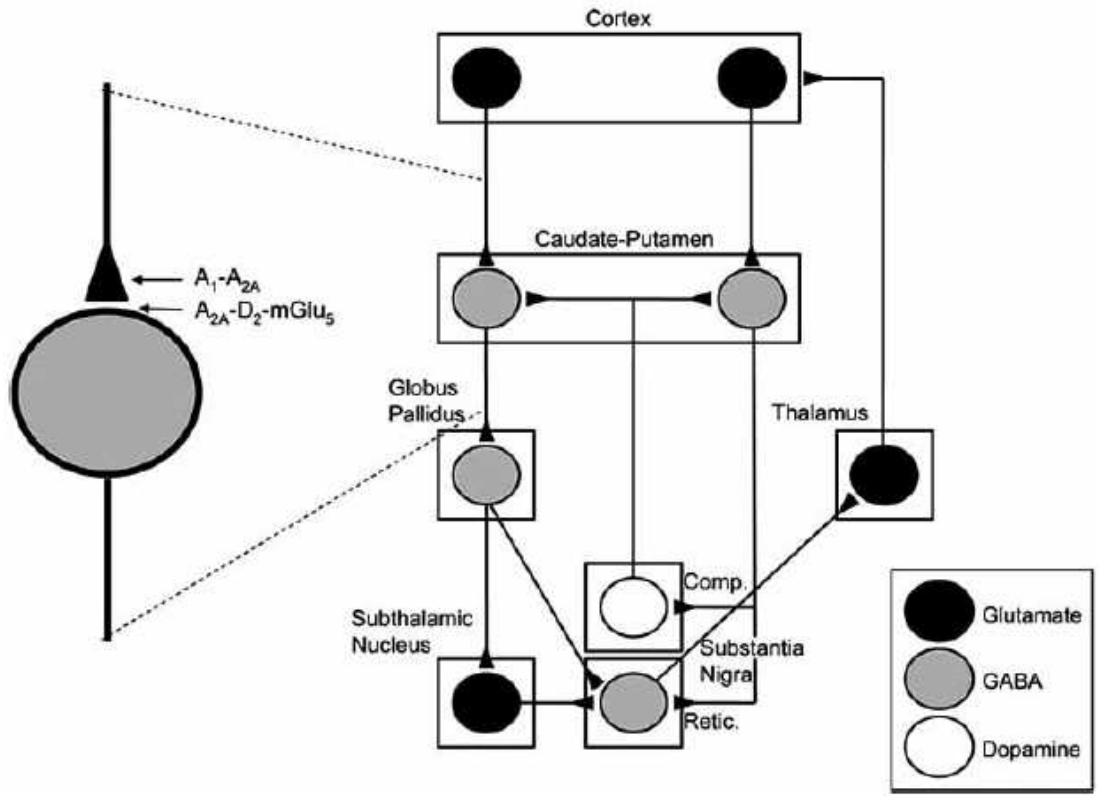


Figure 3: Basal ganglia circuitry showing localization of adenosine A_1-A_{2A} receptor heteromers in glutamatergic cortico-striatal terminals and A_{2A}-D_2-mGlu5 receptor heteromers in the GABAergic striatopallidal enkephalinergic neuron (Müller & Ferré, 2007).

3.2.1 **The adenosine A_{2A} receptor as symptomatic antiparkinsonian therapy**

Two pathways are present in the striata, namely the direct and indirect pathway. The A_{2A}/D_2 heteromers are found in the indirect striato-pallidal GABA pathway which expresses enkephalin (ENK) and globus pallidus externa (GPe,) and the A_1/D_1 heteromers are found in the direct striato-nigral neurons GABA pathway which expresses dynorphin (DYN) (Fuxe *et al.*, 2007). Stimulation of the direct pathway leads to motor activation (initiate movement) and stimulation of the indirect pathway leads to motor inhibition, whereas inhibition of the indirect pathway will alleviate motor inhibition (Fuxe *et al*., 2007).

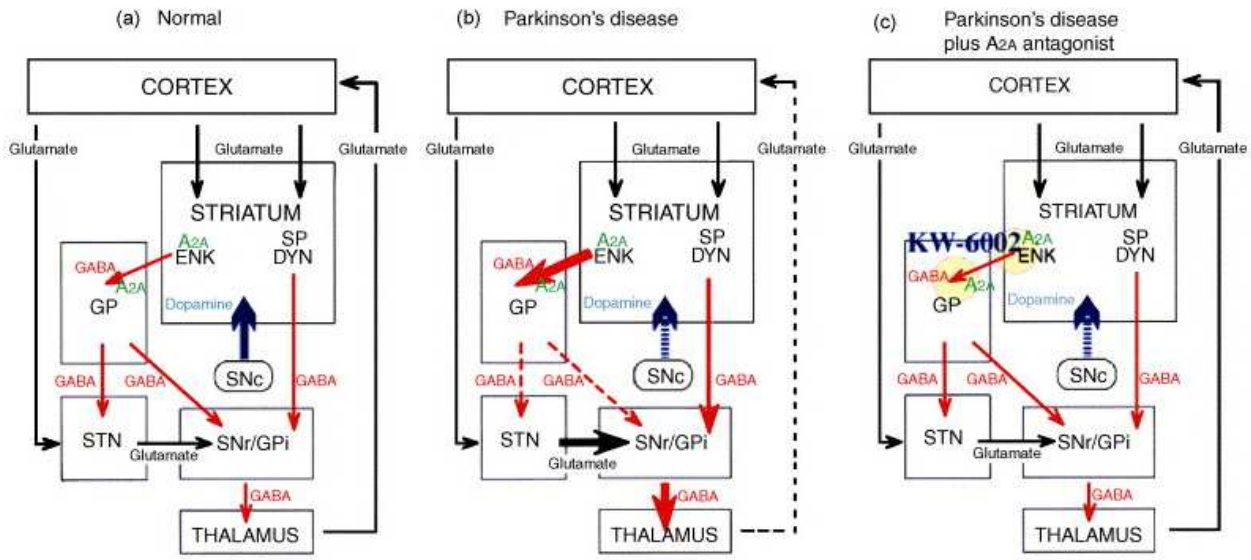


Figure 4: Illustration of the activity (indicated by the thickness of the arrows) of the main pathways of the basal ganglia under (**a**) normal conditions, (**b**) Parkinson's disease and (**c**) Parkinson's disease treated with A_{2A} antagonists (Kase *et al*., 2004).

At **physiological conditions** (figure 4a) (normal state), striatal neurons receive dopaminergic inputs from substantia nigra pars compacta (SNc). 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 cause motor excitation. At the same time, dopamine inhibits the indirect pathway via dopamine D_2 inhibitory receptors which sends GABAergic projection to the SNr/Gpi via GPe and subthalamic nucleus (STN) and causes a removal of motor inhibition (Fuxe *et al.,* 2007; Kase *et al.,* 2004). In this normal state, a balance between direct and indirect pathway exists. As a result, there is removal of GABA inhibition from SNr/Gpi to thalamus resulting in an increased drive from thalamus to cortex hence facilitating normal movements (Fuxe *et al.,* 2007; Kase *et al.,* 2004).

In **PD** (figure 4b), degeneration of SNc 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 GABA 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 (Schwarzschild *et al.,* 2006). This imbalance between the direct and the indirect pathways leads to an increased GABAgenic inhibitory output from the SNr/GPi complex to the thalamus hence a marked loss of motor drive from the thalamus to the cortex thereby

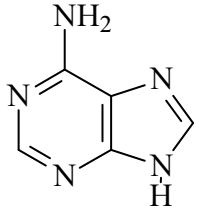
producing a pronounced reduction of movements e.g. bradykinesia and akinesia in PD (Fuxe *et al.,* 2007; Schwarzschild *et al.,* 2006).

Adenosine *via* A_{2A} receptors in the striatum and GPe excites the indirect pathway thus opposing the activation of dopamine D_2 receptors. The dopamine D_2 signalling will therefore be increased by adenosine A_{2A} antagonist, i.e., A_{2A} antagonists increase dopamine D_2 affinity for dopamine. **Treatment with A_{2A} antagonists** (figure 4c) will block the indirect striato pallidal GABA pathway, resulting in the recovery of GPe activity leading to the relief of over activity of STN neurons and thereby restoring/normalizing some balance between the direct and the indirect pathways (Kase *et al*., 2004). However, the movements are only partially restored since insufficient release of DA may be present and the dopamine D_1 activated direct pathway is not substantially modulated by adenosine A_{2A} antagonist. Adenosine A_{2A} antagonists may therefore lead to reversibility of movement dysfunction (Fuxe *et al*., 2007).

3.2.2 Adenosine A_{2A} antagonists

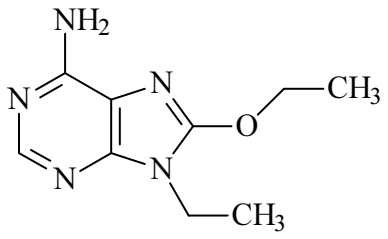
Most adenosine A_{2A} receptor antagonists belong to two different chemical classes, i) amino substituted heterocyclic compounds which are derived from adenine or structurally related to adenine and ii) xanthine derivatives and analogs (Müller & Ferré, 2007).

3.2.2.1 Adenine derivatives

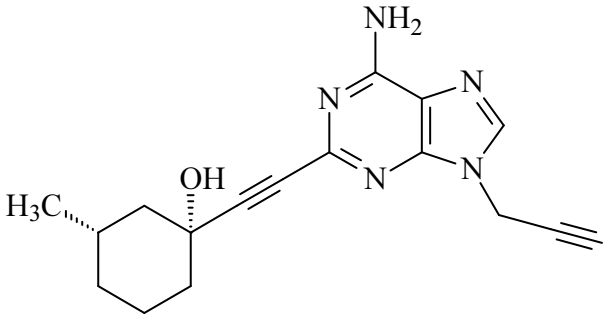


Adenine

Klotz and co-workers (2003) found that low molecular weight compounds, 9-ethyladenine derivatives, displayed good adenosine A_{2A} receptor affinity and selectivity. Substitution at the 8-position with a small, nonpolar substituent appeared to be favourable for high adenosine A_{2A} selectivity, e.g., 8-ethoxy-9-ethyladenine (ANR-94). Patented 2-alkynyl-substituted adenine derivatives (ATL-2) were found to exhibit high A_{2A} receptor affinity but moderate selectivity. The propargyl substituent at N9 appeared to be favourable for high adenosine A_{2A} receptor affinity (Beauglehole *et al.,* 2005).

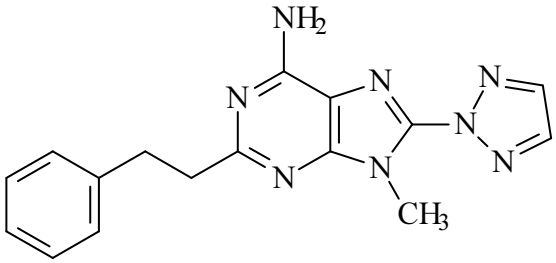


ANR-94



ATL-2

Further, substitution in the 8-position with a 1,2,3-triazole ring yielded potent adenosine A_{2A} receptor antagonists, e.g. 9-methyl-2-phenetyl-8-[1,2,3]triazol-2-yl-adenine (Müller & Ferré, 2007; Minetti *et al.,* 2005).



9-Methyl-2-phenetyl-8-[1,2,3]triazol-2-yl-adenine

Amino-substituted heterocyclic compounds related to adenine also emerged as potent adenosine A_{2A} antagonists. Borroni and co-workers (2003) screened 2-amino-5-cyano-6-(2-furyl)pyrimidine derivatives for A_{2A} affinity and optimized these structures and patented them as potent A_{2A} antagonists. They (Hoffman Le Roche Inc.) developed structure activity relationships for the 2-aminopyrimidine derivatives (figure 5). The unsubstituted amino in position 2 next to the nitrogen ring (N1 - pyrimidine) is essential for A_{2A} affinity. A 2-furyl in position 6 is optimal for affinity, but other aromatic or heteroaromatic rings are also tolerated. An electron withdrawing substituent is essential in the 5-position, where the cyano group gave the best affinity. Bulky substituent can be accommodated in the 4-position, with an oxygen linker being the best. Sulfur or amino linkers gave similar results, but thioethers are not favourable, due to metabolic instability, whereas water solubility is reduced with an NH linker. A methylene bridge led to a decrease in adenosine A_{2A} receptor selectivity (Müller & Ferré, 2007; Borroni *et al.*, 2003).

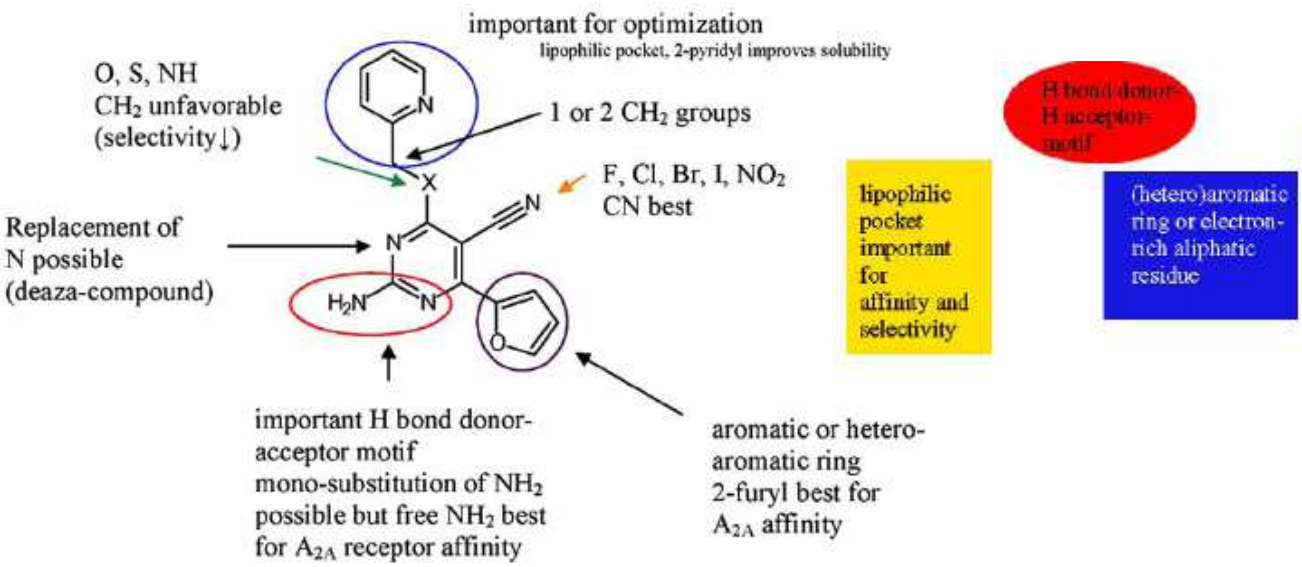
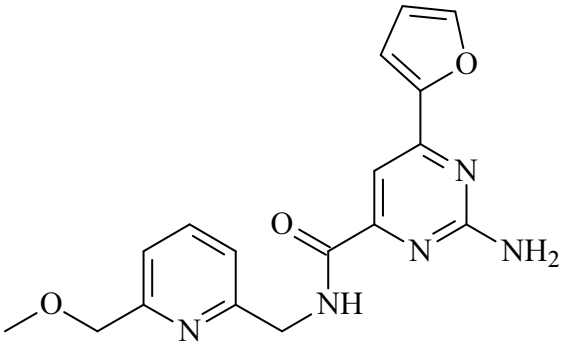


Figure 5: Structure-activity relationships of 2-aminopyrimidine derivatives developed by Hoffmann-La Roche (Müller & Ferré, 2007).

In 2009 Gillespie and co-workers discovered that pyrimidine carboxamide derivatives (**1**) are potent adenosine A_{2A} antagonists (Gillespie *et al.,* 2009a). With further optimization of these pyrimidines they also synthesised triazine carboxamides and found that both potency and selectivity were diminished compared to their original pyrimidine carboxamide. Since the increased nitrogen count was detrimental to activity, they synthesised the corresponding pyridine scaffold and compared the three scaffolds (triazine, pyrimidine and pyridine) against human adenosine A_1 and A_{2A} receptors (table 2).



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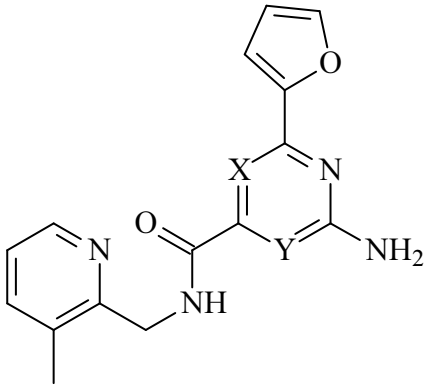
The pyridine derivative was sevenfold less potent than the triazine and 45-fold less potent than the corresponding aminopyrimidine, thus showing that two nitrogens in the ring is optimum for both adenosine A_1 and A_{2A} affinity (Gillespie *et al.*, 2009b).

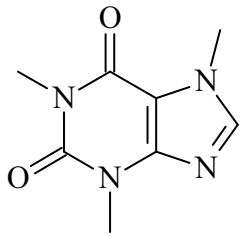
Table 2: Binding affinities of the pyridine, pyrimidine and triazine scaffolds against human adenosine A_{2A} and A_1 receptors (Gillespie *et al.,* 2009b).



	X	Y	A_{2A} K_i (nM)	A_1 K_i (nM)
Pyridine	CH	CH	126	921
Pyrimidine	CH	N	2.8	97
Triazine	N	N	18.6	1112

Since the 2-furyl moiety appeared to be favourable for both potency and selectivity of human adenosine A_1 and A_{2A} receptors, Gillespie also determined whether aryl groups would still retain both potency and selectivity. Simple phenyl derivatives were tolerated, but selectivity across the majority of phenyl derivatives was reduced. Ortho-ethyl and ortho-ethoxy derivatives showed improved adenosine A_{2A} selectivity, while adenosine A_1 affinity was greatly diminished (Gillespie *et al.,* 2009b).

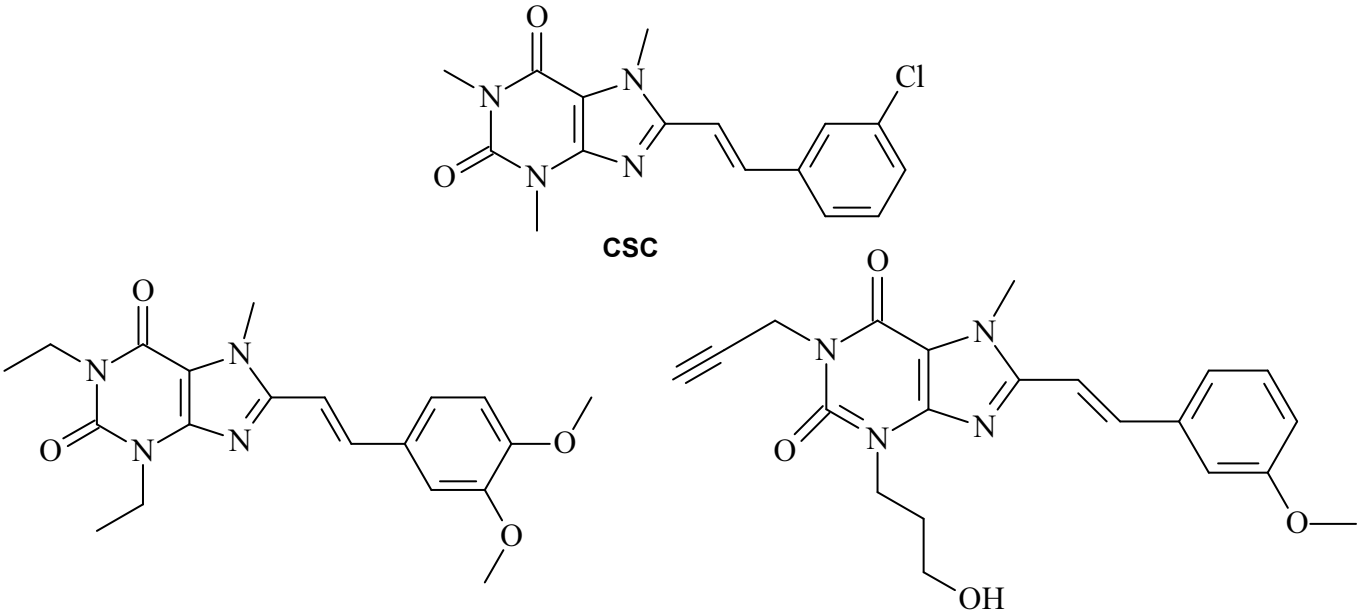
3.2.2.2 Xanthine derivatives



Caffeine

Caffeine, a xanthine derivative, is a nonselective adenosine antagonist but the observation that N7-methylation in 8-substituted xanthine derivatives was better tolerated by the adenosine A_{2A} receptor than the adenosine A_1 receptor (Shamim *et al.,* 1989), and that the 8-substituent had to be coplanar for achieving high A_{2A} receptor affinity (Müller *et al.,* 1997),

led to the highly potent and selective A_{2A} receptor antagonists, CSC, KW6002, and MSX-2 (3-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-propargylxanthine) (Müller *et al.,* 1997).



CSC

KW6002

MSX-2

Müller and Ferré (2007) compared the pharmacophores of xanthenes and of typical adenine-based antagonists (figure 6). The 8-substitued styryl residue in xanthines corresponded to the 2-substituent of adenosine derivatives which will fill the lipophilic pocket. The 2-oxo group and the N1-substituent in xanthines corresponded to the ribose moiety in adenosine derivatives and to the 2-furyl residue found in many antagonists. Electron-rich and hydrogen bond accepting groups are required here.

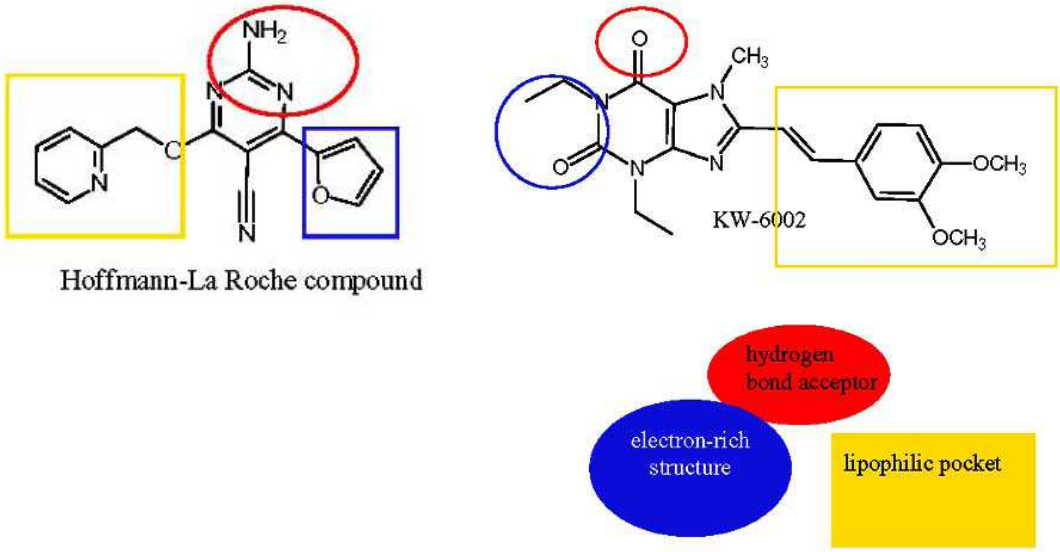


Figure 6: Pharmacophore model for adenosine A_{2A} receptor-selective xanthine derivatives compared to pharmacophore model of amino substituted nitrogen-containing heterocyclic compounds (Müller & Ferré, 2007).

3.3 ADENOSINE A_1 RECEPTOR

As mentioned before there are four different subtypes of adenosine receptors, A_1, A_{2A}, A_{2B} and A_3, of which the adenosine A_1 receptor is found in high density in the human brain, including the hippocampus and prefrontal cortex, which are important areas for cognitive function (Onodera & Kogure, 1988). Adenosine A_1 receptors play an important role in memory formation (Costenla *et al*., 1999; Normile & Barraco, 1991). In fact, administration of an adenosine A_1 agonist before passive avoidance training has shown to impair memory (Normile & Barraco, 1991), and selective adenosine A_1 antagonists enhanced cognition in rodents (Maemoto *et al*., 2004).

3.3.1 The adenosine A_1 receptor as cognitive therapy

Caffeine is the most commonly consumed substance worldwide and is the neuroactive stimulant in coffee. It is a xanthine based nonselective antagonist of adenosine and its behavioural effects result from its stimulating effects via blockade of adenosine A_1 and A_{2A} receptors (Brunyé *et al.,* 2010). These effects include stimulatory actions on alertness, attention, cognitive performance and reduced sleep (Prediger & Takahashi, 2005; Landolt *et al.,* 2004; Suzuki *et al*., 1993).

As mentioned above, caffeine is nonselective, thus antagonizing all types of adenosine receptors which attributes to most of its biological effects. The cognitive effects of caffeine are mostly due to its ability to antagonize adenosine A_1 receptors. Adenosine A_1 antagonists depolarize postsynaptic neurons and presynaptically enhance the release of a number of neurotransmitters, e.g. acetylcholine, glutamate, seretonin and norepinephrine. This release of neurotransmitters has the potential for treating cognitive deficits such as those associated with AD. These transmitters have been implicated in learning and memory, and are also reduced in AD (Rahman, 2009; Suzuki *et al.*, 1993). Several studies have also shown a reduced density of adenosine A_1 receptors along with reduced biding sites for adenosine agonists and antagonists in the molecular layer of the dentate gyrus of postmortem samples of AD patients (Ulas *et al.,* 1993; Kalaria *et al*., 1990). Adenosine A_1 antagonists have been proposed for the treatment of memory disorders (Schingnitz *et al.,* 1991).

3.3.2 Adenosine A_1 antagonists

One of the most important classes of adenosine A_1-selective antagonists are the xanthine based derivates with bulky C8-subtituents. The main requirements for adenosine A_1 receptor antagonist activity are given in figure 7 (Müller, 2001). Increasing the chain length at position N1 and N3, lead to an increase of adenosine A_1 affinity. Dipropyl substitution is optimal. In

the N3-position, more bulky groups are tolerated. Phenyl or cycloalkyl substitution in the 8-position lead to highly selective adenosine A_1 antagonists (figure 7).

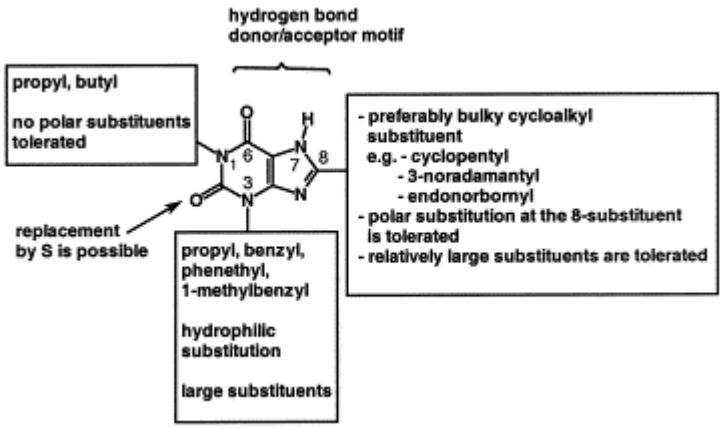
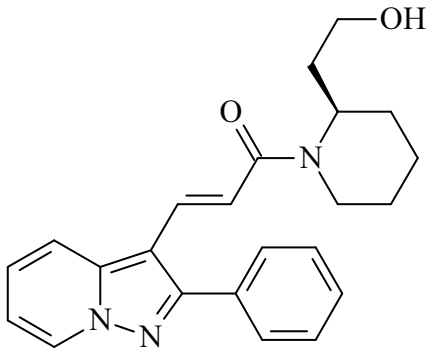


Figure 7: Structure-activity relationships of xanthine based adenosine A_1 antagonists (Müller, 2001).

Numerous other classes of heterocyclic derivatives are also known to bind to the adenosine A_1 receptor. One of these classes, structurally related to the xanthine core, are derivatives of the pyrazolo[1,5-α]pyridine nucleus. These compounds showed good affinity and selectivity for the adenosine A_1 receptor (**2**). Several modifications to this nucleus lead to increased potency and selectivity on the adenosine A_1 receptor. These modifications included constraining the acryloyl amide into a pyridazinone nucleus and substituting the nitrogen with an isobutyryl moiety (**3**) (Kuroda *et al.,* 2001; Kuroda *et al*., 2000; Kuroda *et al.,* 1999). Another nucleus, the naphtyridine (**4)** 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). Pyrimdine derivatives were also investigated and compound **5** was identified as a potent and selective human adenosine A_1 antagonist (Chang *et al*., 2004).



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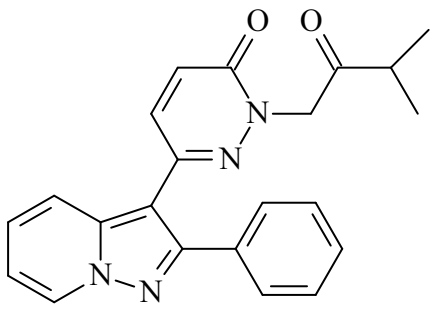

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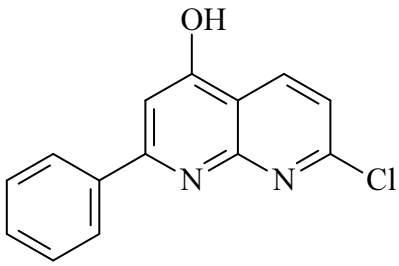
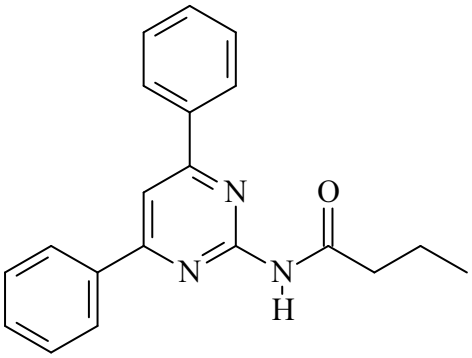


4



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3.4 **CONCLUSION**

Adenosine A_{2A} receptor antagonists exert motor stimulant effects, which play an important role in movement disorders such as PD. In addition, these antagonists are also neuroprotective. Adenosine A_1 receptors are important for cognitive function, and antagonism of these receptors can enhance cognition. Therefore, dual antagonism of both the adenosine A_1 and A_{2A} receptor may be potential for relieving motor and cognitive symptoms of PD.

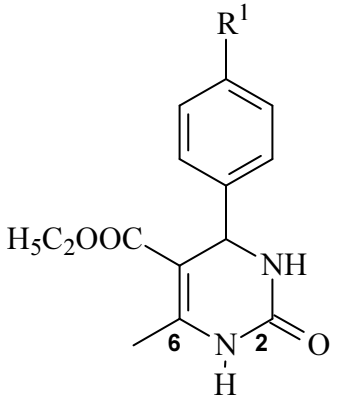
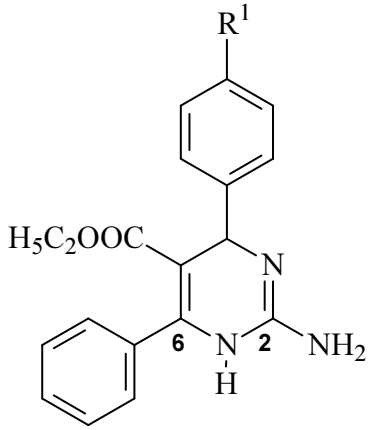
CHAPTER 4

SYNTHESIS

4.1 INTRODUCTION

As mentioned in the introduction, the main aim of this pilot study was to synthesise 3,4-dihydropyrimidin-2(1H)-ones (**1a-i**) and 2-amino-1,4-dihydropyrimidines (**2b-e**) and to evaluate their affinities for adenosine A_1 and A_{2A} receptors. A secondary aim was to investigate the MAO-inhibitory activities of selected compounds. The differentiating structural features of these series included the presence of a carbonyl group in position 2 and a methyl group in position 6 of series 1 (**1a-i**) versus the amino group (position 2) and phenyl group (position 6) in series 2 (**2b-e**). The structures of these compounds are given in table 3.

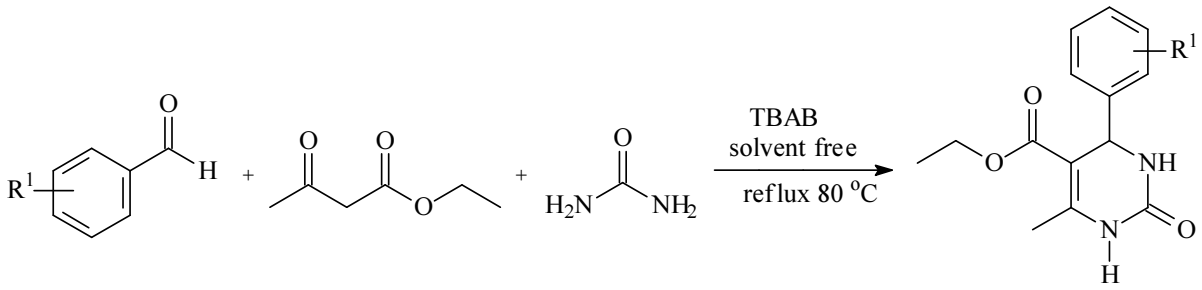
Table 3 The 3,4-dihydropyrimidin-2(1H)-ones (3,4-dihydropyrimidones) and 2-amino-1,4-dihydropyrimidine derivatives that were synthesised in this pilot study.

SERIES 1		**SERIES 2**	
3,4-dihydropyrimidine-2(1H)-ones		**2-amino-1,4-dihydropyrimidines**	
			
Compound	**R^1**	**Compound**	**R^1**
1a	H	2a*	
1b	4-Br	2b	4-Br
1c	4-Cl	2c	4-Cl
1d	4-OCH_3	2d	4-OCH_3
1e	4-CH_3	2e	4-CH_3
1f	4-NO_2	2f*	
1g	4-OH	2g*	
1h	4-F	2h*	
1i	4-CF_3	2i*	

*Synthesis attempted, but reaction failed.

4.2 GENERAL SYNTHETIC APPROACHES

4.2.1 Approach for the synthesis of 3,4-dihydropyrimidones (1a-i)



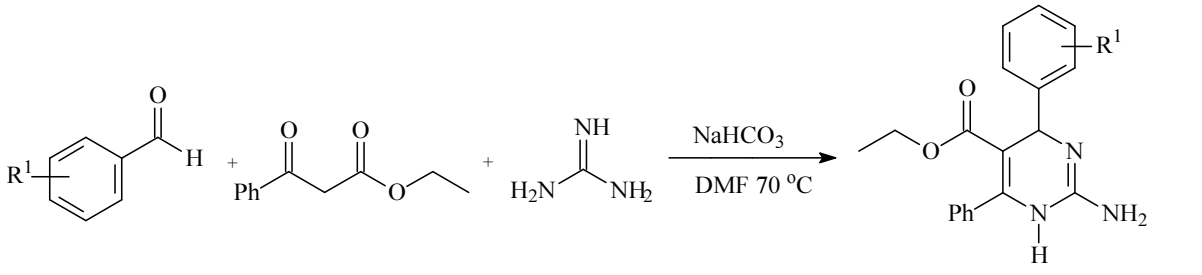
Aldehyde Diketone Urea 3,4-dihydropyrimidone

R^1 = H, F, Cl, Br, etc.
TBAB = Tetra butyl ammonium bromide

Scheme 1: Synthesis of 3,4-dihydropyrimidones.

A modified Biginelli one pot synthesis was used for the preparation of the target compounds under solvent free conditions (Kadre *et al*., 2012). A mixture of β-diketone (10 mmol), aldehyde (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was refluxed for an appropriate time at 80-100 ˚C and monitored by TLC. After completion of the reaction, the product was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford the 3,4-dihydropyrimidones in good yields (scheme 1).

4.2.2 Approach for the synthesis of 2-amino-1,4-dihydropyrimidines (2b-e)



Aldehyde Ethylbenzoylacetate Guanidine 2-amino-1,4-dihydropyrimidine

R^1 = H, F, Cl, Br, etc.
DMF = Dimethylformamide

Scheme 2: Synthesis of 2-amino-6-phenyl substituted 1,4-dihydropyrimidines.

The synthesis of the 2-amino-6-phenyl substituted 1,4-dihydropyrimidines was done according to the method of Vanden Eynde and co-workers (2001). The aldehyde (20 mmol), ethyl benzoylacetate (4.2 g, 3.8 ml, 22 mmol), guanidine hydrochloride (2.3 g, 24 mmol) and sodium hydrogen carbonate (6.7 g, 80 mmol) was suspended in DMF (40 mL) while stirring and heated for an appropriate time and monitored by TLC. The product was poured onto crushed ice (300 mL), filtered and washed with water (3x10 mL) to remove the sodium hydrogen carbonate and recrystallised from ethanol to afford these compounds in good yields (scheme 2).

4.3 **MATERIALS AND INSTRUMENTATION**

Sigma-Aldrich supplied all reagents used.

Thin layer chromatography (TLC):
All reactions were monitored by TLC. Silica gel 60 TLC plates (Merck) with UV_{254} fluorescent indicator was used for TLC. The mobile phase consisted of 98% dichloromethane and 2% methanol. The thin layer plates were visualised under a UV, at a wavelength of 254 nm.

Melting point (MP):
The melting points of the synthesised compounds were determined on a Buchi B-545 apparatus and they correspond to literature values.

Mass spectrometry (MS):
Synthesised compounds were characterised by mass spectra. High resolution mass spectra (HRMS) were recorded with a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionization (APCI), positive mode.

Nuclear magnetic resonance (NMR):
The structures of the dihydropyrimidones and 2-amino-dihydropyrimidines were also verified by proton (^{1}H) and carbon (^{13}C) NMR. A Bruker Avance III 600 spectrometer instrument was used at frequencies of 600 MHz and 150 MHz, for ^{1}H and ^{13}C NMR spectra, respectively. All samples for NMR were dissolved in DMSO-d_6. The chemical shifts (δ) are reported in parts per million (ppm) and were referenced to the residual solvent signal which was 2.50 and 39.5 ppm for ^{1}H and ^{13}C spectra respectively. Spin multiplicities are given as s (singlet), brs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), qn (quintet) or m (multiplet).

Infrared spectroscopy (IR):

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

All structures were confirmed with ^{1}H and ^{13}C NMR (Appendix I), whereas melting points, mass spectrometry (Appendix II) and infrared spectroscopy (Appendix III) were used to support and confirm the NMR results.

4.4 DETAILED SYNTHETIC PROCEDURES

4.4.1 Synthesis of 3,4-dihydropyrimidin-2(1H)-ones (series 1)

*4.4.1.1 Synthesis of 5-(ethoxycarbonyl)-6-methyl-4-(phenyl)-3,4-dihydropyrimidin-2(1H)-one (**1a**)*

A mixture of 4-benzaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 24 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford white crystals.

*4.4.1.2 Synthesis of 5-(ethoxycarbonyl)-4-(4-bromophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1b**)*

A mixture of 4-bromobenzaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 48 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford white crystals.

*4.4.1.3 Synthesis of 5-(ethoxycarbonyl)-4-(4-chlorophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1c**)*

A mixture of 4-chlorobenzaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 24 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was

filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford white crystals.

*4.4.1.4 Synthesis of 5-(ethoxycarbonyl)-4-(4-methoxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1d**)*

A mixture of *p*-anisaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 24 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford white crystals.

*4.4.1.5 Synthesis of 5-(ethoxycarbonyl)-4-(4-methylphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1e**)*

A mixture of *p*-tolualdehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 24 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford white crystals.

*4.4.1.6 Synthesis of 5-(ethoxycarbonyl)-4-(4-nitrophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1f**)*

A mixture of 4-nitrobenzaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 72 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford yellow crystals.

*4.4.1.7 Synthesis of 5-(ethoxycarbonyl)-4-(4-hydroxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1g**)*

A mixture of 4-hydroxybenzaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 48 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent,

was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford light yellow crystals.

*4.4.1.8 Synthesis of 5-(ethoxycarbonyl)-4-(4-fluorophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1h**)*

A mixture of 4-fluorobenzaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 24 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford white crystals.

*4.4.1.9 Synthesis of 5-(ethoxycarbonyl)-4-(4-trifluoromethylphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1i**)*

A mixture of 4-trifluoromethyl benzaldehyde (10 mmol), ethyl acetoacetate (10 mmol), urea (10 mmol) and tetra-butyl ammonium bromide (0.05 g, 1.5 M) was stirred and refluxed for 24 hours at 80 ˚C. Thin layer chromatography using methanol:dichloromethane (2:98) as eluent, was used to monitor the reaction. After completion, the product (which precipitated out), was filtered, washed with water (3x10 mL) to remove TBAB and recrystallised from ethanol to afford white crystals.

4.4.2 **Synthesis of 2-amino-6-phenyl-1,4-dihydropyrimidines**

*4.4.2.1 Synthesis of ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-bromophenyl)-pyrimidine-5-carboxylate (**2b**)*

A mixture of 4-bromobenzaldehyde (20 mmol), ethyl benzoylacetate (4.2 g, 3.8 ml, 22 mmol), guanidine hydrochloride (2.3 g, 24 mmol) and sodium hydrogen carbonate (6.7 g, 80 mmol) was suspended in DMF (40 mL) while stirring and heated for 72 hours at 70 ˚C. The reaction was monitored by TLC and after completion; the reaction mixture was cooled and poured onto crushed ice (300 mL) while stirring vigorously. The product was filtered and washed with water (3x10 mL) to remove the sodium hydrogen carbonate and recrystallised from ethanol to afford yellow crystals.

*4.4.2.2 Synthesis of ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-chlorophenyl)-pyrimidine-5-carboxylate (**2c**)*

A mixture of 4-chlorobenzaldehyde (20 mmol), ethyl benzoylacetate (4.2 g, 3.8 ml, 22 mmol), guanidine hydrochloride (2.3 g, 24 mmol) and sodium hydrogen carbonate (6.7 g, 80 mmol) was suspended in DMF (40 mL) while stirring and heated for 48 hours at 70 ˚C. The reaction was monitored by TLC and after completion; the reaction mixture was cooled and poured onto crushed ice (300 mL) while stirring vigorously. The product was filtered and washed with water (3x10 mL) to remove the sodium hydrogen carbonate and recrystallised from ethanol to afford light yellow crystals.

*4.4.2.3 Synthesis of ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-methoxyphenyl)-pyrimidine-5-carboxylate (**2d**)*

A mixture of *p*-anisaldehyde (20 mmol), ethyl benzoylacetate (4.2 g, 3.8 ml, 22 mmol), guanidine hydrochloride (2.3 g, 24 mmol) and sodium hydrogen carbonate (6.7 g, 80 mmol) was suspended in DMF (40 mL) while stirring and heated for 48 hours at 70 ˚C. The reaction was monitored by TLC and after completion; the reaction mixture was cooled and poured onto crushed ice (300 mL) while stirring vigorously. The product was filtered and washed with water (3x10 mL) to remove the sodium hydrogen carbonate and recrystallised from ethanol to afford light yellow crystals.

*4.4.2.4 Synthesis of ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-methylphenyl)-pyrimidine-5-carboxylate (**2e**)*

A mixture of *p*-tolualdehyde (20 mmol), ethyl benzoylacetate (4.2 g, 3.8 ml, 22 mmol), guanidine hydrochloride (2.3 g, 24 mmol) and sodium hydrogen carbonate (6.7 g, 80 mmol) was suspended in DMF (40 mL) while stirring and heated for 48 hours at 70 ˚C. The reaction was monitored by TLC and after completion; the reaction mixture was cooled and poured onto crushed ice (300 mL) while stirring vigorously. The product was filtered and washed with water (3x10 mL) to remove the sodium hydrogen carbonate and recrystallised from ethanol to afford light yellow crystals.

4.5 PHYSICAL CHARACTERIZATION

4.5.1 Physical data for 3,4-dihydropyrimidin-2(1H)-ones (series 1).

*4.5.1.1 Compound 5-(ethoxycarbonyl)-6-methyl-4-(phenyl)-3,4-dihydropyrimidin-2(1H)-one (**1a**)*

The title compound was prepared from 4-benzaldehyde, ethyl acetoacetate and urea in a yield of 87%: mp 205.8 - 206.6 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.08 (t, 3H, J = 7.15 Hz), 2.24 (s, 3H), 3.97 (q, 2H, J = 7.15 Hz), 5.13 (d, 1H, J = 3.39 Hz), 7.22-7.31 (m, 5H), 7.74 (s, 1H), 9.19 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.1, 17.8, 54.0, 59.2, 99.3, 126.3, 127.3, 128.4, 144.9, 148.4, 152.2, 165.3; APCI-HRMS *m*/*z*: calcd for $C_{14}H_{17}N_2O_3$, 261.1234, found (MH^+) 261.1229.

*4.5.1.2 Compound 5-(ethoxycarbonyl)-4-(4-bromophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1b**)*

The title compound was prepared from 4-bromobenzaldehyde, ethyl acetoacetate and urea in a yield of 90%: mp 222.9 - 223.2 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.08 (t, 3H, J = 7.15 Hz), 2.23 (s, 3H), 3.97 (q, 2H, J = 7.15), 5.11 (d, 1H, J = 3.39 Hz), 7.17 (d, 2H, J = 8.66 Hz), 7.52 (d, 2H, J = 8.66 Hz), 7.77 (s, 1H), 9.24 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.1, 17.8, 53.5, 59.3, 98.7, 120.3, 128.6, 131.3, 144.2, 148.8, 151.9, 165.2; APCI-HRMS *m*/*z*: calcd for $C_{14}H_{16}BrN_2O_3$, 339.0339, found (MH^+) 339.0299.

*4.5.1.3 Compound 5-(ethoxycarbonyl)-4-(4-chlorophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1c**)*

The title compound was prepared from 4-chlorobenzaldehyde, ethyl acetoacetate and urea in a yield of 95%: mp 215.1 - 216.2 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.08 (t, 3H, J = 7.15 Hz), 2.23 (s, 3H), 3.96 (q, 2H, J = 7.15 Hz), 5.13 (d, 1H, J = 3.39 Hz), 7.23 (d, 2H, J = 8.28 Hz), 7.38 (d, 2H, J = 8.28 Hz), 7.76 (s, 1H), 9.23 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.1, 17.9, 53.5, 59.4, 98.9, 128.3, 128.5, 131.9, 143.8, 148.8, 152.0, 165.3; APCI-HRMS *m*/*z*: calcd for $C_{14}H_{16}ClN_2O_3$, 295.0844, found (MH^+) 295.0836.

4.5.1.4 *Compound 5-(ethoxycarbonyl)-4-(4-methoxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1d**)*

The title compound was prepared from *p*-anisaldehyde, ethyl acetoacetate and urea in a yield of 94%: mp 205.3 - 206.4 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.09 (t, 3H, J = 7.15), 2.23 (s, 3H), 3.70 (s,3H), 3.96 (q, 2H, J = 7.15), 5.08 (d, 1H, J = 3.39 Hz), 6.86 (d, 2H, J = 8.66 Hz), 7.13 (d, 2H, J = 8.66 Hz), 7.66 (s, 1H), 9.14 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.1, 17.8, 53.3, 55.1, 59.2, 99.6, 113.7, 127.4, 137.1, 148.0, 152.2, 158.5, 165.4; APCI-HRMS *m*/*z*: calcd for $C_{15}H_{19}N_2O_4$, 291.1339, found (MH^+) 291.1331.

4.5.1.5 *Compound 5-(ethoxycarbonyl)-4-(4-methylphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1e**)*

The title compound was prepared from *p*-tolualdehyde, ethyl acetoacetate and urea in a yield of 93%: mp 216.3 - 216.9 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.08 (t, 3H, J = 7.15 Hz), 2.22 (s, 3H), 2.24 (s, 3H), 3.96 (q, 2H, J = 7.15 Hz), 5.09 (d, 1H, J = 3.39 Hz), 7.10 (s, 4H), 7.68 (s, 1H), 9.14 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.2, 17.8, 20.7, 53.7, 59.3, 99.5, 126.2, 129.0, 136.5, 142.0, 148.2, 152.3, 165.4; APCI-HRMS *m*/*z*: calcd for $C_{15}H_{19}N_2O_3$, 275.1390, found (MH^+) 275.1384.

4.5.1.6 *Compound 5-(ethoxycarbonyl)-4-(4-nitrophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1f**)*

The title compound was prepared from 4-nitrobenzaldehyde, ethyl acetoacetate and urea in a yield of 92%: mp 206.9 - 209.6 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.08 (t, 3H, J = 7.15 Hz), 2.25 (s, 3H), 3.97 (q, 2H, J = 7.15 Hz), 5.26 (d, 1H, J = 3.39 Hz), 7.49 (d, 2H, J = 8.66 Hz), 7.89 (s, 1H), 8.21 (d, 2H, J = 8.66 Hz), 9.35 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.1, 17.9, 53.7, 59.4, 98.2, 123.9, 127.7, 146.7, 149.4, 151.8, 152.0, 165.1; APCI-HRMS *m*/*z*: calcd for $C_{14}H_{16}N_3O_5$, 306.1084, found (MH^+) 306.1078.

4.5.1.7 *Compound 5-(ethoxycarbonyl)-4-(4-hydroxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1g**)*

The title compound was prepared from 4-hydroxybenzaldehyde, ethyl acetoacetate and urea in a yield of 90%: mp 232.7 - 234.2 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.08 (t, 3H J = 7.15 Hz), 2.21 (s, 3H), 3.96 (q, 2H, J = 7.15), 5.02 (d, 1H, J = 3.01 Hz), 6.67

(d, 2H, J = 8.66 Hz), 7.0 (d, 2H, J = 8.66 Hz), 7.61 (s, 1H), 9.10 (s, 1H), 9.36 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.2, 17.8, 53.5, 59.2, 99.8, 115.1, 127.5, 135.5, 147.8, 152.3, 156.6, 165.5; APCI-HRMS *m*/*z*: calcd for $C_{14}H_{17}N_2O_4$, 277.1183, found (MH^+) 277.1172.

*4.5.1.8 Compound 5-(ethoxycarbonyl)-4-(4-fluorophenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1h**)*

The title compound was prepared from 4-fluorobenzaldehyde, ethyl acetoacetate and urea in a yield of 91%: mp 182.2 - 183.2 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.07 (t, 3H, J = 7.15 Hz), 2.24 (s, 3H), 3.95 - 3.98 (m, 2H), 5.13 (d, 1H, J = 3.01 Hz), 7.14 (t, 2H), 7.24 - 7.26 (m, 2H), 7.74 (s, 1H), 9.22 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.1, 17.8, 53.3, 59.2, 99.1, 115.1, 115.2, 128.2, 128.3, 141.1, 148.5, 152.0, 160.5, 162.1, 165.3; APCI-HRMS *m*/*z*: calcd for $C_{14}H_{16}FN_2O_3$, 279.1139, found (MH^+) 279.1129.

*4.5.1.9 Compound 5-(ethoxycarbonyl)-4-(4-trifluoromethylphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (**1i**)*

The title compound was prepared from 4-trifluoromethyl benzaldehyde, ethyl acetoacetate and urea in a yield of 92%: mp 179.0 - 180.9°C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 1.07 (t, 3H, J = 7.15), 2.25 (s, 3H), 3.96 - 3.99 (m, 2H), 5.22 (d, 1H, J = 3.39 Hz), 7.44 (d, 2H, J = 8.28 Hz), 7.70 (d, 2H, J = 8.28 Hz), 7.84 (s, 1H), 9.30 (s, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 14.1, 17.9, 53.8, 59.4, 98.6, 123.4, 125.2, 125.5, 127.2, 127.9, 128.1, 149.1, 149.3, 151.9, 165.2, 165.2; APCI-HRMS *m*/*z*: calcd for $C_{15}H_{16}F_3N_2O_3$, 329.1108, found (MH^+) 329.1090.

4.5.2 Physical data for 2-amino-6-phenyl-1,4-dihydropyrimidines (series 2)

*4.5.2.1 Compound ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-bromophenyl)-pyrimidine-5-carboxylate (**2b**)*

The title compound was prepared from 4-bromobenzaldehyde, ethyl benzoylacetate and guanidine hydrochloride in a yield of 92 %: mp 227.0 - 227.1 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 0.73 (t, 3H, J = 7.15), 3.67 (q, 2H, J = 7.15), 5.28 (s, 1H), 6.44 (bs, 2H), 7.22 - 7.27 (m, 6H), 7.31 (d, 2H), 7.54 (d, 2H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 13.6, 52.3, 58.2, 97.2, 120.1, 126.9, 127.2, 128.1, 128.6, 131.2, 142.1, 145.5, 155.2, 166.0, 166.1; APCI-HRMS *m*/*z*: calcd for $C_{19}H_{19}BrN_3O_2$, 400.0655, found (MH^+) 400.0633.

4.5.2.2 *Compound ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-chlorophenyl)-pyrimidine-5-carboxylate (**2c**)*

The title compound was prepared from 4-chlorobenzaldehyde, ethyl benzoylacetate and guanidine hydrochloride in a yield of 94%: mp 236.5 - 238.1 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 0.73 (t, 3H, J = 7.15), 3.67 (q, 2H, J = 7.15), 5.28 (s, 1H), 6.37 (bs, 2H), 7.22 - 7.41 (m, 9H), 7.56 (bs, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 13.7, 52.2, 58.2, 97.0, 126.8, 127.1, 128.1, 128.2, 128.3, 131.6, 142.5, 145.2, 155.4, 161.6, 166.1; APCI-HRMS *m*/*z*: calcd for $C_{19}H_{19}ClN_3O_2$, 356.1160, found (MH^+) 356.1143.

4.5.2.3 *Compound ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-methoxyphenyl)-pyrimidine-5-carboxylate (**2d**)*

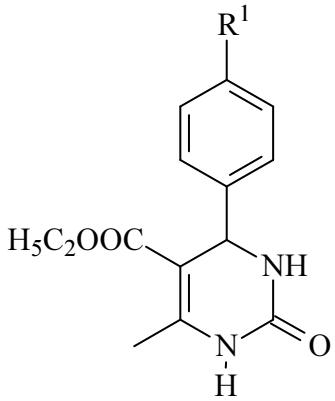
The title compound was prepared from p-anisaldehyde, ethyl benzoylacetate and guanidine hydrochloride in a yield of 93%: mp 234.1 - 235.2 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 0.73 (t, 3H, J = 7.15), 3.66 (q, 2H, J = 7.15), 3.72 (s 3H) 5.24 (s, 1H), 6.26 (bs, 2H), 6.89 (d, 2H), 7.22 - 7.28 (m, 7 H), 7.40 (bs, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 13.7, 52.3, 55.0, 58.0, 97.8, 113.7, 126.8, 126.9, 127.5, 128.1, 138.5, 142.7, 155.3, 158.4, 161.0, 166.2; APCI-HRMS *m*/*z*: calcd for $C_{20}H_{22}N_3O_3$, 352.1656, found (MH^+) 352.1649.

4.5.2.4 *Compound ethyl-2-amino-1,4-dihydro-6-phenyl-4-(4-methylphenyl)-pyrimidine-5-carboxylate (**2e**)*

The title compound was prepared from p-tolualdehyde, ethyl benzoylacetate and guanidine hydrochloride in a yield of 90%: mp 235.5 - 241.7 °C (ethanol). ^{1}H NMR (Bruker Avance III 600, DMSO-d_6) δ 0.73 (t, 3H, J = 7.15), 2.26 (s, 3H), 3.66 (q, 2H, J = 7.15), 5.24 (s, 1H), 6.27 (bs, 2H), 7.13 (d, 2H) 7.21 - 7.25 (m, 7H), 7.43 (bs, 1H). ^{13}C NMR (Bruker Avance III 600, DMSO-d_6) δ 13.7, 20.7, 52.6, 58.1, 97.6, 126.2, 126.8, 127.0, 128.1, 128.9, 136.2, 142.7, 143.4, 155.4, 161.1, 166.2; APCI-HRMS *m*/*z*: calcd for $C_{20}H_{20}N_3O_2$, 334.1550, found (MH^-) 334.1566.

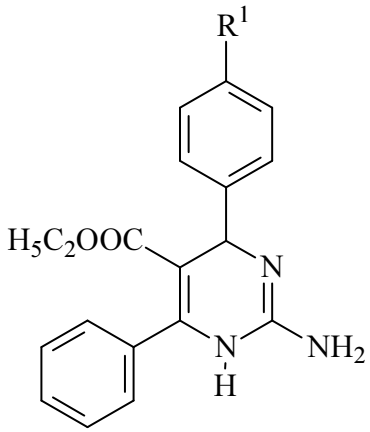
4.5.3 **Interpretation of IR spectra (see Appendix III)**

*4.5.3.1 3,4-Dihydropyrimidin-2(1H)-ones (**1a-i**).*



IR (cm^{-1}): 3260 cm^{-1} (NH stretch), 3165 cm^{-1} (NH), 1750 - 1680 cm^{-1} (C=O), 1670 - 1640 cm^{-1} (C=O) 1100 - 1200 cm^{-1} (C-O-C)

*4.5.3.2 2-Amino-6-phenyl-1,4-dihydropyrimidines (**2b-e**)*



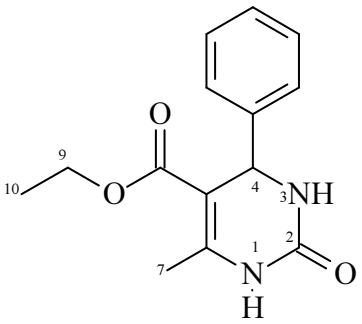
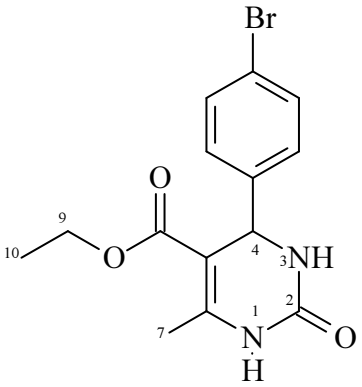
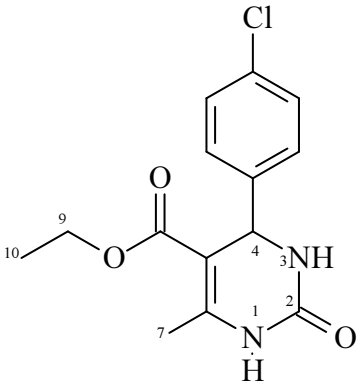
IR (cm^{-1}): 3450, 3350 cm^{-1} (NH_2), 1600 - 1650 cm^{-1} (C=O) 1450 cm^{-1} (phenyl ring), 1100 - 1200 cm^{-1} (C-O-C)

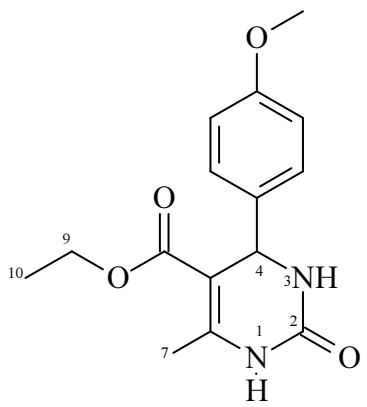
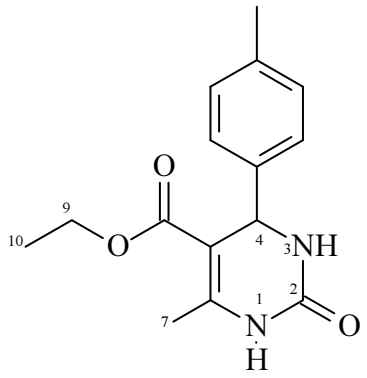
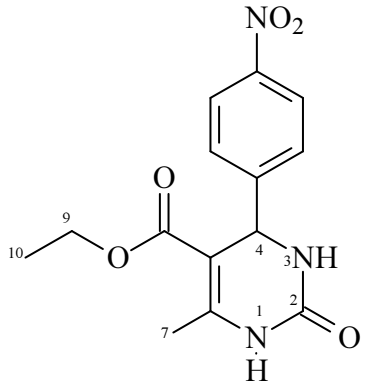
4.5.4 **Interpretation of the NMR spectra (see Appendix I)**

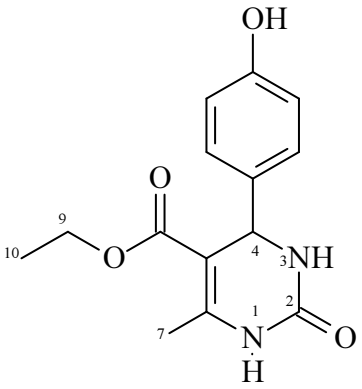
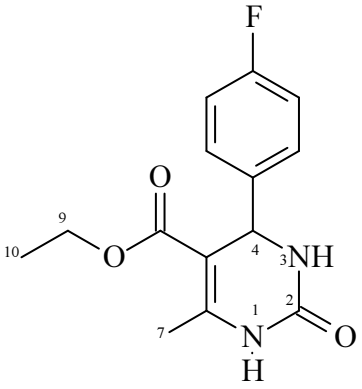
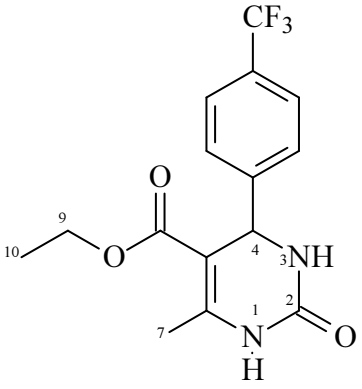
In table 4 the structures of the 3,4-dihydropyrimidone and 2-amino-1,4-dihydropyrimidine analogues are given and in conjunction with the ^{1}H NMR assignments. All the signals expected (with appropriate integrals and coupling constants) for these compounds, **1a**-**i** and **2b**-**e** were observed. These include the proton signals of the aliphatic chain, the two NH, a singlet for the methyl substituent on the pyrimidine ring (series 1), the H-4 proton signal and

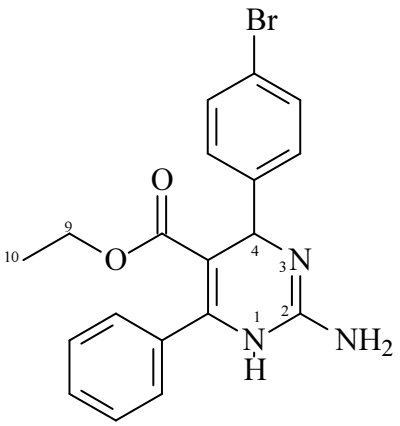
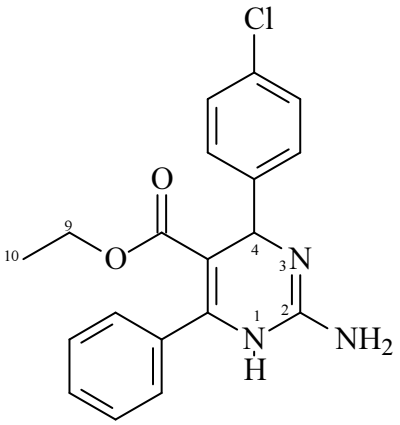
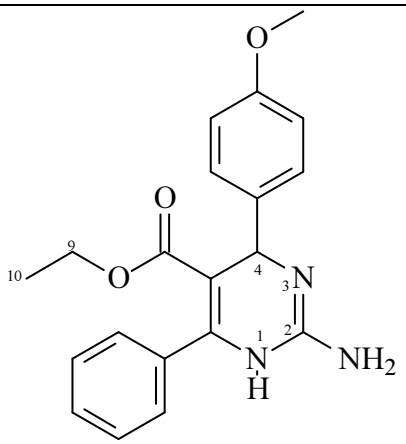
the signals of the aromatic protons. In addition, the ^{13}C NMR data (not tabulated) also correlated with the proposed structures and their expected chemical shifts.

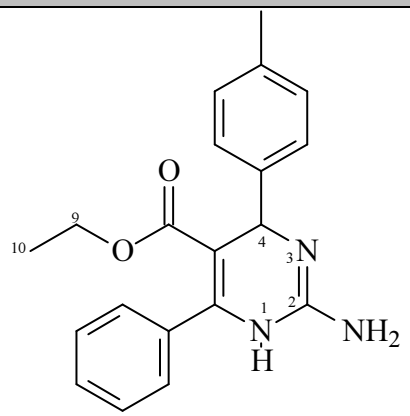
Table 4 Correlation of the ^{1}H NMR data with the structures of 3,4-dihydropyrimidone (**1a-i**) and the 2-amino-1, 4-dihydropyrimidine (**2b-e**) derivatives.

	Structure	**^{1}H NMR signal assignment**
1a		• Methyl (CH_3) groups at position 10 and 7 - signals at 1.08 (t, 3H), 2.24 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.97 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.13 (d, 1H) ppm. • Aromatic protons - signals at 7.22 - 7.31 (m, 5H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.74 (s, 1H), 9.19 (s, 1H) ppm.
1b		• Methyl (CH_3) groups at position 10 and 7 - signals at 1.08 (t, 3H), 2.23 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.97 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.11 (d, 1H) ppm. • Aromatic protons - signals at 7.17 (d, 2H), 7.52 (d, 2H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.77 (s, 1H), 9.24 (s, 1H) ppm.
1c		• Methyl (CH_3) groups at position 10 and 7 - signals at 1.08 (t, 3H), 2.23 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.96 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.13 (d, 1H) ppm. • Aromatic protons - signals at 7.23 (d, 2H), 7.38 (d, 2H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.76 (s, 1H), 9.23 (s, 1H) ppm.

	Structure	^{1}H NMR signal assignment
1d		• Methyl (CH_3) groups at position 10 and 7, and methoxy (OCH_3) group - signals at 1.09 (t, 3H), 2.23 (s, 3H), 3.70 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.96 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.08 (d, 1H) ppm. • Aromatic protons - signals at 6.86 (d, 2H), 7.13 (d, 2H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.66 (s, 1H), 9.14 (s, 1H) ppm.
1e		• Methyl (CH_3) groups at position 10 and 7, and on the para aromatic ring - signals at 1.08 (t, 3H), 2.22 (s, 3H), 2.24 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.96 (q, 2H). • Pyrimidine proton at position 4 - signal at 5.09 (d, 1H) ppm. • Aromatic protons - signal at 7.10 (s, 4H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.68 (s, 1H), 9.14 (s, 1H) ppm.
1f		• Methyl (CH_3) groups at position 10 and 7 - signals at 1.08 (t, 3H), 2.25 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.97 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.26 (d, 1H) ppm. • Aromatic protons - signals at 7.49 (d, 2H), 8.21 (d, 2H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.89 (s, 1H), 9.35 (s, 1H) ppm.

	Structure	^{1}H NMR signal assignment
1g		• Methyl (CH_3) groups at position 10 and 7 - signals at 1.08 (t, 3H), 2.21 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.96 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.02 (d, 1H,) ppm. • Aromatic protons - signals at 6.67 (d, 2H), 7.0 (d, 2H), ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.61 (s, 1H), 9.10(s, 1H) ppm. • Hydroxyl proton - signal at 9.36 (s, 1H) ppm.
1h		• Methyl (CH_3) groups at position 7 and 10 - signals at 1.07 (t, 3H), 2.24 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.95 - 3.98 (m, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.13 (d, 1H) ppm. • Aromatic protons - signals at 7.14 (t, 2H) 7.24 - 7.26 (m, 2H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.74 (s, 1H), 9.22 (s, 1H) ppm.
1i		• Methyl (CH_3) groups at position 7 and 10 - signals at 1.07 (t, 3H), 2.25 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.96 - 3.99 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.22 (d, 1H) ppm. • Aromatic protons - signals at 7.44 (d, 2H), 7.70 (d, 2H) ppm. • Amine groups (NH) at position 1 and 3 - signals at 7.84 (s, 1H), 9.30 (s, 1H) ppm.

	Structure	^{1}H NMR signal assignment
2b		• Methyl (CH_3) group at position 10 - signal at 0.73 (t, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.67 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.28 (s, 1H) ppm. • Amine group (NH_2) at position 2 - signal at 6.44 (bs, 2H) ppm. • Aromatic protons and amine group (NH) at position 1 - signals at 7.22 - 7.71 (m, 6H), 7.31 (d, 2H), 7.54 (d, 2H) ppm.
2c		• Methyl (CH_3) group at position 10 - signal at 0.73 (t, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.67 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.28 (s, 1H) ppm. • Amine group (NH_2) at position 2 - signal at 6.37 (bs, 2H) ppm. • Aromatic protons - signals at 7.22 - 7.41 (m, 9H) ppm. • Amine group (NH) at position 1 - signal at 7.56 (bs, 1H) ppm.
2d		• Methyl (CH_3) group at position 10 - signal at 0.73 (t, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.66 (q, 2H) ppm. • Methoxy group - signal at 3,72 (s, 3H). • Pyrimidine proton at position 4 - signal at 5.24 (s, 1H) ppm. • Amide group (NH_2) at position 2 - signal at 6.26 (bs, 2H) ppm. • Aromatic protons - signals at 6.89 (d, 2H), 7.22 - 7.28 (m, 7 H) ppm. • Amine groups (NH) at position 1 - signal at 7.40 (bs, 1H) ppm.

	Structure	^{1}H NMR signal assignment
2e		• Methyl (CH_3) group at position 10 and the aromatic methyl group - signals at 0.73 (t, 3H), 2.26 (s, 3H) ppm. • Methylene group (CH_2) at position 9 - signal at 3.66 (q, 2H) ppm. • Pyrimidine proton at position 4 - signal at 5.24 (s, 1H) ppm. • Amide group (NH_2) at position 2 - signal at 6.27 (bs, 2H) ppm. • Aromatic protons - signals at 7.13 (d, 2H), 7.21 - 7.25 (m, 7H) ppm. • Amine groups (NH) at position 1 - signal at 7.43 (bs, 1H) ppm.

4.6 **CONCLUSION**

This chapter described the successful synthesis of the target 3,4-dihydropyrimidine-2(1H)-one (**1a-i**) and 2-amino-1,4-dihydropyrimidine (**2b-e**) derivatives. The structures of compounds were confirmed by NMR spectroscopy and supported by MS and IR data. Both the ^{1}H NMR and ^{13}C NMR spectra corresponded with the proposed structures and the melting points recorded for each compound corresponded with those reported in literature.

CHAPTER 5

MAO-B INHIBITION STUDIES

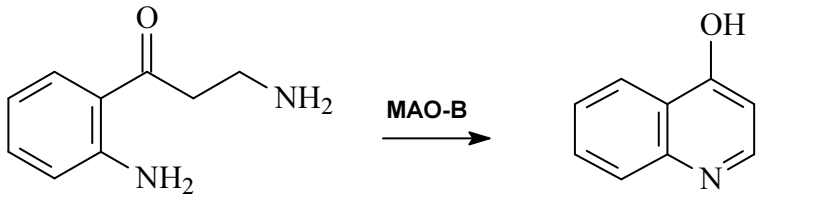
5.1 INTRODUCTION

In addition to its adenosine A_{2A} receptor blocking activity, CSC (see 2.7.5 for structure) has been reported to be a potent MAO-B inhibitor (Vlok *et al.,* 2006; Chen *et al.,* 2002). Therefore, the current pilot study also evaluated the dihydropyrimidones and amines as inhibitors of MAO-B.

In this study a fluorometric assay was used to measure the catalytic activity of MAO-B. For this purpose, kynuramine was used as substrate B. The extent by which the inhibitor reduces the MAO-catalyzed oxidation of kynuramine to 4-hydroxyquinoline, a fluorescent metabolite, was measured with a fluorescence spectrophotometer.

5.2 INSTRUMENTATION AND CHEMICALS

A Varian Cary Eclipse fluorescence spectrophotometer was used at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. Insect cell microsomes containing recombinant human MAO-B (5 mg/ml) and kynuramine.2HBr were obtained from Sigma-Aldrich. The Graphpad Prism 5 software package was used to construct sigmoidal dose-response curves and to determine IC_{50} values. The IC_{50} value is the concentration of the inhibitor at which 50% of the enzyme is inhibited. Fluorometric assays are generally more sensitive than spectrophotometric assays (Zhou *et al*., 1996; Matsumoto *et al*., 1985).



Kynuramine **4-Hydroxyquinoline**

Figure 8 The oxidation of kynuramine to 4-hydroxyquinoline by MAO-B.

5.3 METHOD

Recombinant human MAO-B (5 mg/mL) was pre-aliquoted and stored at -80 ºC. For the enzyme activity measurements, the enzyme was diluted to 0.15 mg/mL. This stock solution of the enzyme was prepared in potassium phosphate buffer (100 mM, pH 7.4).

The reactions were prepared in 500 µL potassium phosphate buffer (100 mM, pH 7.4) to which was added various concentrations of the test inhibitor, kynuramine (substrate) and the MAO-B enzyme. The concentrations of the test inhibitors were 0, 0.003, 0.01, 0.1, 1, 10, and 100 µM. Kynuramine was added to the incubations to yield a final concentration of 30 µM. DMSO, 4% (v/v), was added as co-solvent and the final enzyme concentration was 0.0075 mg/mL .

The reactions were incubated at 37 ºC for 20 minutes and subsequently terminated by the addition of 400 µL NaOH (2 N). Distilled water (1000 µL) were added to each incubation to make up the final volume, and the resulting reactions were centrifuged for 10 minutes at 16 000 g. The fluorescence intensities of the supernatant fractions were measured at an excitation wavelength of 310 nm and an emission wavelength of 400 nm to determine the concentration of 4-hydroxyquinoline. The PMT voltage was set to medium with an excitation sit width of 5 nm and emission slit width of 10 nm.

To quantify the amounts of 4-hydroxyquinoline formed in the reactions, a calibration curve was prepared by measuring the fluorescence of increasing concentrations of 4-hydroxyquinoline (0.0469 - 1.5 µM). Each of these calibration standards contained 4% (v/v) DMSO in potassium phosphate buffer to a final volume of 500 µL. To each calibration standard were added 400 µL of NaOH and 1000 µL of distilled water. Control samples were included to confirm that the test inhibitors do not fluoresce or quench the fluorescence of 4-hydroxyquinoline.

Employing Prism 5, a sigmoidal dose-response curve was constructed to determine the IC_{50} values. For this purpose the initial rate of oxidation versus the logarithm of the inhibitor concentration was plotted. The IC_{50} values were determined in triplicate and are expressed as mean ± standard deviation (SD).

5.4 RESULTS AND CONCLUSION

None of the dihydropyrimidones or amines suppressed MAO-B catalytic activity, thus indicating that these compounds did not exhibit any MAO-B inhibitory activities.

To provide a possible explanation for the lack of MAO-B inhibition of the test compounds, the architecture of the MAO-B active site may be considered. The active site of MAO-B has a substrate cavity interconnected to an entrance cavity, with Ile199 as a conformational gate separating these two cavities (Binda *et al.,* 2003). The substrate cavity has a number of aromatic residues, where two tyrosyl side chains (Tyr398 and Tyr435) form a tight “aromatic

sandwich" perpendicular to the flavin (Figure 9). A substrate must first be able to pass through this "aromatic sandwich" before the substrate amino group is able to interact with the flavin moiety of the flavin adenine dinucleotide (FAD) cofactor for amine oxidation (Hubálek *et al.,* 2005). In general, it has been observed that compounds with larger structures often are more potent MAO-B inhibitors than smaller compounds. For example, CSC (a relatively large structure) is a highly potent MAO-B inhibitor, while caffeine (a smaller structure) is reported to be several orders of magnitude weaker as an MAO-B inhibitor. The higher potency inhibition observed for CSC compared to caffeine may be explained by the proposal that CSC interacts with both the substrate and entrance cavities of MAO-B, while caffeine is thought to bind only to the substrate cavity. The additional productive interactions with the residues of the entrance cavity may explain the high affinity of CSC for the MAO-B active site. For CSC to interact with both the substrate and entrance cavities of MAO-B, the caffeine and phenyl moieties should be co-planar.

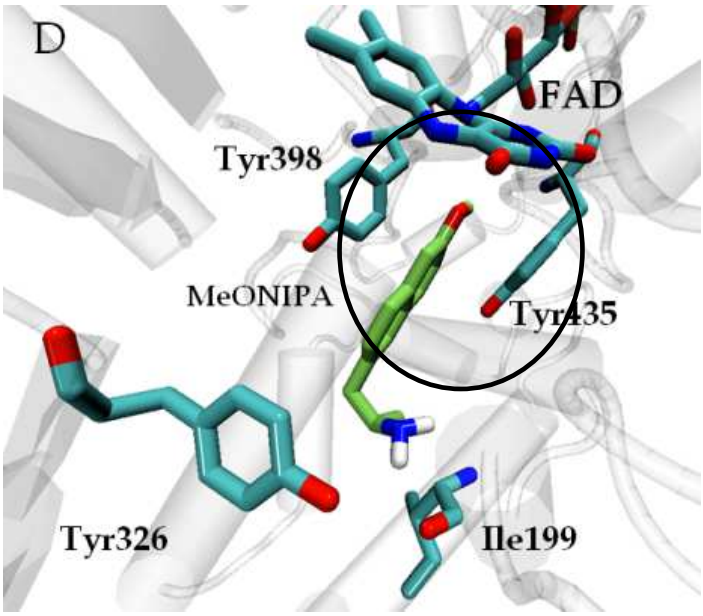


Figure 9: Molecular docking of 6-methoxy-2-naphthylisopropylamine into the MAO-B substrate cavity, showing the tight aromatic sandwich (circled) formed by Tyr398 and Tyr435 (Fierro *et al.,* 2013).

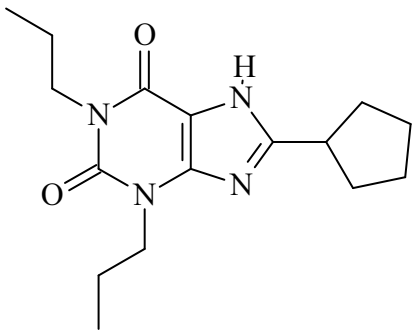
If co-planarity is also a requirement for binding of the dihydropyrimidones or amines to the MAO-B active site, it may be expected that these compound will not be MAO-B inhibitors. The phenyl rings of the dihydropyrimidones or amines examined here are not expected to be co-planar, which may prevent these compounds from binding simultaneously with the two MAO-B active site cavities.

CHAPTER 6

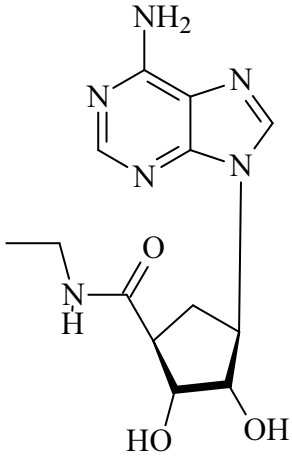
RADIOLIGAND BINDING STUDIES

6.1 INTRODUCTION

Antagonism of adenosine A_1 and A_{2A} receptors mediates several important physiological effects such as cognitive enhancement and motor activation, respectively. The adenosine A_1 receptor inhibits adenylate cyclase via the inhibitory guanine nucleotide binding protein G_i, while the adenosine A_{2A} receptor stimulates adenylate cyclase via Gs. Adenosine receptors have been characterized in different tissues by both functional and binding studies. Using the rank order affinities of agonists and antagonists it is possible to distinguish between receptor subtypes via radioligand binding assays. Radioligand binding assays for adenosine A_1 and A_{2A} receptors are well characterised and are routinely used (Bruns *et al.,* 1986).



DPCPX



NECA

The synthesised 3,4-dihydropyrimidones and 2-amino-1,4-dihydropyrimidines (table 3) were investigated for both adenosine A_1 and A_{2A} affinity and compared to the 1,4-dihydropyrimidines examined previously (van Rhee *et al.,* 1996). The experimental methods as well as the results of *in vitro* evaluations are discussed in this chapter.

6.3 EXPERIMENTAL PROCEDURE:

6.3.1 Materials used in adenosine A_1 and A_{2A} radioligand binding assays

Reagents obtained from Sigma Aldrich included the following: Anhydrous magnesium chloride ($MgCl_2$), silicone solution (Sigma-cote®), adenosine deaminase (0.15 mL, 11 mg protein/mL, 151 units/mg protein), N^6-cyclopentyladenosine (CPA), Trizma hydrochloride

and Trizma base. The radioligands, 5'-N-ethylcarboxamido[^{3}H]adenosine ([^{3}H]NECA) and 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) were obtained from Amersham Biosciences. Dimethyl sulfoxide (DMSO) and Whatman GF/B 25 mm diameter filters were sourced from Merck), while scintillation vials and filter count scintillation fluid were obtained from Perkin Elmer.

Table 5 Radioligands and tissue types used in the adenosine A_{2A} and A_1 assay

	A_{2A} radioligand binding assay	**A_1 radioligand binding assay**
Tissue	Rat striatal membranes	Rat whole brain membranes
Radioligand	[^{3}H]NECA	[^{3}H]DPCPX

6.3.2 **Adenosine A_{2A} radioligand binding assay**

The affinities of the synthesised compounds for the adenosine A_{2A} receptor were evaluated with a radioligand binding protocol described in literature (Van der Walt *et al*., 2013; Bruns *et al.,* 1986). For this radioligand binding assay rat striatum was used as receptor source and [^{3}H]NECA as the radioligand. [^{3}H]NECA is non selective and binds to both the adenosine A_1 and A_{2A} receptor subtypes. In order to exclude binding of [^{3}H]NECA to the adenosine A_1 receptor, the radioligand binding study was done in the presence of CPA. CPA has a higher affinity for the adenosine A_1 receptor than [^{3}H]NECA and displaces [^{3}H]NECA from this receptor, leaving only the adenosine A_{2A} receptors available for binding. The radioactivity measured is therefore an indication of only the adenosine A_{2A} receptor binding. The affinity of the test compound is determined by its ability to displace [^{3}H]NECA from the A_{2A} receptors. If a compound has a high affinity for the A_{2A} receptor, [^{3}H]NECA will be displaced from the receptor, and the residual radioactivity will be low, while the opposite is true for a compound with weak or no affinity for the A_{2A} receptor.

Tissue preparation

Approval for the collection of rat tissue samples (striatum and whole brain) was obtained from the North-West University Research Ethics Committee (**NWU-0035-10-A5**). The Vivarium provided male Sprague Dawley rats weighing between 200 - 500 g for dissection to obtain the rat striata. Dissection was done on ice and the tissue samples were immediately snap frozen with liquid nitrogen and stored at -70 °C.

When required, the rat striata and whole brains were thawed on ice, weighed and then suspended in 10 volumes (10 volumes of original tissue weight) of ice cold 50 mM Tris (pH 7.7, 25 °C, pH 8.26 at 5 °C) and homogenised (30 s for striata and 90 s for whole brains)

with a Polytron PT-10 homogeniser (Brinkman). The resulting homogenate was centrifuged for 10 min at 50 000 x g at 4 ^{o}C. The supernatant was decanted and the remaining pellet was resuspended in 10 volumes of ice cold Tris and homogenised as before. The resulting homogenate was centrifuged as previously described and the final pellet was suspended in ice cold Tris to yield a suspension containing 1 g/5 mL of the original tissue weight. The membrane suspension was aliquoted to eppendorf tubes and stored at -70 °C until needed.

Assay preparation:

All stock solutions of the test compounds (10 mM) and CPA (10 mM) were prepared in DMSO. [^{3}H]NECA and CPA were diluted with 50 mM Tris to concentrations of 40 nM and 500 nM, respectively. The membrane suspension, for use in the assay, was prepared as follows: the prepared rat striatal membranes were thawed on ice at room temperature. To a 50 ml Falcon tube were added Tris buffer (40 ml), $MgCl_2$ (48.21 mg), adenosine deaminase (9.06 µl) and the prepared rat striatal membranes (to yield a concentration of 506 mg/40 ml or 12.56 g/L). All polypropylene tubes, caps and tips were coated with sigma-cote® to prevent adhesion to the tubes.

Radioligand binding assay protocol

All incubations were performed in duplicate in polypropylene vials. The order of addition was as follows: test compound (10 µL), membrane suspension (790 µL), 500 nM CPA (100 µL) and 40 nM [^{3}H]NECA (100 µL). Each incubation vial thus contained the test compound (in concentrations of 0 µM - 300 µM), 1 mL of Tris (pH 7.7 at 25 °C), 10 mg of original tissue weight of the rat stiatal membranes, 4 nM [^{3}H]NECA, 50 nM CPA, 10 mM $MgCl_2$, 0.2 unit/mL adenosine deaminase and 1% DMSO. After all additions were done, each vial was vortexed, and incubated for one hour at 25 °C in a shaking water bath. Half an hour after incubation started, the incubations were vortexed again. Incubations were terminated via filtration through prewetted filters under reduced pressure using a Hoffeler vacuum system. Each tube was rinsed twice with 4 mL Tris and the contents passed through the filter. Finally each filter was washed with another 4 mL of Tris buffer.

Non-specific binding incubations were also done in duplicate, but 10 µl of a 10 mM CPA solution was added instead of the test compound.

After filtration, the filters were placed in scintillation vials and 4 mL of scintillation fluid (Filter-count) was added to each vial. The vials were shaken by hand and left to stand for 2 hours. Vials were then placed in the Packard Tri-CARB 2100 TR scintillation counter to count the

radioactivity retained on the filters. Specific binding (total binding - non specific binding) was expressed as counts per minute (CPM).

6.3.3 **Adenosine A_1 radioligand binding assay**

The affinities of the synthesised compounds for the adenosine A_1 receptor were evaluated by a radioligand binding protocol that was previously described (Van der Walt *et al.,* 2013, Bruns *et al.,* 1987). For this radioligand binding assay rat whole brain (excluding the cerebellum and brain stem) was used as receptor source and the highly A_1 selective [^{3}H]DPCPX used as radioligand. The affinity of the test compound is therefore determined by its ability to displace [^{3}H]DPCPX from the adenosine A_1 receptors. A compound with high adenosine A_1 receptor affinity will displace the radioligand from the receptor, resulting in a low residual radioactivity (CPM), while the opposite is true for a compound with weak or no affinity for the adenosine A_1 receptor.

Assay preparation:

Preparation for the adenosine A_1 affinity assay was similar to that of the A_{2A} radioligand binding assay. All stock solutions of the test compounds (10 mM) and CPA (10 mM, used to determine non-specific binding) were prepared in DMSO. [^{3}H]DPCPX was diluted with 50 mM Tris to a concentration of 1 nM. The membrane suspension, for use in the assay, was prepared as follows: the prepared rat whole membranes were thawed on ice at room temperature. To a 50 ml Falcon tube were added: Tris buffer (50 ml), adenosine deaminase (3.38 µl) and the prepared rat whole brain membranes (to yield a concentration of 281 mg/ 50 ml). All polypropylene tubes, caps and tips were coated with sigma-cote® to prevent adhesion to the tubes.

Radioligand binding assay protocol

The radioligand binding assays for adenosine A_1 affinity was carried out in a manner similar to that of the A_{2A} radioligand binding assay. All incubations were performed in duplicate in polypropylene vials. The order of addition was as follows: test compound (10 µL), 1 nM [^{3}H]DPCPX (100 µL) and membrane suspension (890 µL). Each vial thus contained: 1 mL of 50 mM Trisbuffer, 5 mg of original whole brain tissue weight, 0.1 nM [^{3}H]DPCPX, 0.1 unit/mL adenosine deaminase and 1% DMSO. After all additions, each vial was vortexed and incubated for one hour at 25 °C in a shaking water bath. Half an hour after incubation started the incubations were vortexed again. Incubations were terminated via filtration through prewetted 2.5 cm GF/B filters under reduced pressure. Each tube was also rinsed twice with 4 mL of Tris, which was filtered and the filters finally washed with another 4 mL of Tris.

Non-specific binding incubations were done in triplicate, but 10 µL of 10 mM CPA was added (to yield a final concentration of 0.1 mM) instead of the test compound.

After filtration, each filter was placed into a scintillation vial and 4 mL of scintillation fluid (Filter count) was added. The vials were shaken by hand and left to stand for 2 hours. Vials were then placed in the Packard Tri-CARB 2100 TR scintillation counter to count the radioactivity retained on the filters. Specific binding was expressed as CPM.

6.4 **DATA ANALYSIS**

CPM values were plotted against the logarithm of the inhibitor concentrations to obtain a sigmoidal dose-response curve that were used to determine the IC_{50} values. The data obtained was fitted to the one site competition model of the Prism software package (GraphPad Software Inc.).

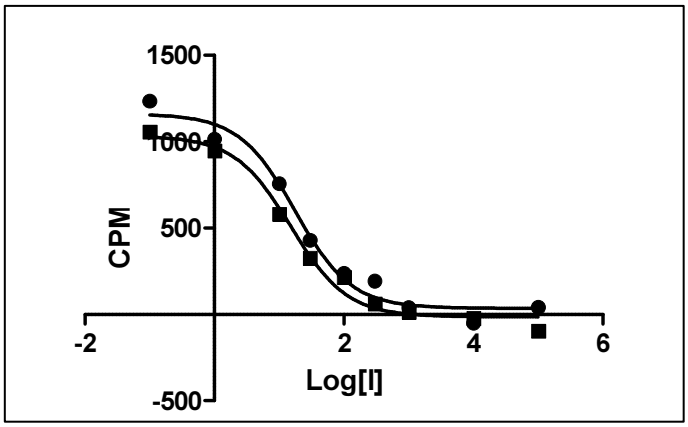


Figure 10: Example of a sigmoidal dose-response curve.

On a sigmoidal dose-response curve the X-axis is the logarithm of the concentration of the unlabeled compound, and the Y-axis is the CPM in the presence of the labeled compound (figure 10).

All incubations were carried out in duplicate and the IC_{50} values were expressed as mean ± S.E.M. The IC_{50} value for the sigmoidal plot was obtained using a non-linear regression equation as indicated below:

$$\boldsymbol{Y} = Bottom \times \frac{Top - Bottom}{1 + 10^{x - log IC_{50}}}$$

Y = CPM represents radioligand binding as counts per minute

X = the concentration of the unlabeled ligand

"Top" = top part of the sigmoidal curve

"Bottom"= the bottom part of the sigmoidal curve

For the adenosine A_1 radioligand binding studies the K_i-values of the test compounds were calculated by using the Cheng and Prussof equation below (Cheng & Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1+\frac{[radioligand]}{K_D}}$$

[Radioligand] = concentration of [^{3}H]DPCPX (0.1 nM)
K_D = dissociation constant of [^{3}H]DPCPX (0.36 nM)

However, because the adenosine A_{2A} radioligand binding studies were performed in the presence of CPA, an adapted version of the Cheng-Prusoff equation (Bruns et al., 1986), as indicated below, was used to calculate the K_i-values:

$$K_i = \frac{IC_{50}}{1+\frac{L}{K_D}+\frac{C}{K_C}}$$

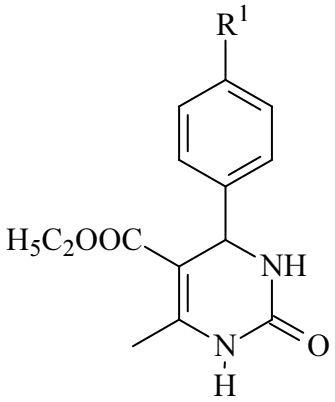
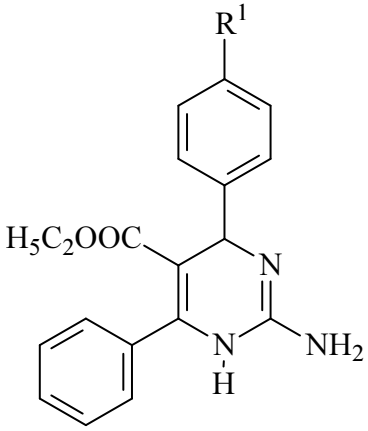
C = concentration of CPA (50 nM)
K_C = dissociation constant of CPA (685 nM)
L = concentration of [^{3}H]NECA (4 nM)
K_D = dissociation constant of [^{3}H]NECA (15.3 nM)

The K_i-values were expressed in µM, where a lower K_i-value is indicative of a higher affinity for the receptor and vice versa.

6.5 **RESULTS**

As previously described, the IC_{50} values of the synthesised compounds (**1a-i** and **2b-e)** (table 6) were determined from the sigmoidal dose response curves and the corresponding K_i-values (table 7) were calculated with the Cheng-Prusoff equation.

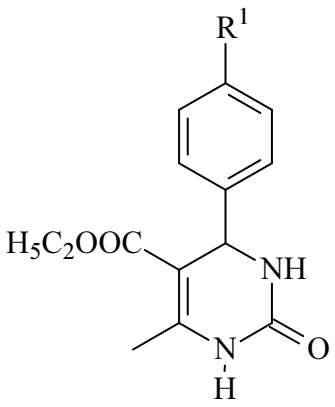
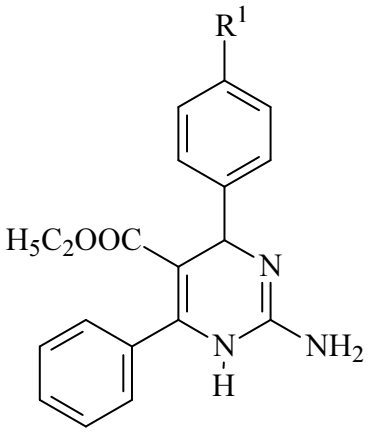
Table 6: IC_{50} values of the 3,4-dihydropyrimidone and 2-amino-1,4-dihydropyrimidine derivatives obtained for adenosine A_1 and A_2 receptors, expressed as IC_{50} ± S.E.M. µM.

Series 1				**Series 2**			
3,4 Dihydropyrimidones				**2-Amino-1,4-dihydropyrimidines**			
							
	R^1	**A_{2A} IC_{50} [a] (µM)**	**A_1 IC_{50} [a] (µM)**		**R^1**	**A_{2A} IC_{50} [a] (µM)**	**A_1 IC_{50} [a] (µM)**
1a	H	126.40 ± 28.5	12.89 ±0.15				
1b	4-Br	263.35 ± 60.25	9.45 ± 2.66	2b	4-Br	251.8 ± 26.20	10.10 ± 0.85
1c	4-Cl	139.50 ± 21.0	20.04 ± 4.25	2c	4-Cl	51.49 ± 0.52	> 300 µM [b]
1d	4-OCH_3	> 300 µM [b]	10.9 ± 2.19	2d	4-OCH_3	160.05 ± 32.95	32.08 ± 3.32
1e	4-CH_3	> 300 µM [b]	23.77 ± 1.01	2e	4-CH_3	63.83 ± 0.18	12.36 ± 2.91
1f	4-NO_2	> 300 µM [b]	95.79 ± 18.51				
1g	4-OH	> 300 µM [b]	174.4 ± 10.80				
1h	4-F	38.32 ± 1.76	14.32 ± 1.65				
1i	4-CF_3	> 300 µM [b]	53.33 ± 0.20				

[a] All IC_{50} values were determined in duplicate and are expressed as mean ± S.E.M.

[b] The IC_{50} values > 300 µM.

Table 7: Affinities of the 3,4-dihydropyrimidone and 2-amino-1,4-dihydropyrimidine derivatives for adenosine A_1 and A_{2A} receptors expressed as Ki ± S.E.M. µM.

Series 1				**Series 2**			
3,4 Dihydropyrimidones				**2-Amino-1,4-dihydropyrimidines**			
							
	R^1	**A_{2A} affinity K_i (µM)**	**A_1 affinity K_i (µM)**		**R^1**	**A_{2A} affinity K_i (µM)**	**A_1 affinity K_i (µM)**
1a	H	94.72 ± 21.36	10.09 ± 0.12				
1b	4-Br	197.4 ± 45.08	7.39 ± 2.08	2b	4-Br	188.7 ± 79.63	7.90 ± 0.66
1c	4-Cl	104.5 ± 15.74	15.68 ± 3.33	2c	4-Cl	38.59 ± 0.39	no affinity [c]
1d	4-OCH_3	no affinity [c]	8.53 ± 1.71	2d	4-OCH_3	119.9 ± 24.69	25.11 ± 2.59
1e	4-CH_3	no affinity [c]	18.6 ± 0.79	2e	4-CH_3	47.83 ± 0.11	9.67 ± 2.28
1f	4-NO_2	no affinity [c]	74.97 ± 14.49				
1g	4-OH	no affinity [c]	136.5 ± 8.45				
1h	4-F	28.71 ±1.33	11.21 ± 1.29				
1i	4-CF_3	no affinity [c]	41.74 ± 0.16				

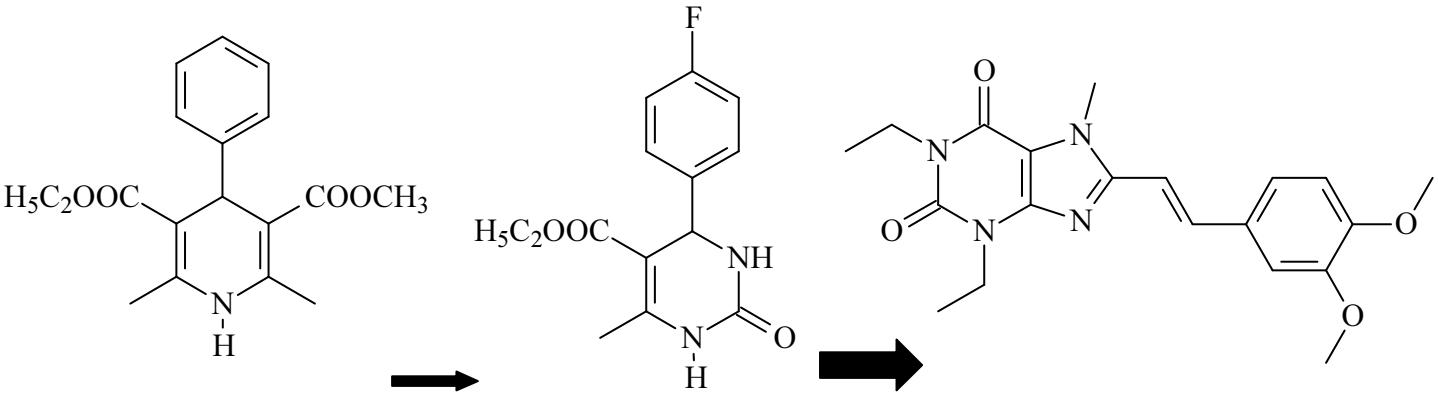
[c] IC_{50} value not available *for* K_i calculation (see table 6)

6.6 **DISCUSSION**

The compounds from both series 1 (**1a-i**) and 2 (**2b-e**) showed weak adenosine A_{2A} affinity, with the *p*-fluorophenyl substituted dihydropyrimidone derivative (**1h**) in series 1 exhibiting the highest affinity for the adenosine A_{2A} receptor (K_i = 28.7 µM), followed by the *p*-chlorophenyl substituted dihydropyrimidine derivative (**2c**) of series 2 (K_i = 38.59 µM). Compounds from both series 1 and 2 showed more promising adenosine A_1 affinity in the low micromolar range and *p*-bromophenyl substitution in both series resulted in the highest affinity for adenosine A_1 with K_i-values of 7.39 µM (**1b**) and 7.9 µM (**2b**). The *p*-

methoxyphenyl substituted dihydropyrimidone (**1d**) and *p*-methyl dihydropyrimidine (**2e**) also exhibited moderate affinity for the adenosine A_1 receptor with K_i-values of 8.53 µM and 9.67 µM, respectively. Generally, these results indicate that most of the compounds synthesised in this study have adenosine A_1 selective affinity, rather than dual adenosine A_1 and A_{2A} affinity.

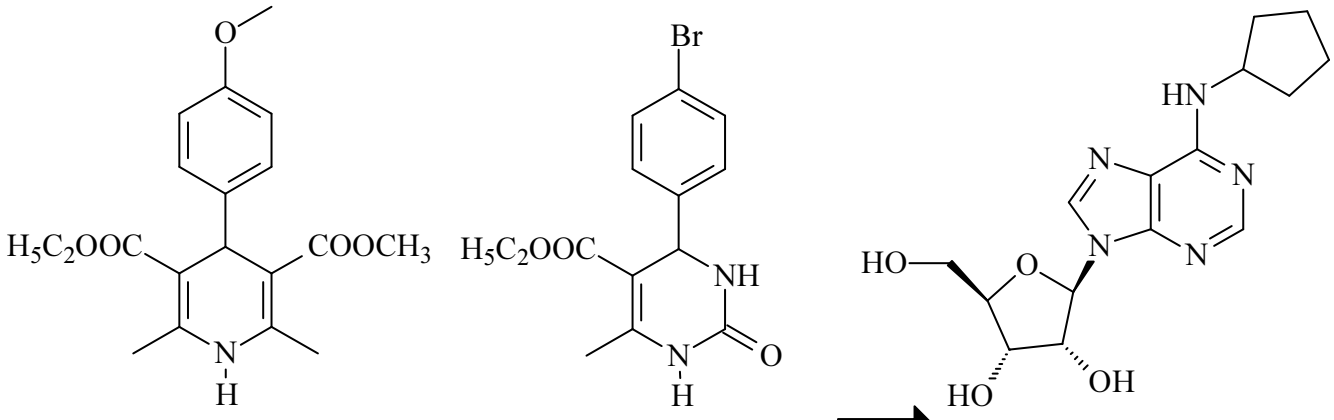
A comparison of the adenosine A_{2A} affinity of the highest affinity derivative obtained in this pilot study (**1h,** K_i = 28.7 µM), with the highest affinity derivative of Van Rhee and co-workers (1996) (**9**, Ki = 2.74 mM) indicates that adenosine A_{2A} affinity showed an approximate 100-fold improvement. However, KW6002, a known adenosine A_{2A} antagonist, that has already reached clinical trials, has a K_i-value of 7.94 nM (Van der Walt *et al*., 2013).



9 **1h** **KW6002**

K_i = 2.74 **mM** K_i = 28.7 **µM** K_i = 7.94 **nM**

The same trend was observed for adenosine A_1 affinity, where the highest affinity derivative of this study showed improved adenosine A_1 affinity in the micromolar range (**1b**, K_i = 7.39 µM) when compared to that of Van Rhee and co-workers (1996) which had K_i-values in the millimolar range (**10**, K_i = 2.75 mM). CPA, the reference compound, had a K_i-value of 10.4 nM (Van der Walt *et al.,* 2013).



10 **1b** **CPA**

K_i = 2.75 **mM** K_i = 7.39 **µM** K_i = 10.4 **nM**

The increase in affinity is probably due (at least in part), to the increase in the number of nitrogen atoms present in the heterocyclic ring. Similar results were obtained by Gillespie and co-workers in 2009 who found that increasing the number of nitrogens in the heterocyclic ring (from a pyridine to a pyrimidine) enhanced the affinities for both the adenosine A_{2A} and adenosine A_1 receptor subtypes (see table 2 section 3.2.2.1). They compared the affinities of three scaffolds (triazine, pyrimidine and pyridine) using human adenosine A_1 and A_{2A} receptors and observed that the pyridine scaffold was sevenfold less potent than the triazine and 45-fold less potent than the corresponding aminopyrimidine, thus showing that two nitrogens in the ring are optimum for both adenosine A_1 and A_{2A} affinity (Gillespie *et al*., 2009 a,b).

The compounds in this study, however only displayed poor to moderate affinity, when compared to the reference compounds, KW6002 and CPA. The poor adenosine A_{2A} affinity can probably be attributed to the absence of a central aromatic heterocyclic ring. The amino acid, Phe-168, plays an important role, in the binding site of the adenosine A_{2A} receptor. In the crystal structure of the adenosine A_{2A} receptor, co-crystallised with the known antagonist, ZM241385, surface calculations showed that Phe-168 has the highest contact area (of all the amino-acids present in the active site) and undergoes an aromatic π-π-stacking interaction with the central aromatic triazolotriazine core (figure 11) (Jaakola *et al*., 2010). Since the dihydropyrimidine ring present in both series 1 and 2 of this pilot study is not aromatic it most likely adapts a nonplanar conformation which is unlikely to undergo an aromatic π-π-stacking interaction with Phe-168.

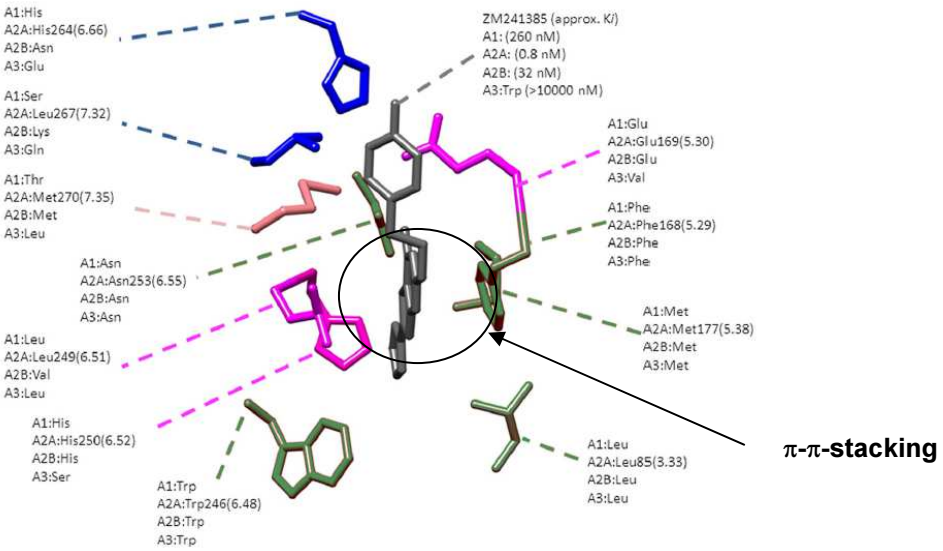


Figure 11: Docking study showing the aromatic interaction of Phe-168 with the central aromatic triazolotriazine core of ZM241385 (Jaakola *et al*., 2010)

One way in which the affinity of the current compounds for the adenosine A_{2A} receptor could possibly be improved would be by incorporating the structural features associated with high affinity as described by Hoffman Le Roche. The phenyl ring at position 6, could for example, be replaced with a 2-furyl ring. Another possibility is the replacement of the dihydropyrimidine ring with an aromatic pyrimidine ring to improve the chances for an aromatic π-π-stacking interaction with Phe168 (see figure 12).

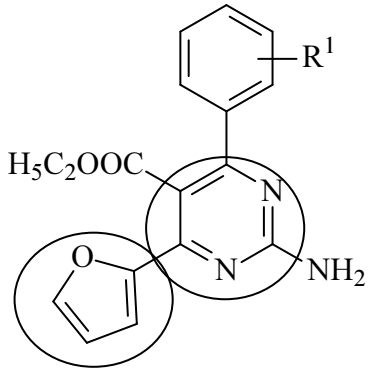


Figure 12: Structural changes to series 2 of this study to improve A_{2A} affinity.

The possibility further exists that series 2 (**2a-e**) may have promising adenosine A_3 affinity. This is based on results obtained by Jiang and co-workers (1996) for a series of 1,4-dihydropyridine derivates. In this case both the 4-trans-β-styryl and a 6-phenyl substituent enhanced affinity for adenosine A_3 receptors in the micromolar range. It was especially the 6-phenyl substituent that was noted to be a better enhancer of the A_3 receptor affinity (figure 13) (Jiang *et al.,* 1996).

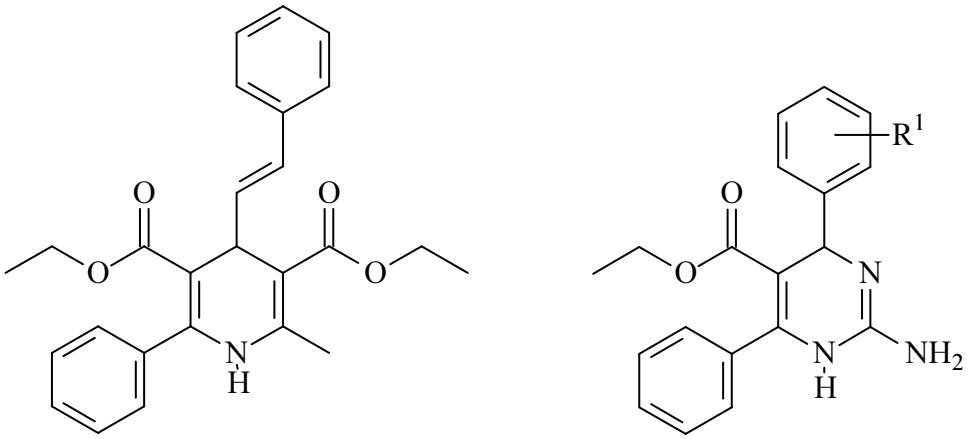
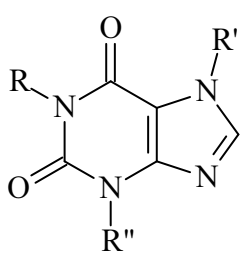
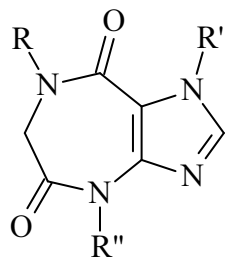


Figure 13: Structural similarity between the structures of Jiang on the left and that of series 2 on the right (Jiang *et al.,* 1996).

Jiang and co-workers also found that pyridine derivatives, which had a more planar geometry, lost affinity for A_3 receptors, but in return gained affinity for adenosine A_1 receptors. This finding also supports our theory that the compounds in this pilot study, which are most likely nonplanar, which showed only weak to moderate affinity for both adenosine A_1 and A_{2A} receptors, thus making them possible candidates for binding to adenosine A_3 receptors. This trend was also observed in a survey of non-xanthine derivates as adenosine receptor ligands where Siddiqi and co-workers (1996) found that all antagonists for the adenosine A_1 receptor tend to have a planar geometry (Siddiqi *et al*., 1996). In another study, a series of imidazodiazepinediones had lower affinity than the corresponding xanthine analogues. The imidazodiazepinediones adopted a boat shaped seven-membered ring conformation compared to the planar heteroaryl ring system of the xanthines (Daly *et al*., 1990). It therefore seems that the adenosine A_3 receptor does not share this preference for planarity.



Xanthines



Imidazodiazepinediones

In conclusion the 3,4-dihydropyrimidone and 2-amino,1,4-dihydropyrimidine scaffolds can be used as a lead for the design of novel adenosine A_1 and A_{2A} antagonists, although further structural modifications will be required to obtain clinical viability and to increase affinity for the adenosine receptor.

CHAPTER 7

CONCLUSION

Signalling at adenosine receptors plays an important role in several diseases (Moro *et al*., 2006) and antagonism of the adenosine A_{2A} receptor has emerged as a potential nondopaminergic antiparkinsonian treatment strategy. Its potential in the treatment of Parkinson's disease is inter alia due to its role in neurocircuits involved in the regulation of movement. Adenosine receptors are also a major target of caffeine, which is a non-selective antagonist of adenosine A_1 and A_{2A} receptors and evokes its effects via blockade of these receptors. These effects include stimulatory actions such as heightened alertness and attention, increased cognitive performance and reduced sleep. The cognitive effects of caffeine are mostly due to its ability to antagonise adenosine A_1 receptors (Ribeiro and Sebastião, 2010). Adenosine A_1 antagonists depolarize postsynaptic neurons and presynaptically enhance the release of a number of neurotransmitters, e.g. acetylcholine, glutamate, serotonin and norepinephrine. This release of neurotransmitters could find application in the treatment of cognitive deficits such as those associated with Alzheimer's and PD.

Van Rhee and co-workers (1996) synthesised 1,4-dihydropyridine derivatives and reported that these derivatives had affinity for three adenosine receptor subtypes (A_1,A_{2A} and A_3) in the millimolar range. These results prompted this pilot study which aimed to investigate the potential of the structurally related 3,4-dihydropyrimidones and 2-amino-1,4-dihydropyrimidines as adenosine A_1 and A_{2A} antagonists.

Nine 3,4-dihydropyrimidone and four 2-amino-6-phenyl-1,4-dihydropyrimidine derivates have been synthesised successfully via a Biginelli one pot synthesis. In vitro evaluation of the synthesised compounds included an assessment of their MAO-B inhibitory activities and their affinities for adenosine A_1 and A_{2A} receptors.

No MAO-B inhibitory activity was observed for compounds of either series. Overall, the 3,4-dihydropyrimidones and 2-amino-1,4-dihydropyrimidines had weak adenosine A_{2A} affinity with only a few compounds showing affinity in the micromolar range. However, both the 3,4-dihydropyrimidones and 2-amino-1,4-dihydropyrimidines showed more promising adenosine A_1 affinity in the micromolar range, with the *p*-bromophenyl substituted derivatives (**1b** and

2b) in both series showing the highest adenosine A_1 affinity with K_i-values of 7.39 μM and 7.9 μM, respectively.

A comparison between the compounds of this study and those previously synthesised by Van Rhee and co-workers (1996), revealed that adenosine A_{2A} affinity in both series improved. For example, the most potent compound in this study (**1h**) had a K_i-value of 28.7 μM compared to their highest affinity derivative which had a K_i-value of 2.7 mM. However KW6002, a known adenosine A_{2A} antagonist has a K_i-value of 7.9 nM (Van der Walt *et al.,* 2013). The same trend was observed for adenosine A_1 affinity, where the highest affinity derivative (**1b**) showed improved adenosine A_1 affinity in the micromolar range when compared to that of Van Rhee and co-workers (1996) which had a K_i-value in the millimolar range. However, CPA, the reference compound had a K_i-value in the nanomolar range (Van der Walt *et al.,* 2013).

It thus appears that increasing the number of nitrogen atoms in the heterocyclic ring, (e.g. dihydropyridine vs. dihydropyrimidine), results in enhanced affinity for both adenosine A_1 and A_{2A} receptor subtypes. This trend was also observed by Gillespie and co-workers (2009 a,b) in their series of pyridines and pyrimidines. The low micromolar range affinity (rather weak affinity) can be attributed to the dihydropyrimidine ring most likely having a nonplanar conformation, thus making it unsuitable for an aromatic π-π-stacking interaction with Phe-168 in the adenosine A_{2A} binding site. A correlation between a lack of planarity and decreased adenosine affinity was also found by Daly and co-workers (1990) when they enlarged the six-membered planar pyrimidinedione ring of a xanthine scaffold to a seven-membered nonplanar diazepinedione ring.

In conclusion, although the adenosine A_1 and A_{2A} affinities of the 3,4-dihydropyrimidones and 2-amino-1,4-dihydropyrimidines of this study, were in the micromolar range, the affinities were increased when compared to the lead 1,4-dihydropyridines of Van Rhee and co-workers (1996). These two scaffolds can thus be used as leads in the design of novel adenosine A_1 and A_{2A} antagonists, although further structural modifications will be required to improve affinity for the adenosine receptor and to identify a viable candidate for further development and clinical viability.

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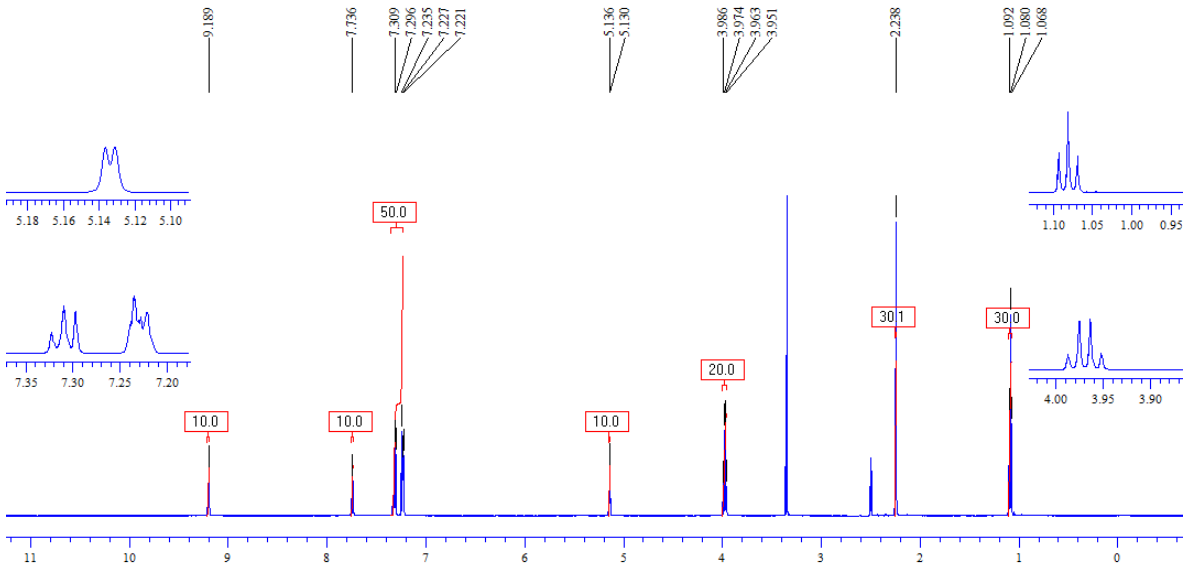
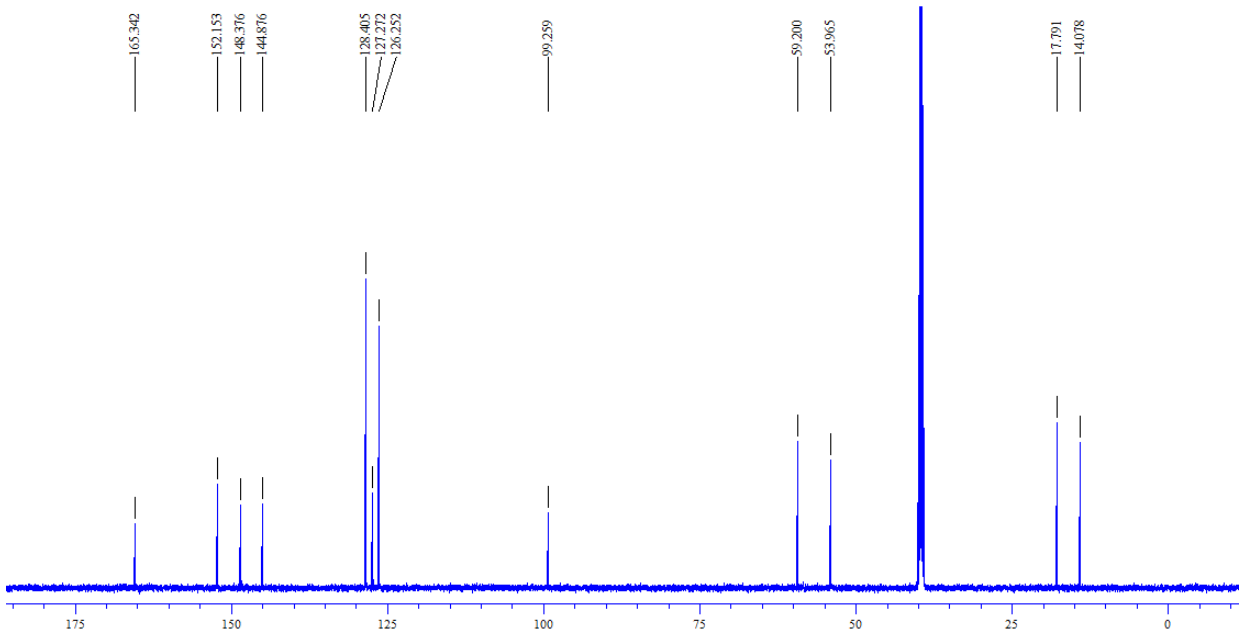
APPENDIX I

NMR

^{1}H NMR - compound 1a



^{13}C NMR - compound 1a



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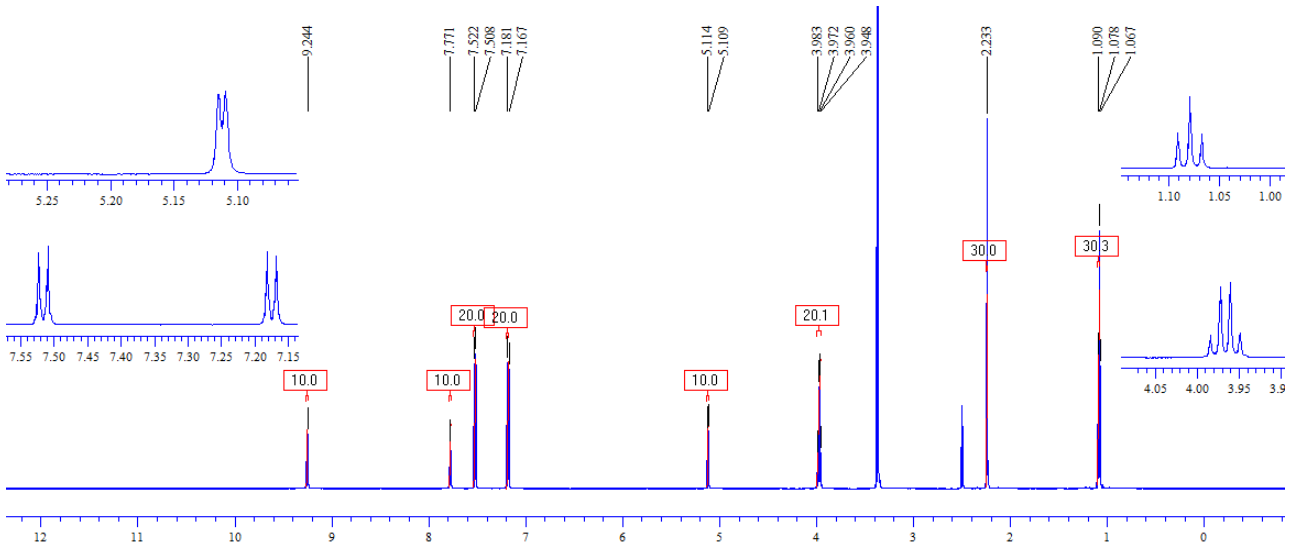

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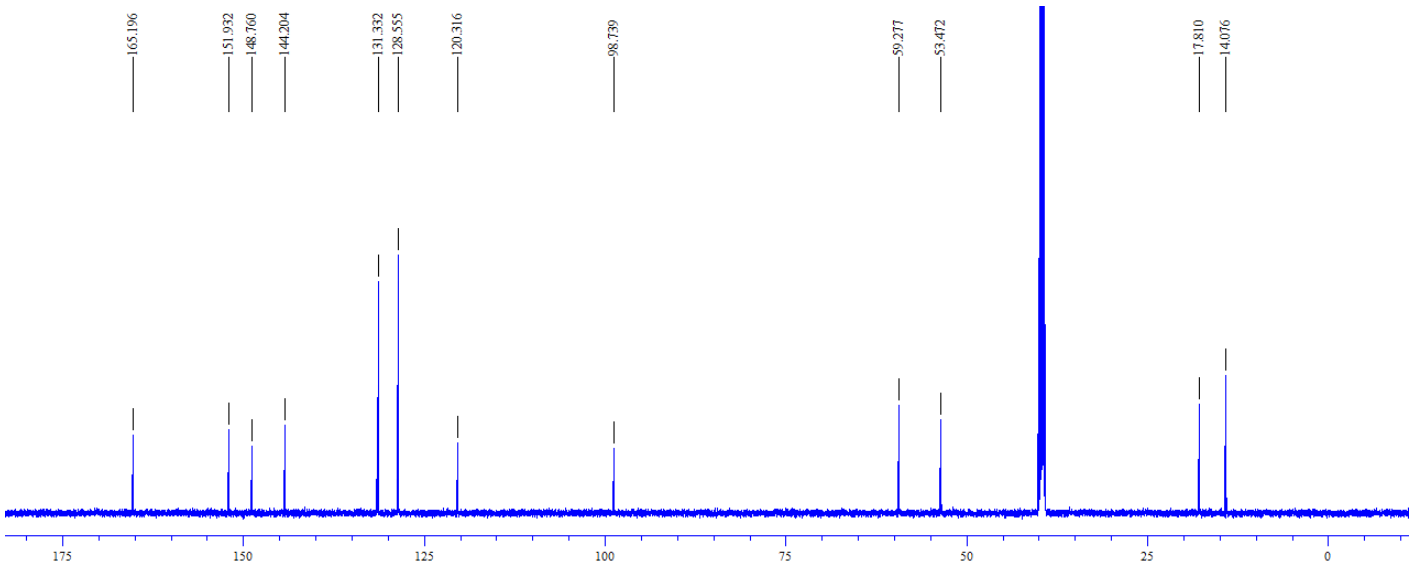

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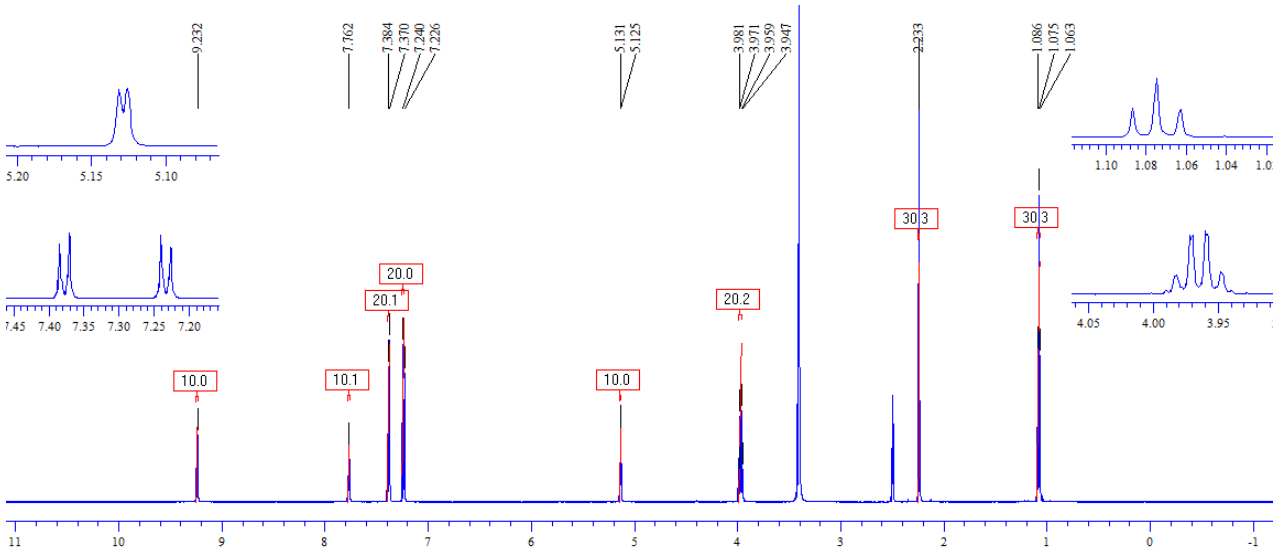
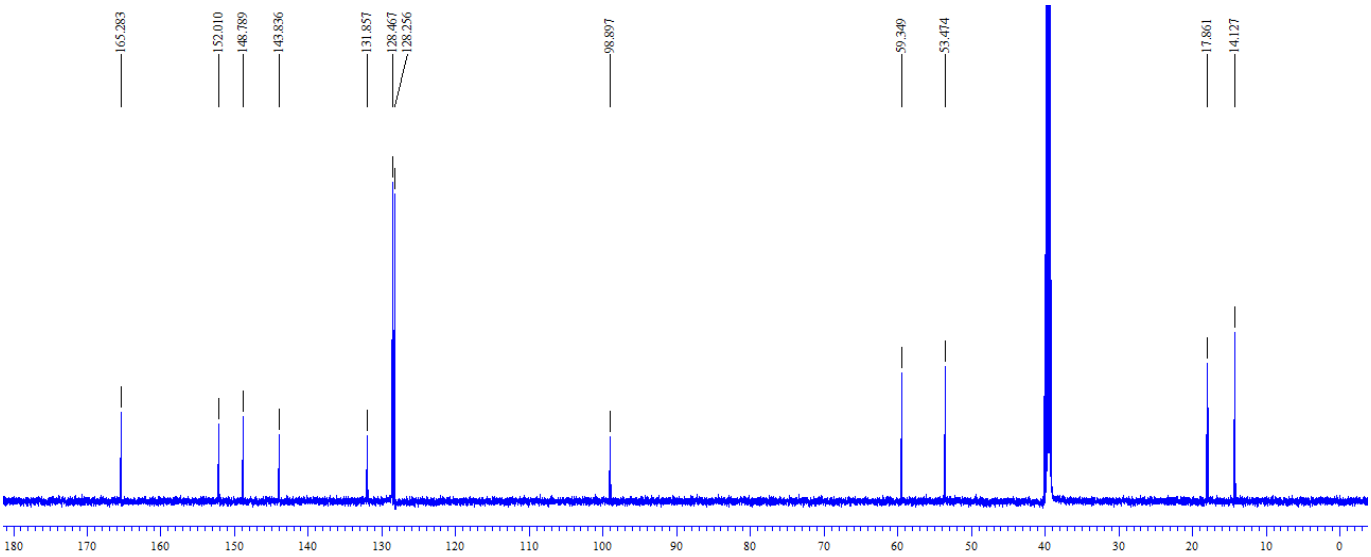


^{13}C NMR - compound 1c



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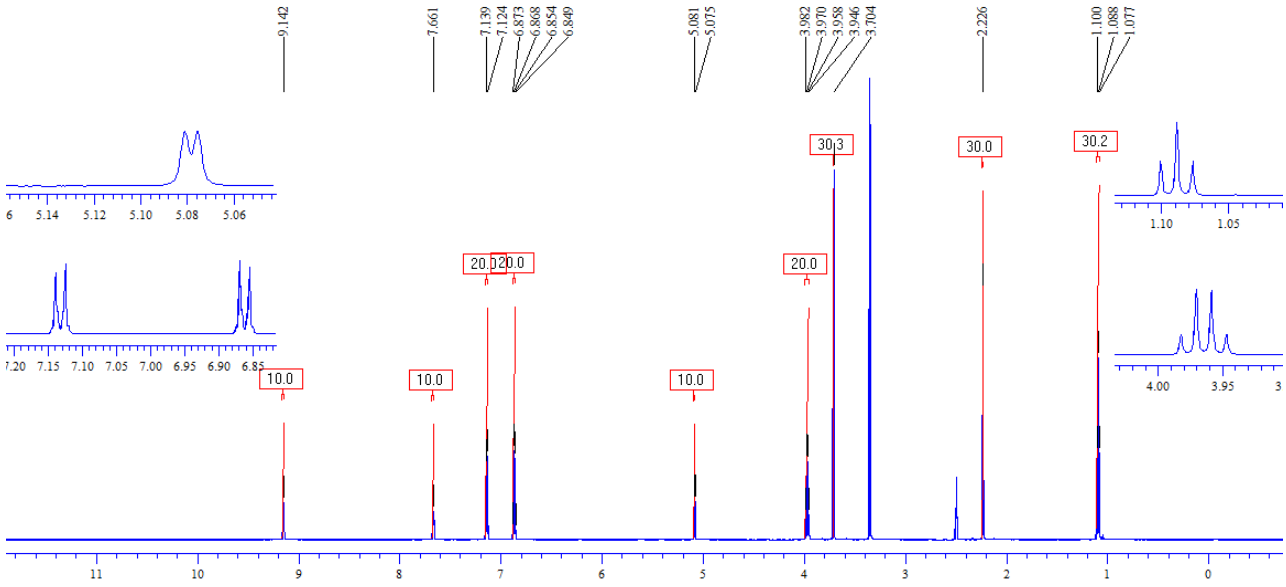

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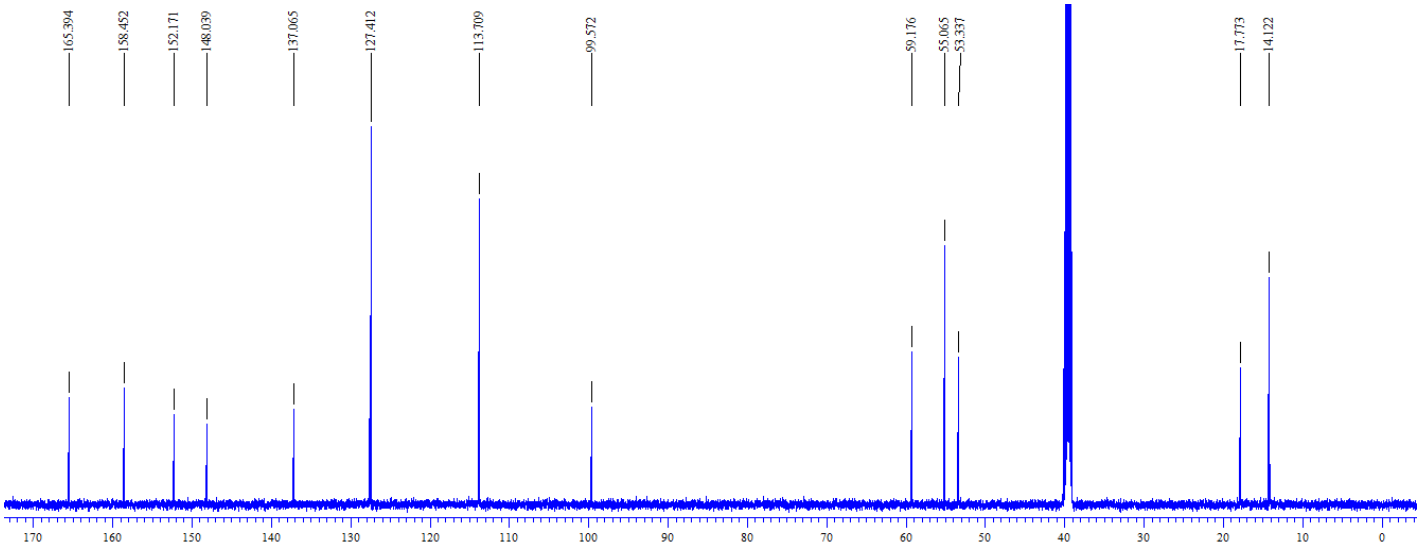

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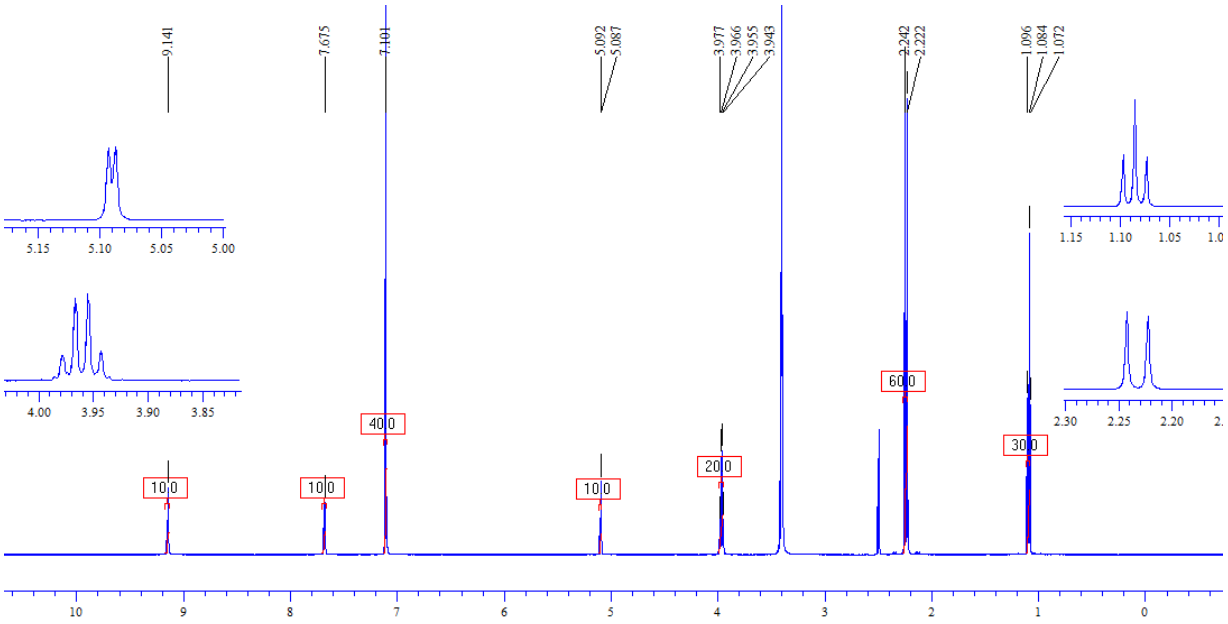

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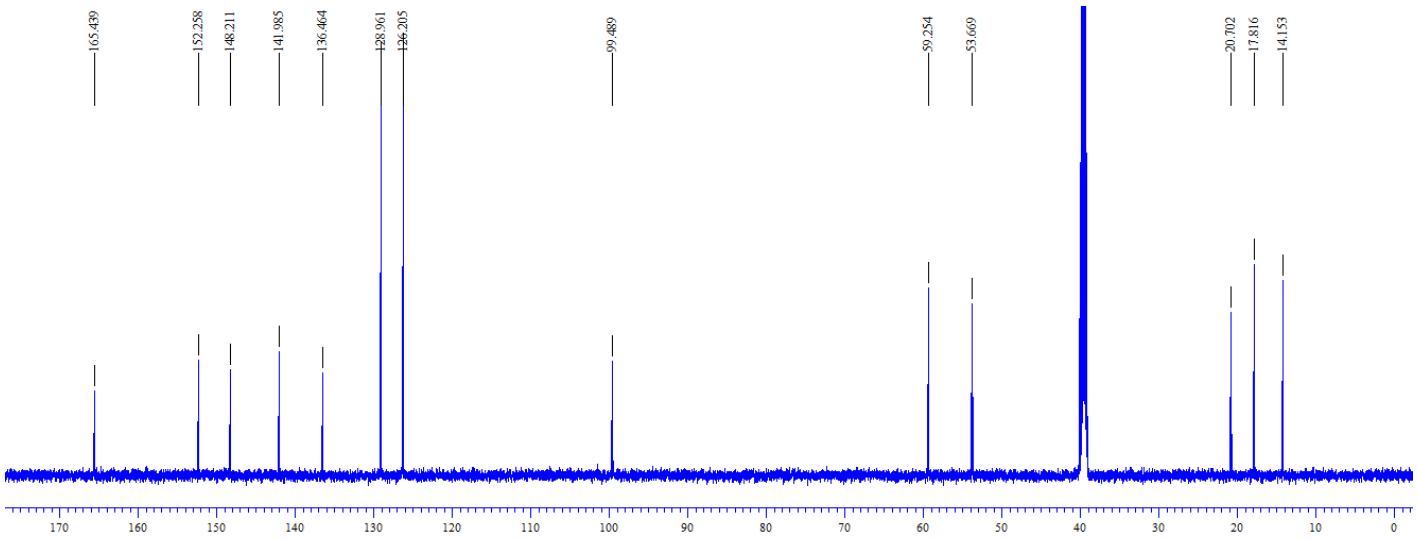

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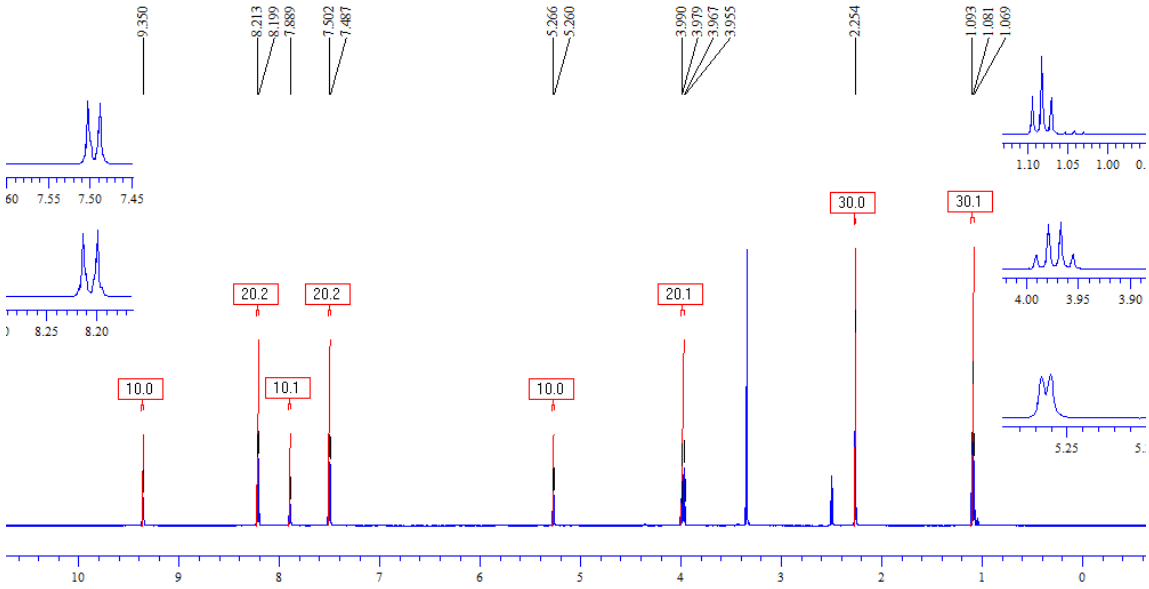

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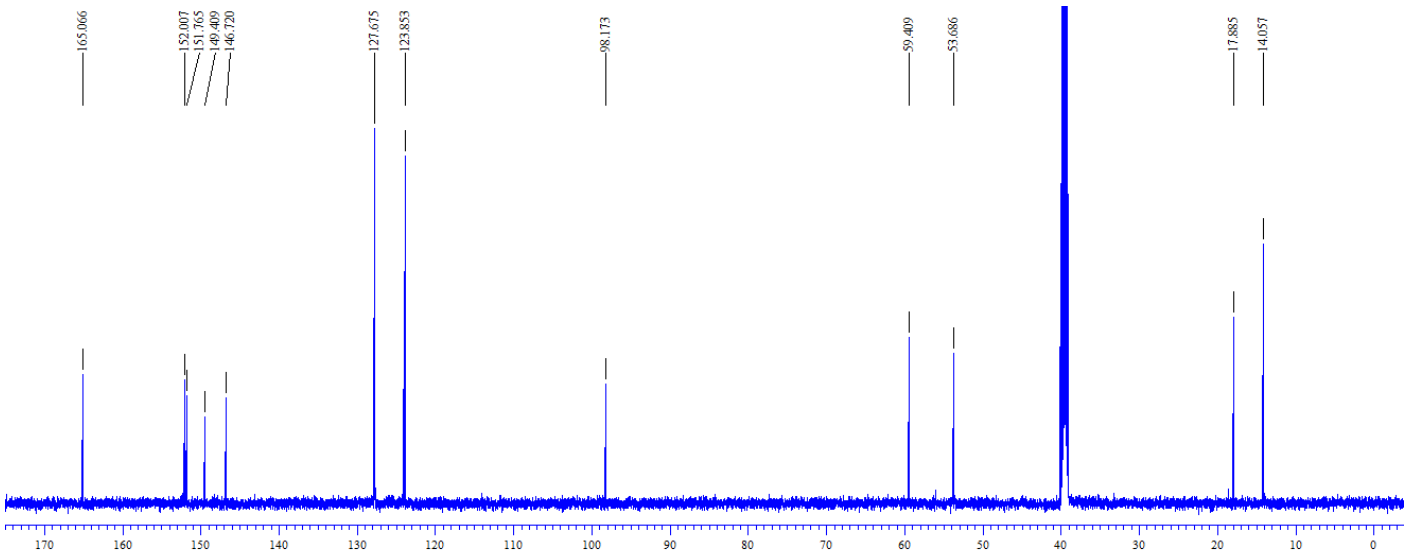

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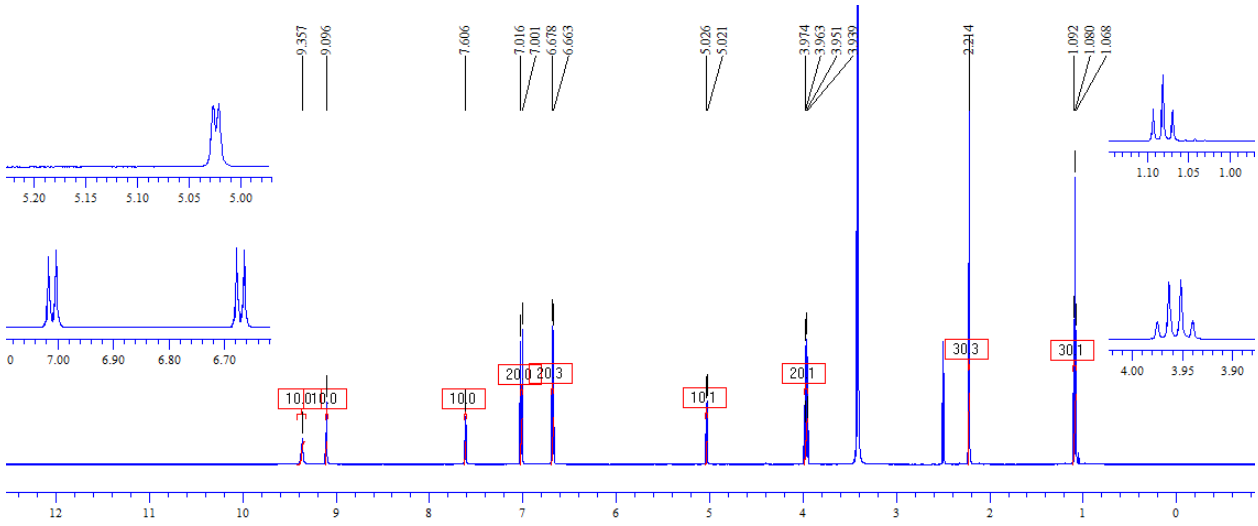

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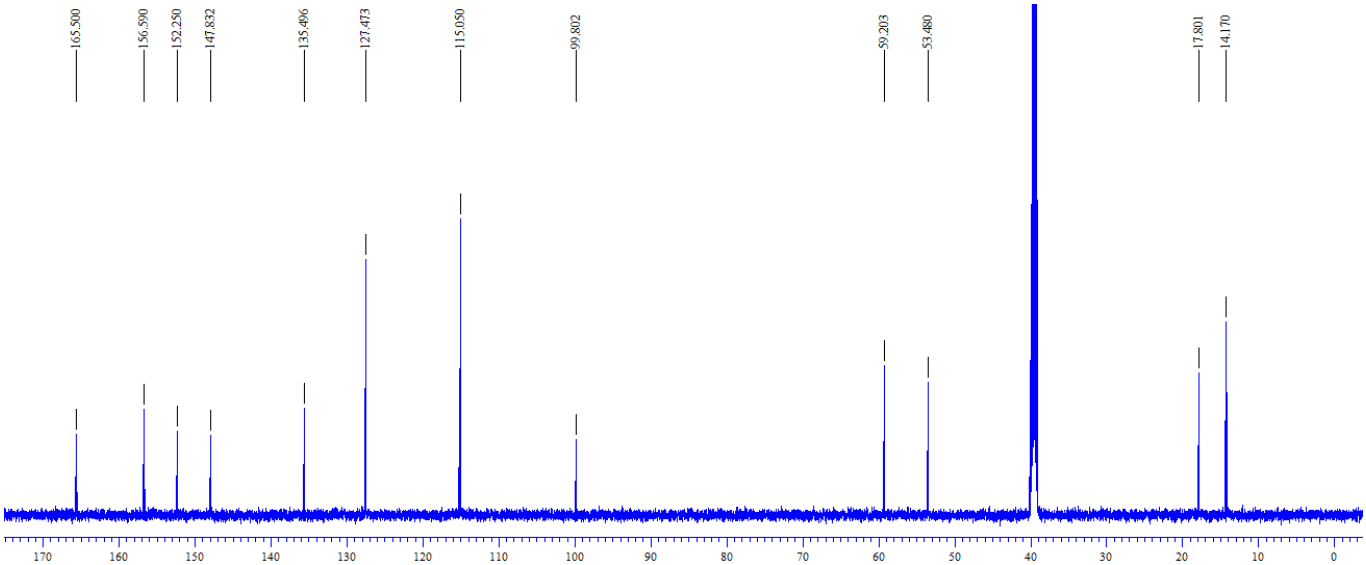

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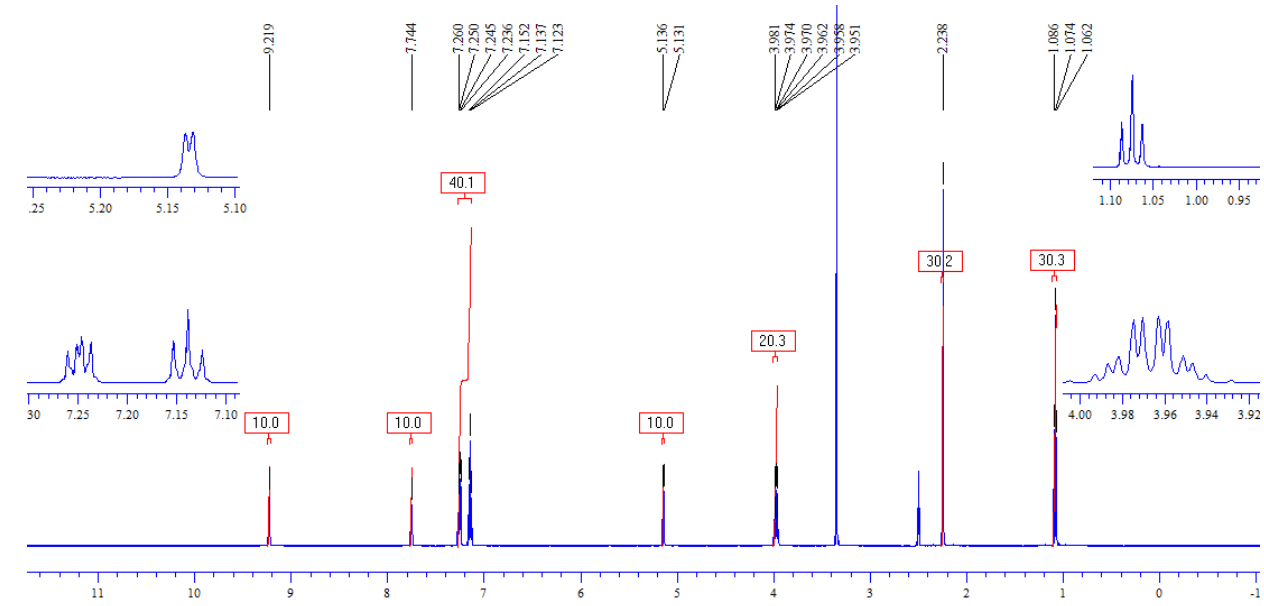

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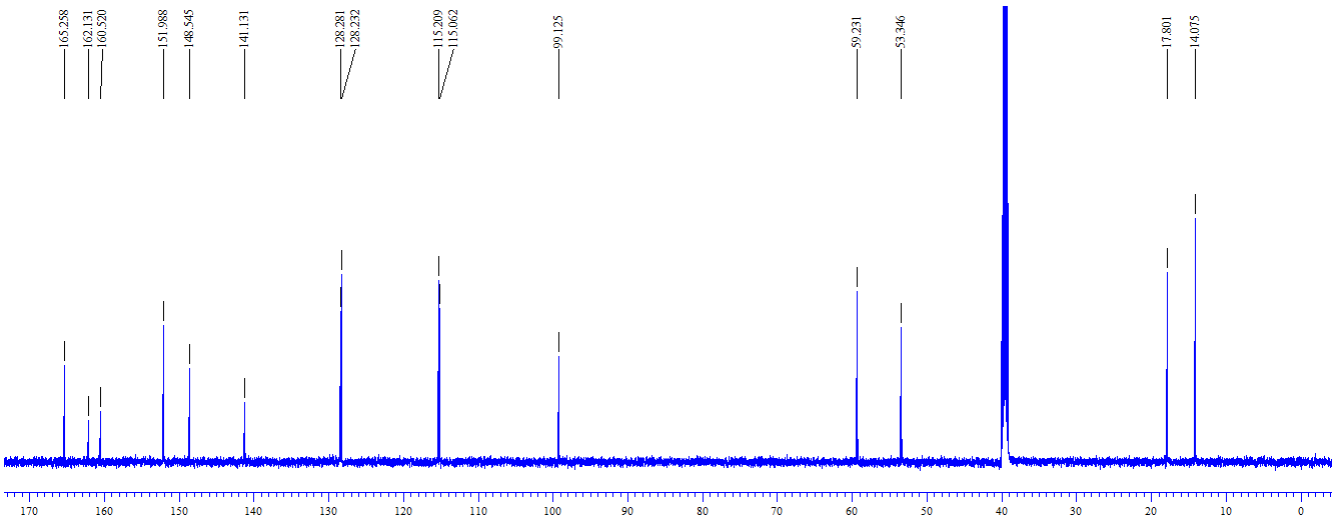

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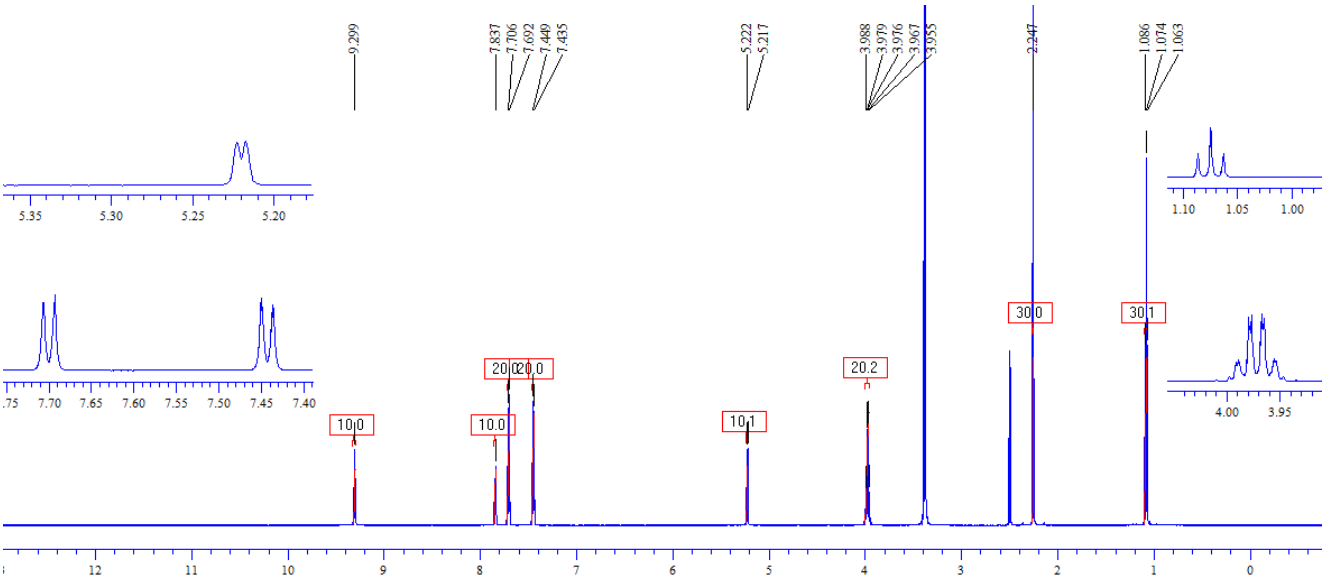

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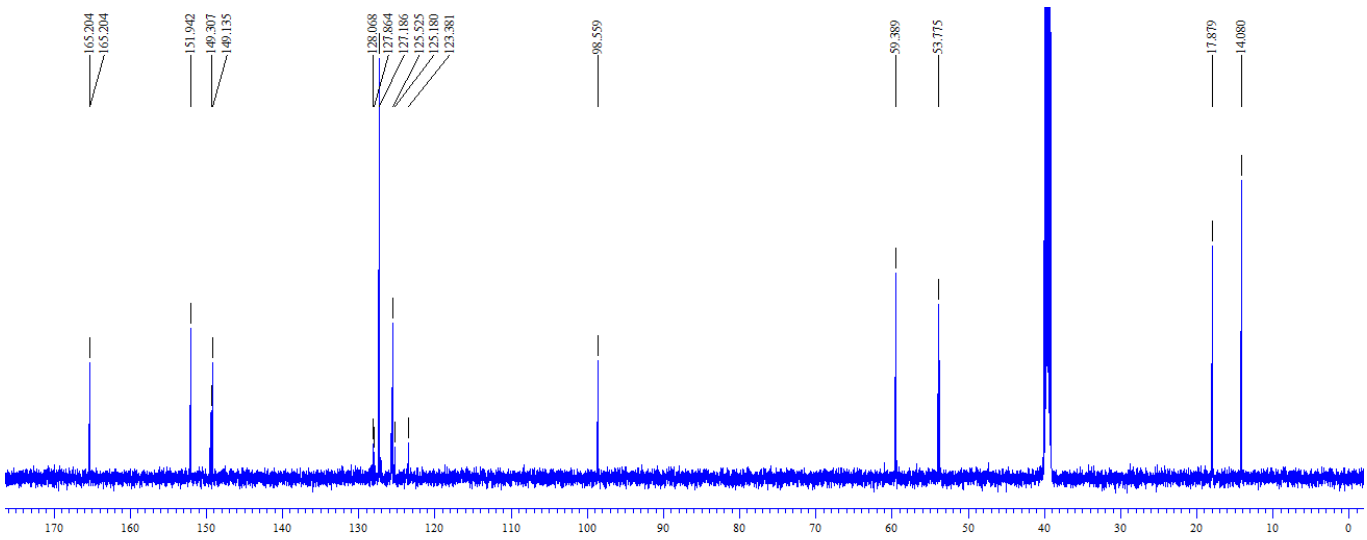

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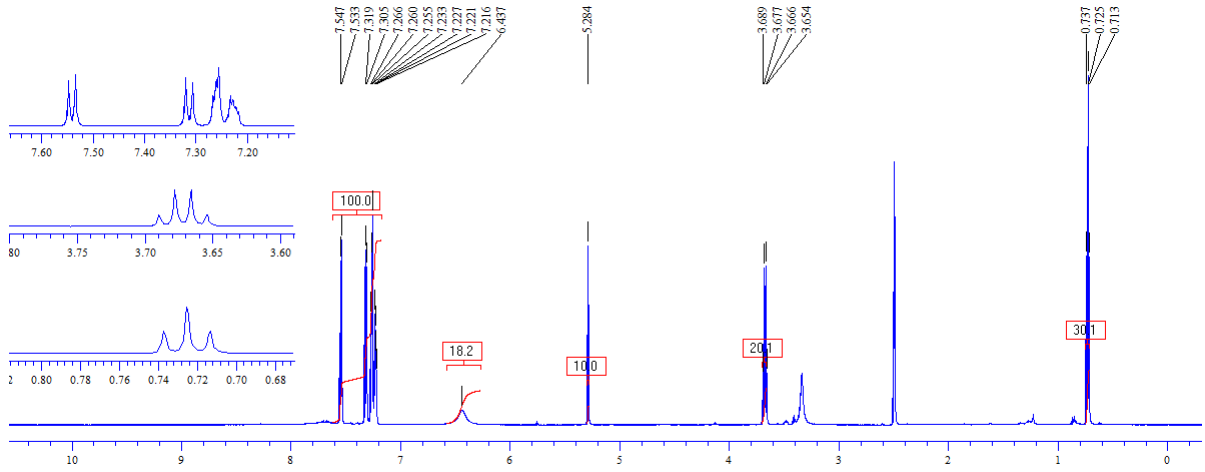

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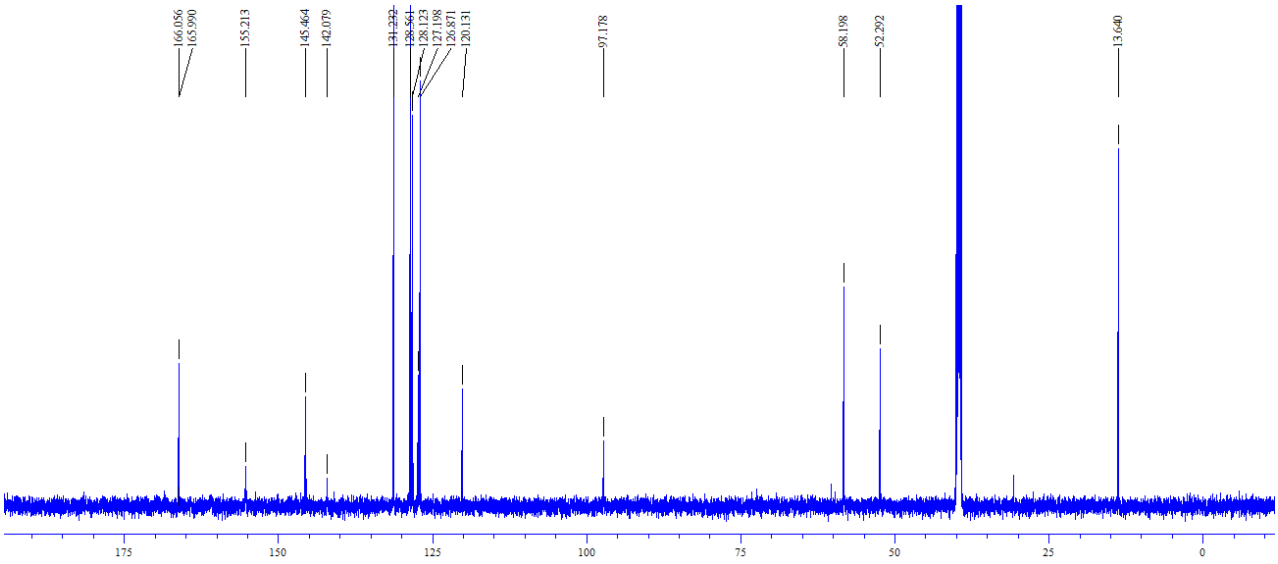

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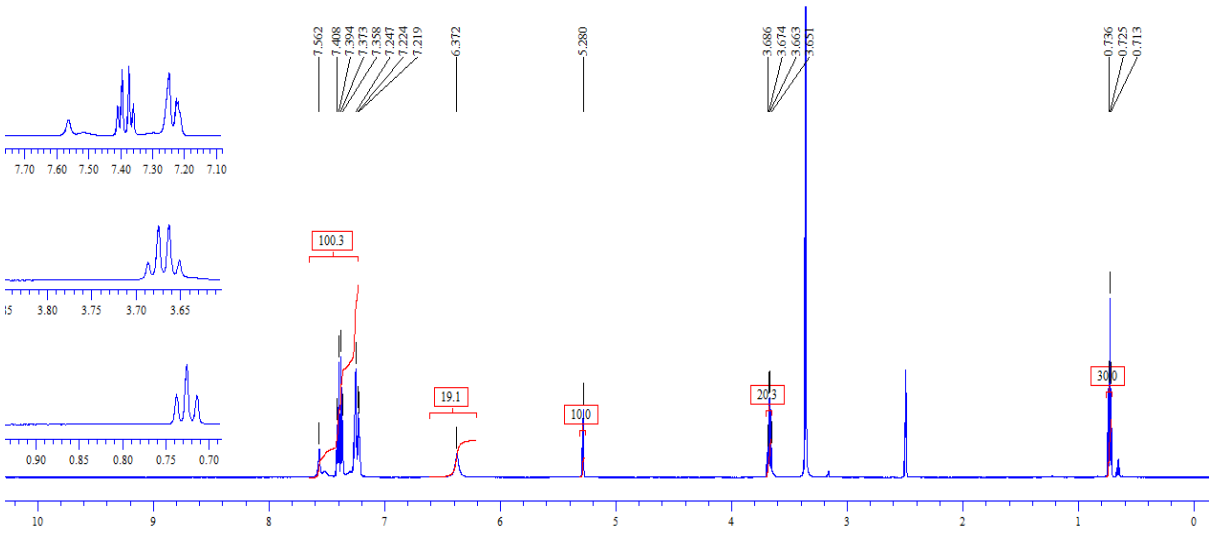

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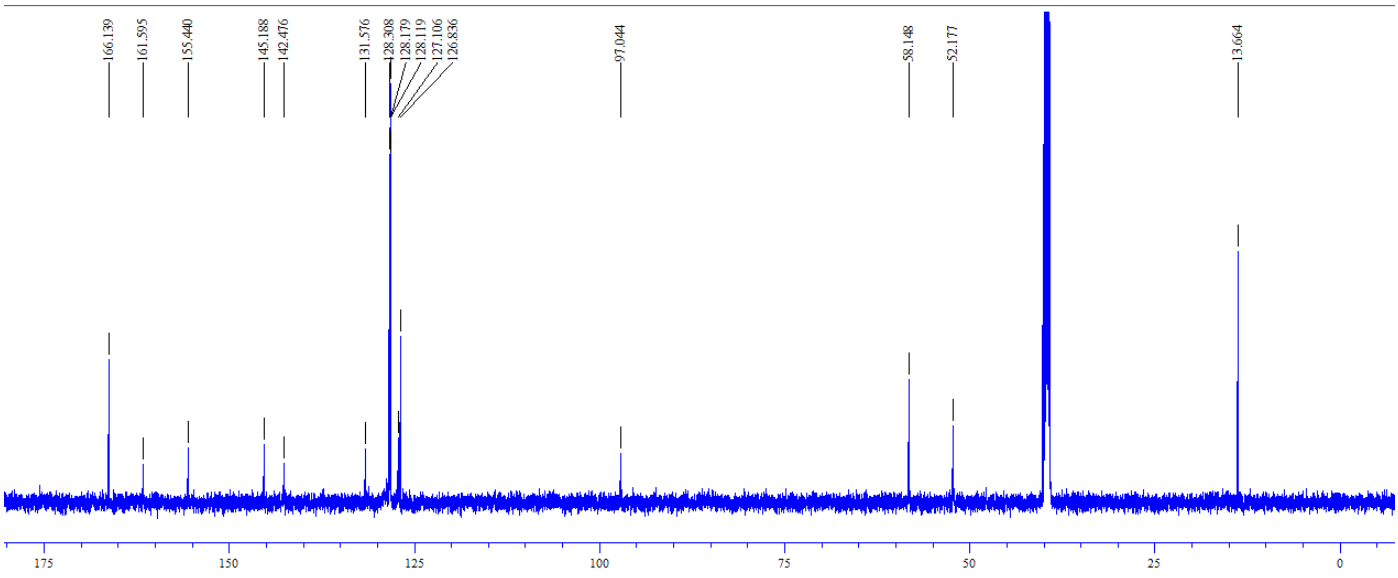

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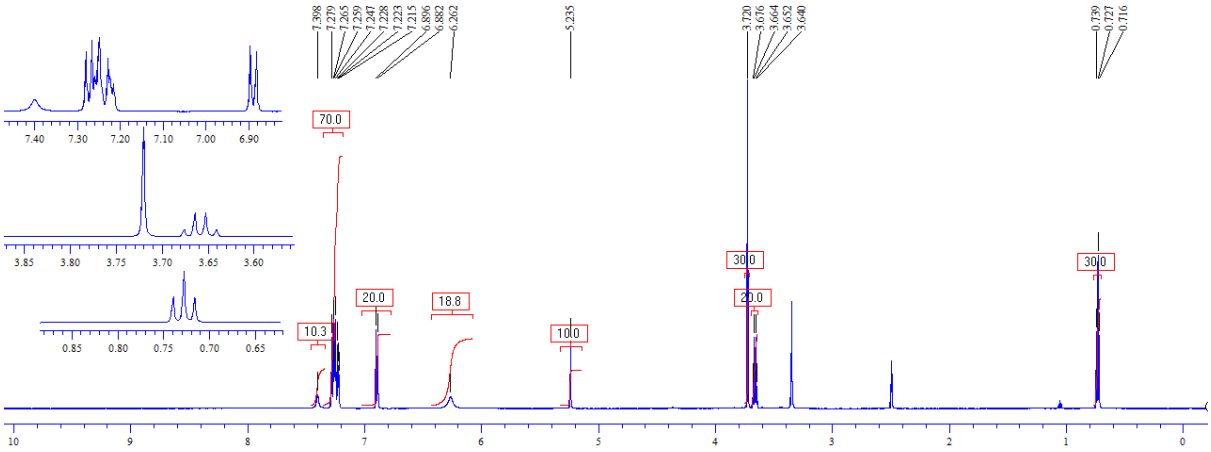

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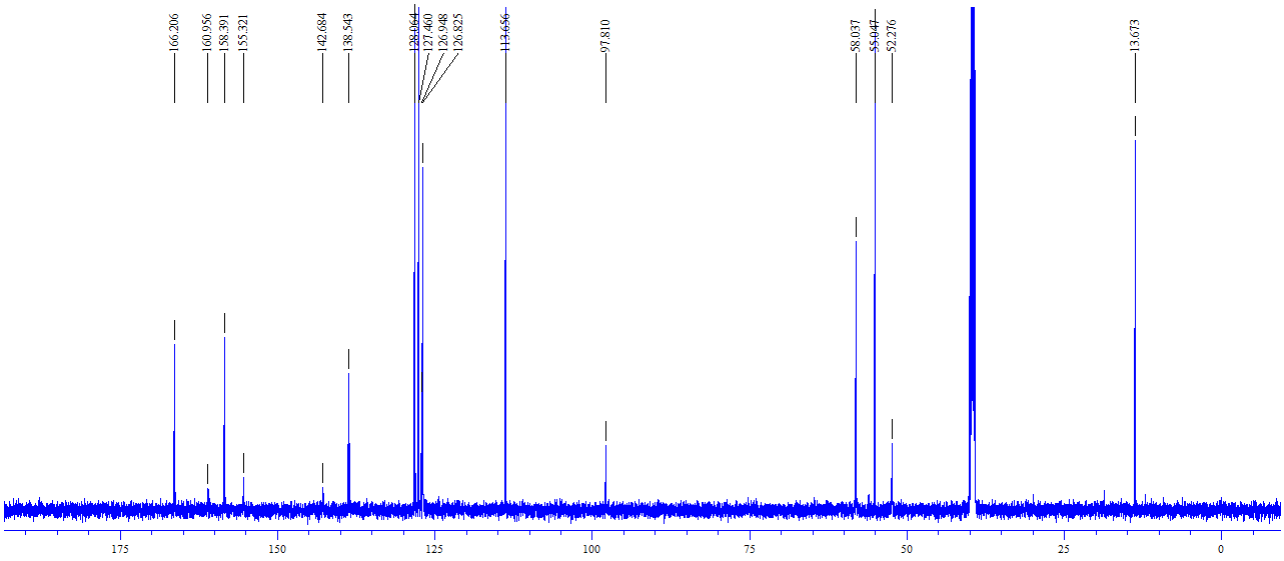

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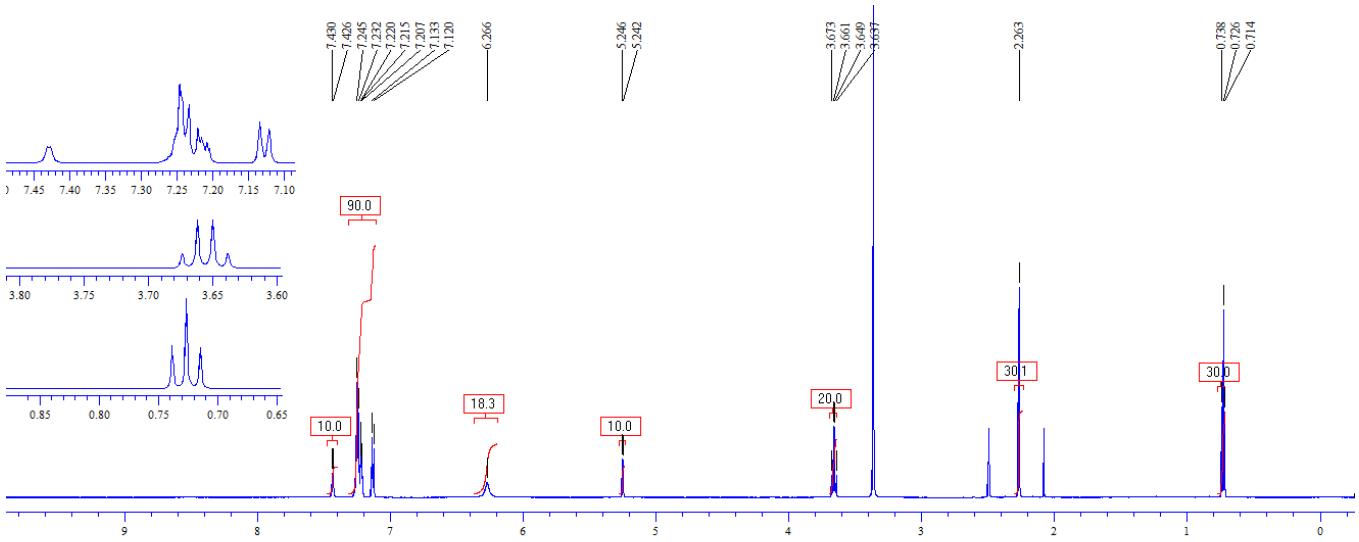

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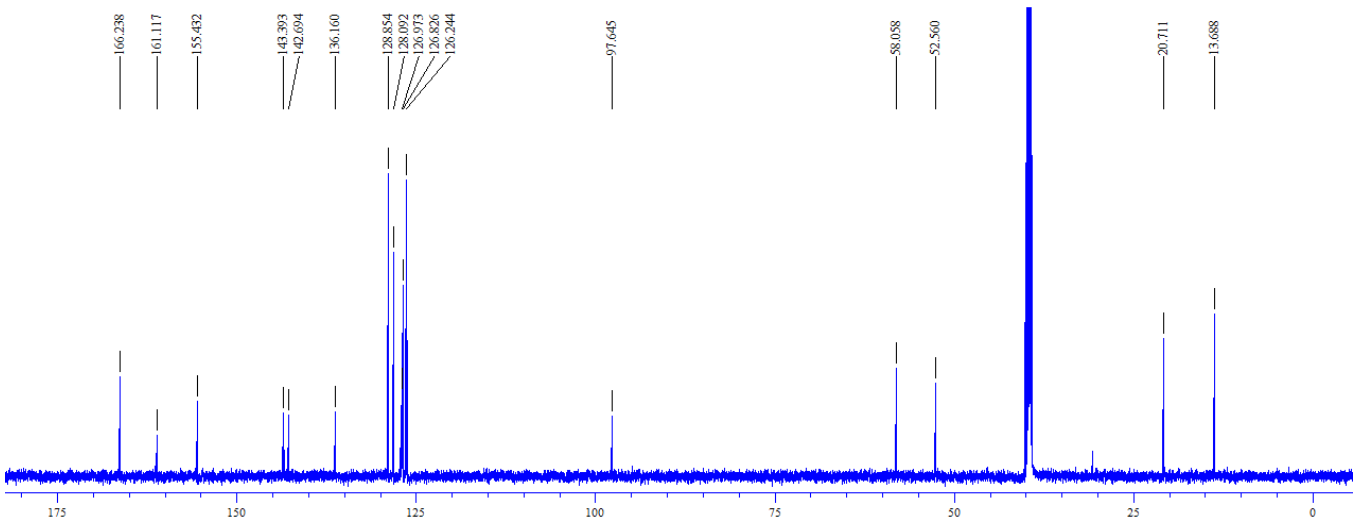
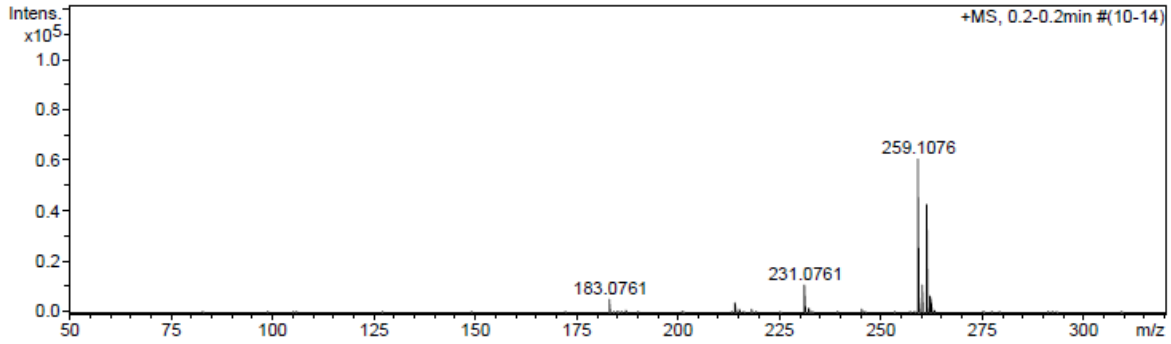


APPENDIX II

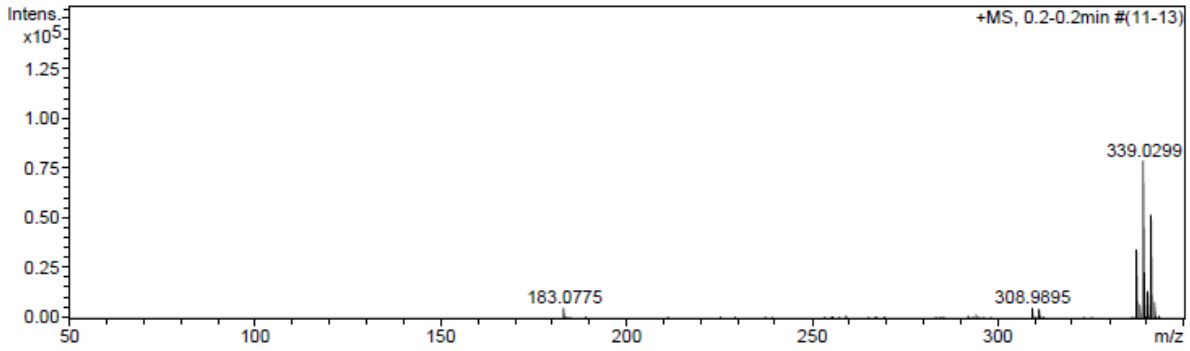
MS

Compound 1a



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
259.1076	1	C 14 H 15 N 2 O 3	100.00	259.1077	0.1	0.4	396.3	8.5	ok	even
261.1229	1	C 14 H 17 N 2 O 3	100.00	261.1234	0.5	1.8	2.8	7.5	ok	even

Compound 1b



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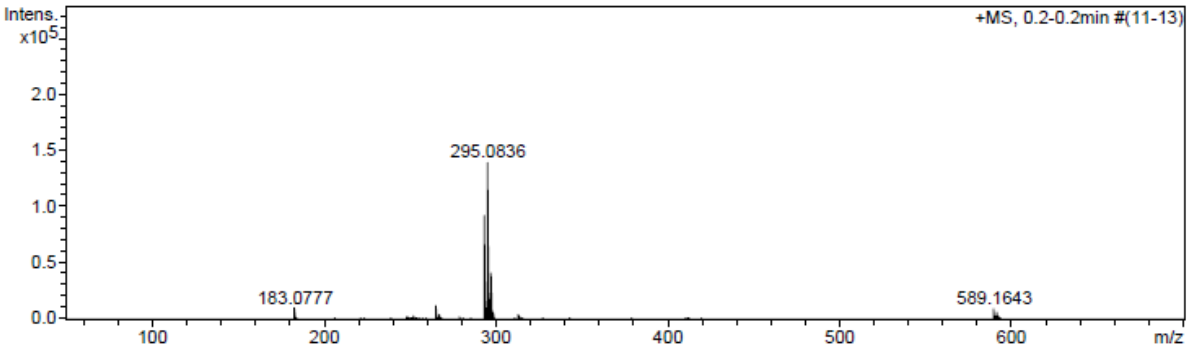
337.0209	1	C 14 H 14 Br N 2 O 3	100.00	337.0182	-2.7	-8.0	381.9	8.5	ok	even
	2	C 14 H 9 O 10	0.00	337.0190	-1.9	-5.7	647.4	10.5	ok	even
	3	C 12 H 7 N 3 O 9	0.00	337.0177	-3.3	-9.7	649.0	11.0	ok	odd
	4	C 13 H 3 N 7 O 5	0.00	337.0190	-1.9	-5.7	652.2	16.0	ok	odd
338.0241	1	C 13 H 24 Br 2	100.00	338.0239	-0.2	-0.5	554.7	1.0	ok	odd
	2	C 14 H 15 Br N 2 O 3	0.00	338.0261	2.0	5.8	702.9	8.0	ok	odd
339.0299	1	C 14 H 16 Br N 2 O 3	100.00	339.0339	4.0	11.9	151.7	7.5	ok	even
	2	C 13 H 25 Br 2	0.00	339.0318	1.9	5.6	293.8	0.5	ok	even

Compound 1c



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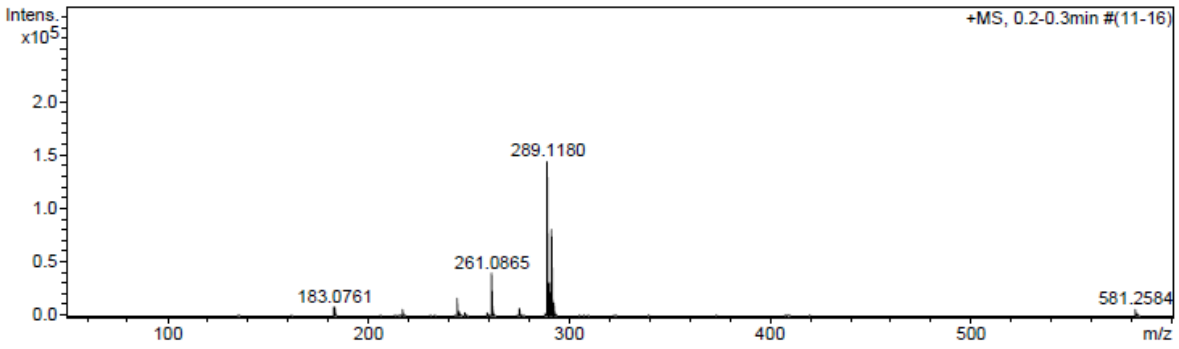
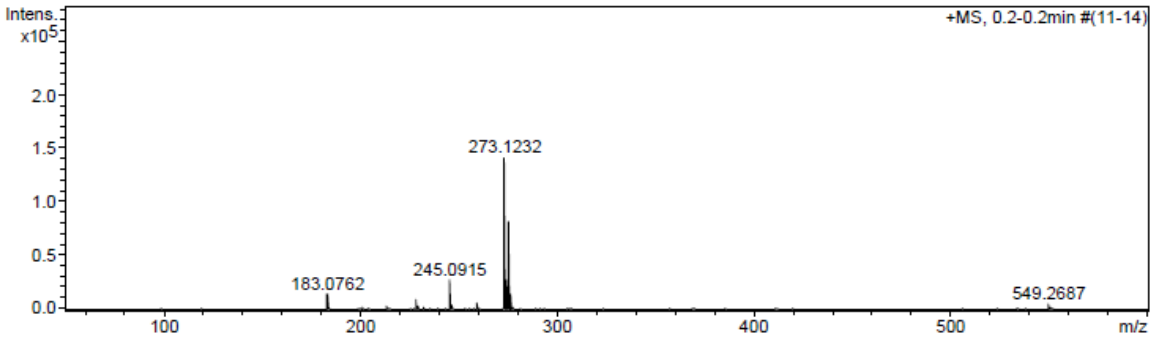
293.0712	1	C 14 H 14 Cl N 2 O 3	100.00	293.0687	-2.5	-8.5	377.1	8.5	ok	even
294.0746	1	C 14 H 15 Cl N 2 O 3	100.00	294.0766	2.0	6.7	628.5	8.0	ok	odd
	2	C 13 H 8 N 7 O 2	0.00	294.0734	-1.2	-4.1	707.1	13.5	ok	even
	3	C 14 H 14 O 7	0.00	294.0734	-1.2	-4.1	712.5	8.0	ok	odd
295.0836	1	C 14 H 16 Cl N 2 O 3	100.00	295.0844	0.8	2.6	22.8	7.5	ok	even
	2	C 14 H 15 O 7	0.10	295.0812	-2.4	-8.1	154.8	7.5	ok	even
	3	C 13 H 9 N 7 O 2	0.07	295.0812	-2.4	-8.1	159.4	13.0	ok	odd
589.1643	1	C 28 H 31 Cl 2 N 4 O 6	100.00	589.1615	-2.8	-4.7	21.0	14.5	ok	even
	2	C 28 H 25 N 6 O 9	0.00	589.1678	3.4	5.8	304.7	19.5	ok	even

Compound 1d



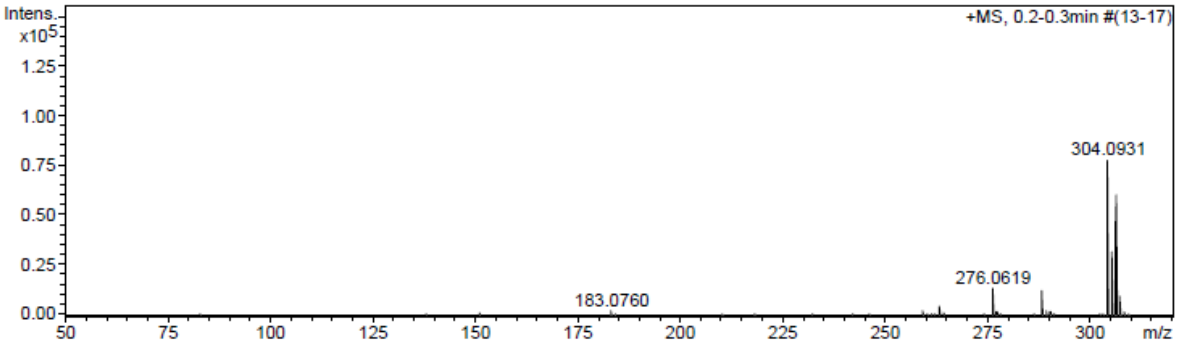
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
289.1180	1	C 15 H 17 N 2 O 4	100.00	289.1183	0.3	0.9	312.0	8.5	ok	even
291.1331	1	C 15 H 19 N 2 O 4	100.00	291.1339	0.8	2.9	7.3	7.5	ok	even
581.2584	1	C 30 H 37 N 4 O 8	43.73	581.2606	2.2	3.7	7.1	14.5	ok	even
	2	C 29 H 31 N 11 O 3	41.75	581.2606	2.2	3.7	9.9	20.0	ok	odd
	3	C 28 H 35 N 7 O 7	100.00	581.2592	0.8	1.4	10.8	15.0	ok	odd
	4	C 31 H 33 N 8 O 4	11.23	581.2619	3.5	6.0	12.8	19.5	ok	even
	5	C 29 H 41 O 12	94.33	581.2593	0.8	1.4	13.7	9.5	ok	even

Compound 1e



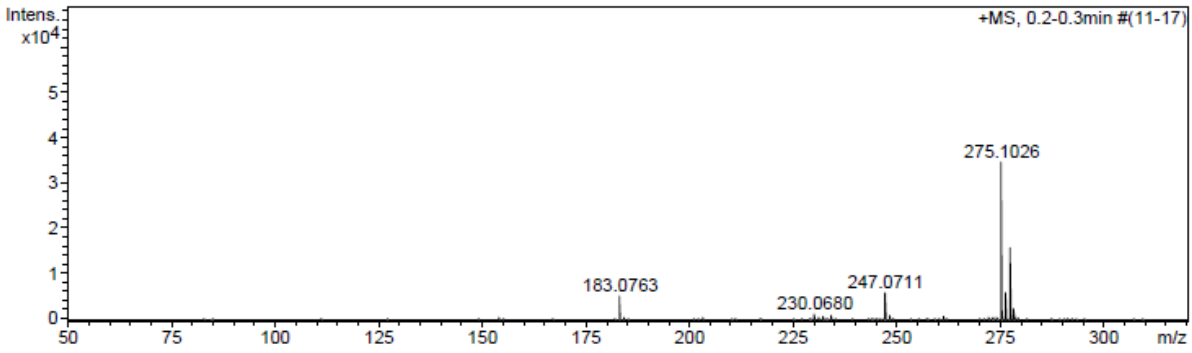
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
273.1232	1	C 15 H 17 N 2 O 3	100.00	273.1234	0.2	0.8	322.8	8.5	ok	even
275.1381	1	C 15 H 19 N 2 O 3	100.00	275.1390	0.9	3.4	0.4	7.5	ok	even
549.2687	1	C 30 H 37 N 4 O 6	45.63	549.2708	2.0	3.7	6.1	14.5	ok	even
	2	C 29 H 41 O 10	100.00	549.2694	0.7	1.3	10.2	9.5	ok	even

Compound 1f



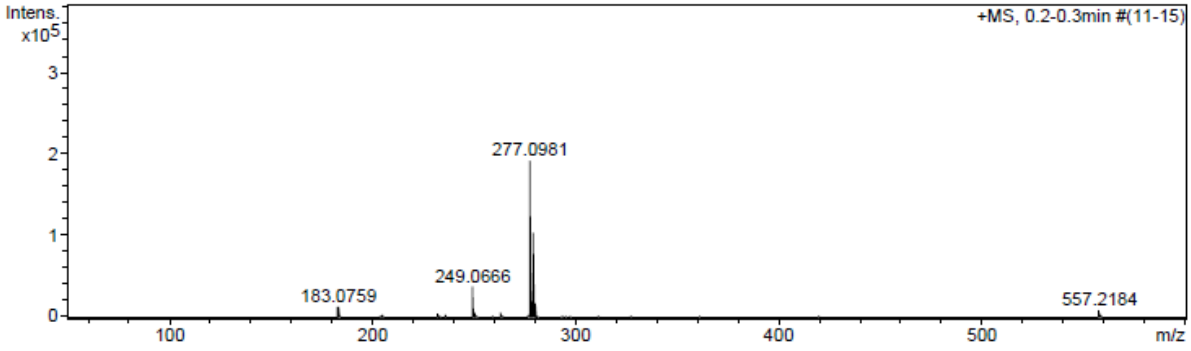
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
304.0931	1	C 14 H 14 N 3 O 5	100.00	304.0928	-0.3	-0.9	459.3	9.5	ok	even
	2	C 13 H 8 N 10	69.71	304.0928	-0.3	-0.9	461.3	15.0	ok	odd
306.1078	1	C 14 H 16 N 3 O 5	100.00	306.1084	0.6	2.1	3.3	8.5	ok	even
	2	C 13 H 10 N 10	83.77	306.1084	0.6	2.1	13.2	14.0	ok	odd

Compound 1g



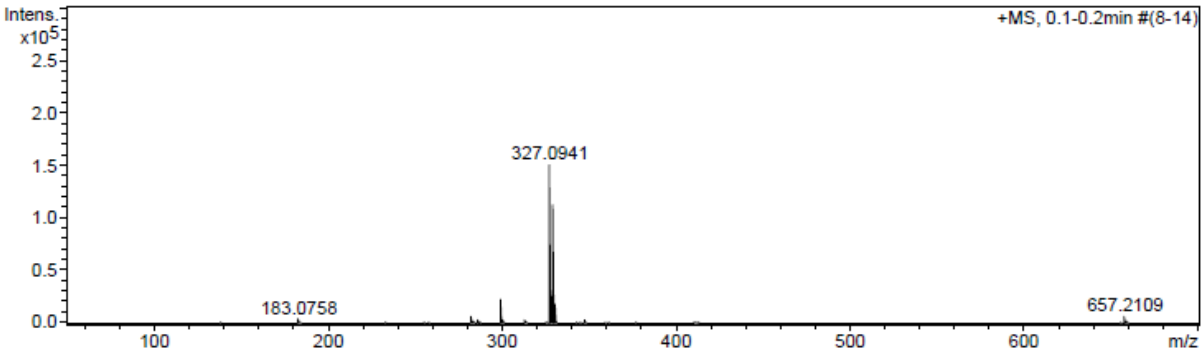
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
275.1026	1	C 14 H 15 N 2 O 4	100.00	275.1026	0.0	0.1	252.4	8.5	ok	even
277.1172	1	C 14 H 17 N 2 O 4	100.00	277.1183	1.1	4.0	3.4	7.5	ok	even

Compound 1h



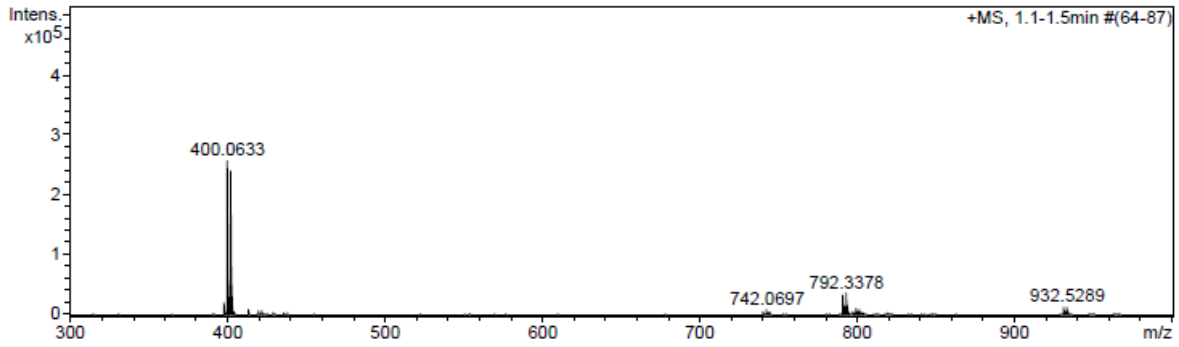
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
277.0981	1	C 14 H 14 F N 2 O 3	100.00	277.0983	0.2	0.6	299.9	8.5	ok	even
279.1129	1	C 14 H 16 F N 2 O 3	100.00	279.1139	1.0	3.7	1.9	7.5	ok	even
	2	C 14 H 17 N O 5	27.03	279.1101	-2.8	-10.0	2.5	7.0	ok	odd
	3	C 15 H 13 N 5 O	62.15	279.1115	-1.4	-5.2	14.5	12.0	ok	odd
557.2184	1	C 28 H 31 F 2 N 4 O 6	31.42	557.2206	2.2	4.0	5.5	14.5	ok	even
	2	C 28 H 32 F N 3 O 8	49.22	557.2168	-1.6	-2.9	7.4	14.0	ok	odd
	3	C 30 H 33 F 2 N O 7	8.27	557.2220	3.6	6.4	8.8	14.0	ok	odd
	4	C 30 H 34 F O 9	100.00	557.2181	-0.2	-0.4	9.5	13.5	ok	even
	5	C 29 H 28 F N 7 O 4	99.22	557.2181	-0.3	-0.5	9.8	19.0	ok	odd
	6	C 29 H 27 F 2 N 8 O 2	8.03	557.2220	3.6	6.4	10.7	19.5	ok	even
	7	C 31 H 30 F N 4 O 5	57.59	557.2195	1.1	2.0	15.7	18.5	ok	even
	8	C 31 H 31 N 3 O 7	16.54	557.2157	-2.7	-4.9	16.8	18.0	ok	odd
	9	C 30 H 25 N 10 O 2	15.37	557.2156	-2.7	-4.9	20.1	23.5	ok	even
	10	C 30 H 24 F N 11	51.21	557.2195	1.1	2.0	21.4	24.0	ok	odd

Compound 1i



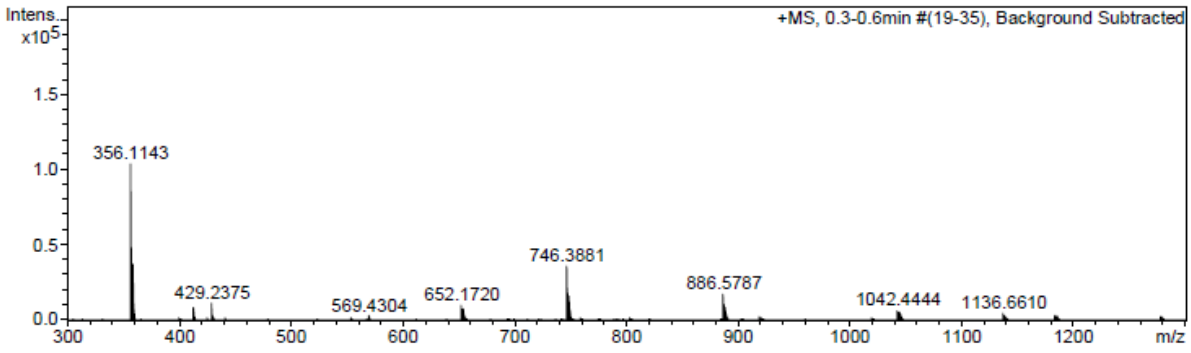
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
327.0941	1	C 15 H 14 F 3 N 2 O 3	100.00	327.0951	1.0	2.9	422.0	8.5	ok	even
329.1090	1	C 15 H 16 F 3 N 2 O 3	100.00	329.1108	1.7	5.3	3.3	7.5	ok	even
657.2109	1	C 28 H 30 F 5 N 6 O 7	50.53	657.2091	-1.9	-2.8	5.4	14.5	ok	even
	2	C 29 H 35 F 6 O 10	45.01	657.2129	2.0	3.0	6.6	9.5	ok	even
	3	C 28 H 28 F 3 N 10 O 6	16.94	657.2140	3.1	4.7	7.9	18.5	ok	even
	4	C 29 H 35 F 2 N 2 O 13	95.07	657.2102	-0.7	-1.1	8.9	12.5	ok	even
	5	C 30 H 31 F 6 N 4 O 6	12.97	657.2142	3.3	5.0	9.2	14.5	ok	even
	6	C 28 H 37 N 2 O 16	20.00	657.2138	2.8	4.3	10.9	11.5	ok	even
	7	C 29 H 26 F 5 N 10 O 3	100.00	657.2104	-0.5	-0.8	12.4	19.5	ok	even
	8	C 30 H 31 F 2 N 6 O 9	93.59	657.2115	0.6	0.9	13.7	17.5	ok	even
	9	C 29 H 26 F N 12 O 6	12.28	657.2077	-3.2	-4.9	16.3	22.5	ok	even
	10	C 30 H 22 F N 16 O 2	30.40	657.2090	-1.9	-2.9	28.5	27.5	ok	even

Compound 2b



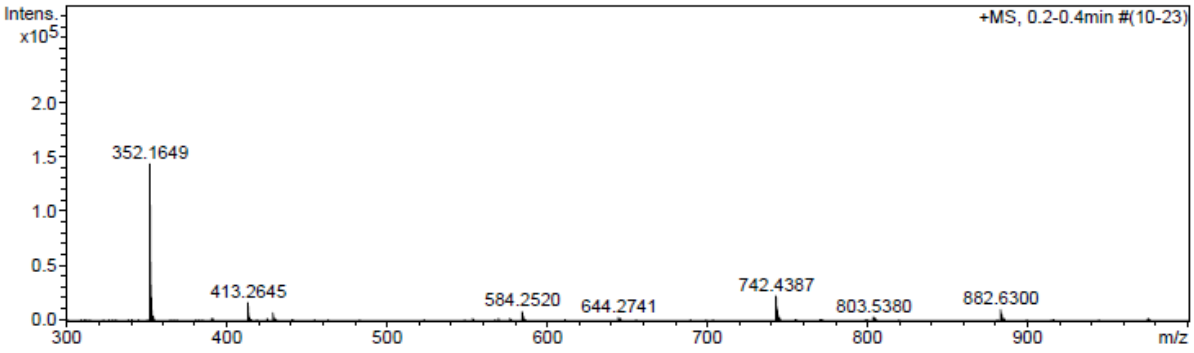
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
400.0633	1	C 19 H 19 Br N 3 O 2	100.00	400.0655	2.2	5.4	29.3	11.5	ok	even
	2	C 19 H 14 N O 9	0.00	400.0663	3.0	7.4	458.6	13.5	ok	even

Compound 2c



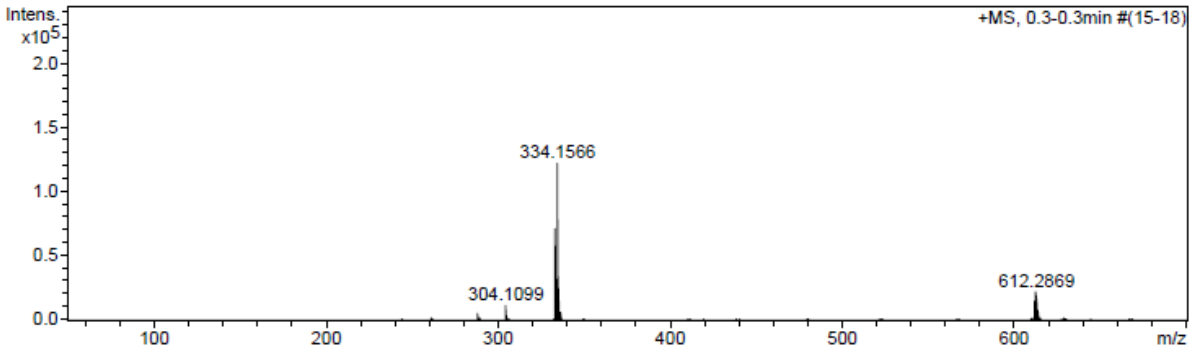
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
356.1143	1	C 19 H 19 Cl N 3 O 2	100.00	356.1160	1.8	4.9	9.0	11.5	ok	even
	2	C 19 H 18 N O 6	0.02	356.1129	-1.4	-4.0	188.1	11.5	ok	even
	3	C 20 H 14 N 5 O 2	0.05	356.1142	-0.1	-0.2	189.3	16.5	ok	even

Compound 2d



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
352.1649	1	C 20 H 22 N 3 O 3	100.00	352.1656	0.6	1.8	2.6	11.5	ok	even

Compound 2e

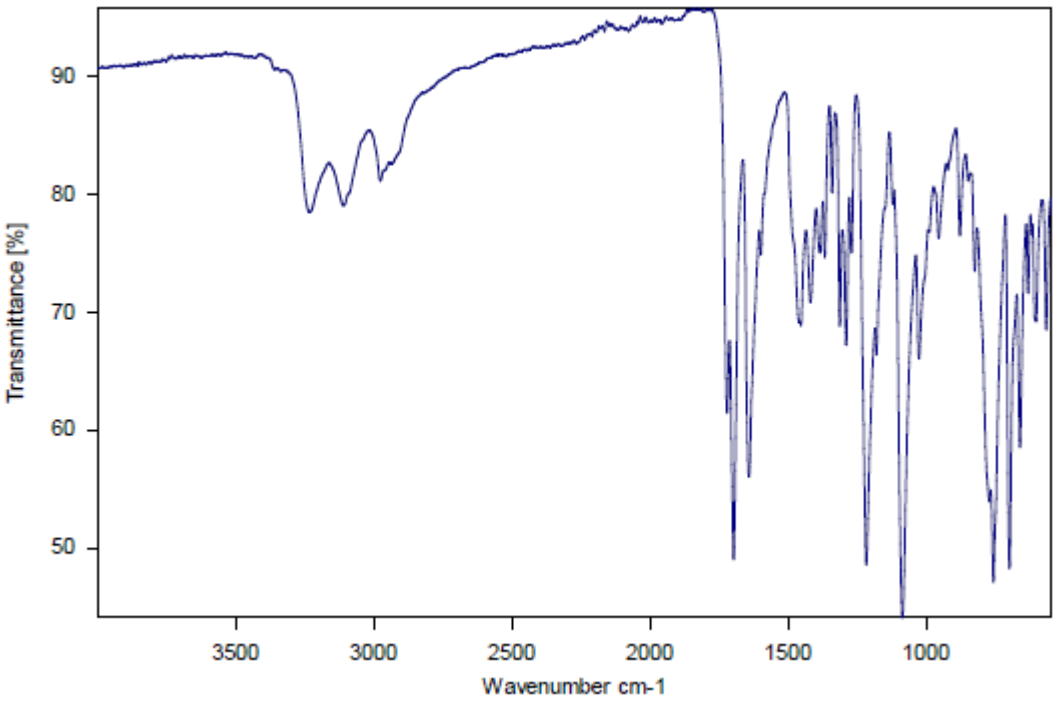


Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
333.1494	1	C 20 H 19 N 3 O 2	100.00	333.1472	-2.3	-6.8	520.8	13.0	ok	odd
334.1566	1	C 20 H 20 N 3 O 2	100.00	334.1550	-1.6	-4.8	12.5	12.5	ok	even
610.2706	1	C 39 H 36 N 3 O 4	100.00	610.2700	-0.6	-0.9	679.6	23.5	ok	even
	2	C 37 H 34 N 6 O 3	16.79	610.2687	-1.9	-3.1	683.1	24.0	ok	odd
611.2799	1	C 39 H 37 N 3 O 4	100.00	611.2779	-2.0	-3.3	353.2	23.0	ok	odd
	2	C 38 H 37 N 5 O 3	0.00	611.2891	9.2	15.1	355.3	23.0	ok	odd
	3	C 37 H 35 N 6 O 3	12.87	611.2765	-3.4	-5.5	358.9	23.5	ok	even
612.2869	1	C 37 H 36 N 6 O 3	42.92	612.2843	-2.5	-4.1	25.1	23.0	ok	odd
	2	C 39 H 38 N 3 O 4	100.00	612.2857	-1.2	-1.9	31.1	22.5	ok	even
613.2898	1	C 37 H 37 N 6 O 3	100.00	613.2922	2.3	3.8	103.1	22.5	ok	even
	2	C 38 H 37 N 4 O 4	0.00	613.2809	-8.9	-14.5	105.4	22.5	ok	even
	3	C 39 H 39 N 3 O 4	19.90	613.2935	3.7	6.0	109.4	22.0	ok	odd

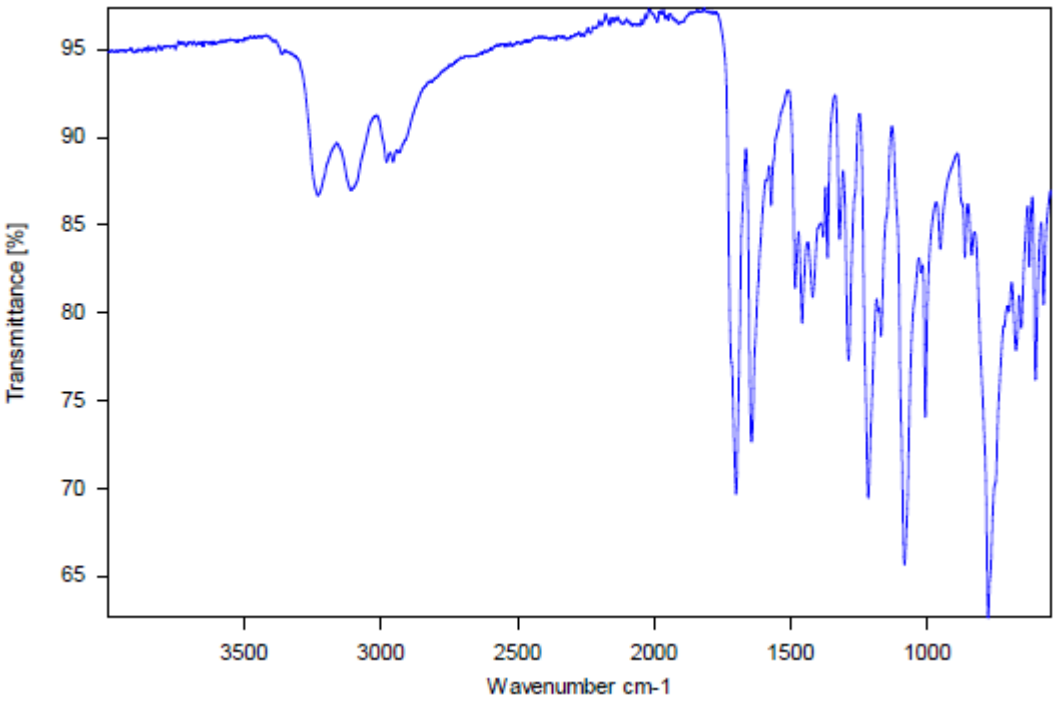
APPENDIX III

IR

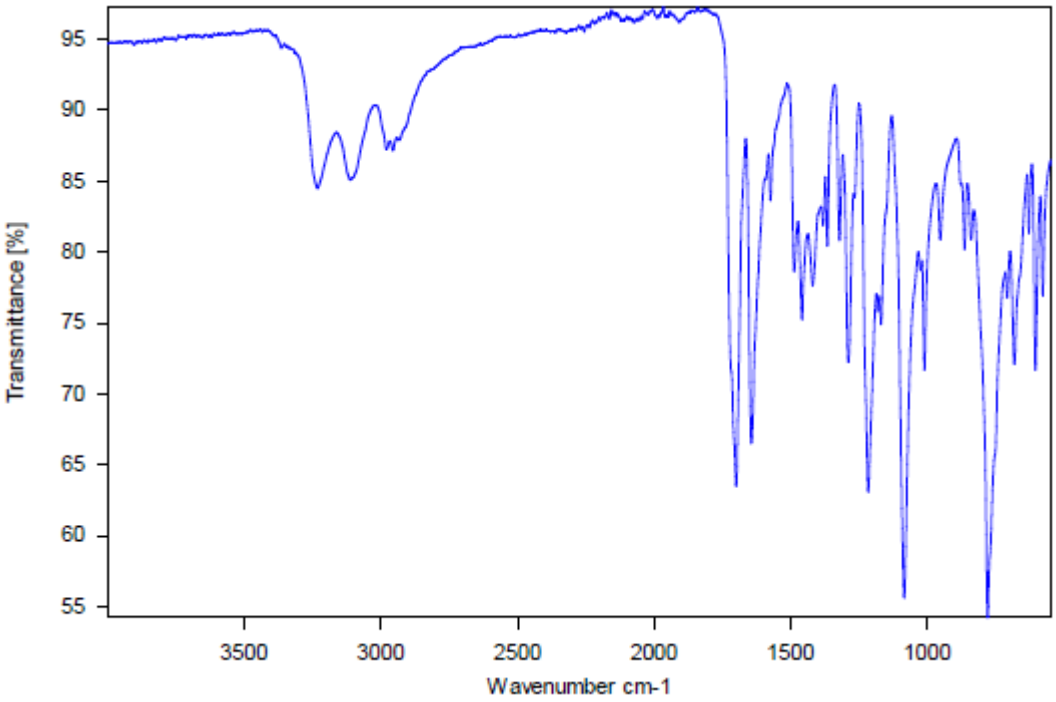
Compound 1a



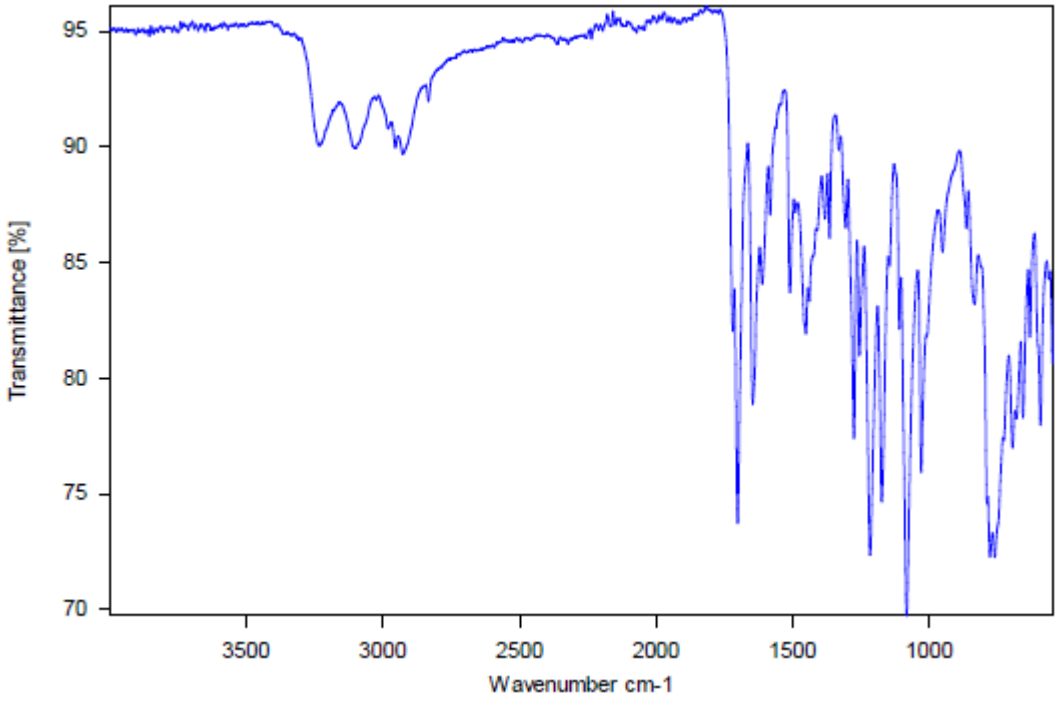
Compound 1b



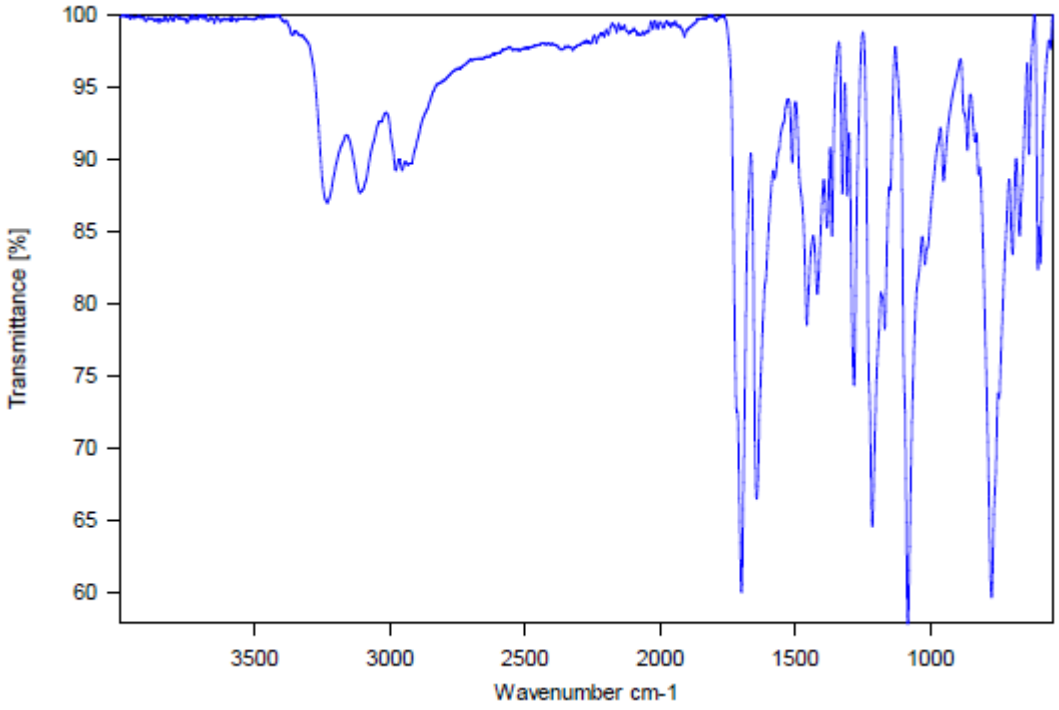
Compound 1c



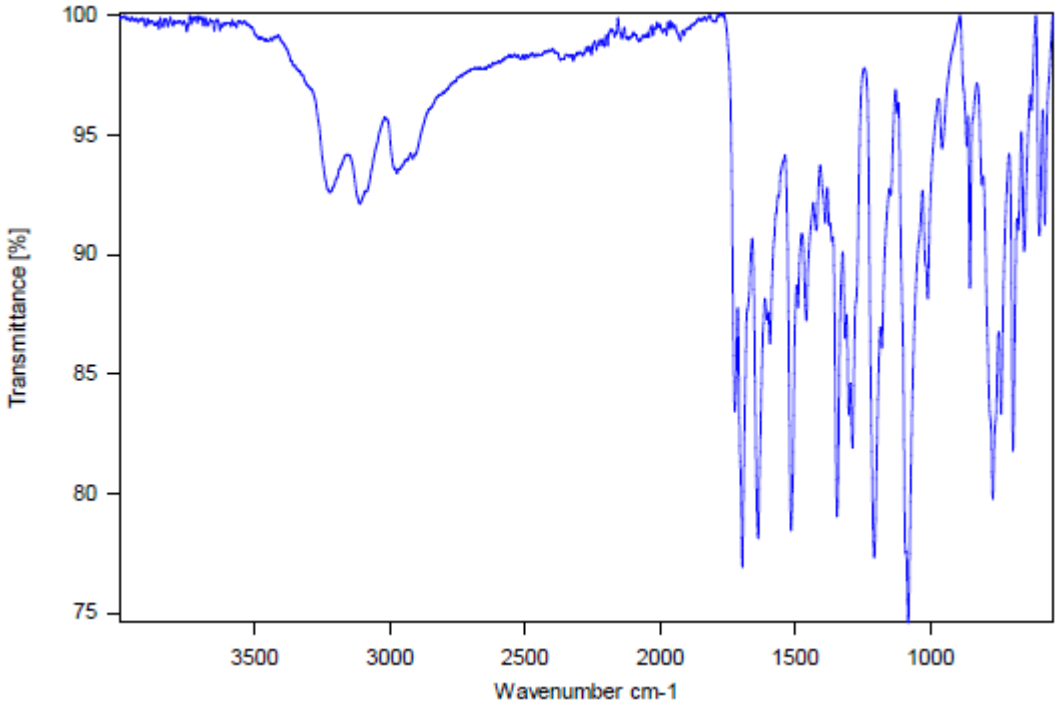
Compound 1d



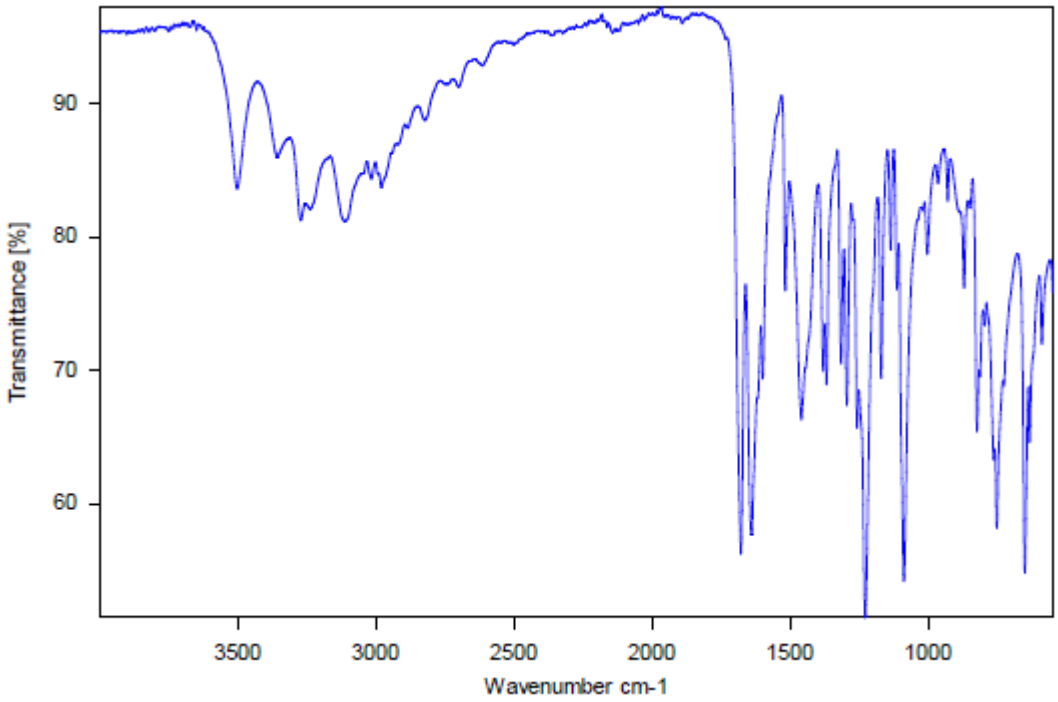
Compound 1e



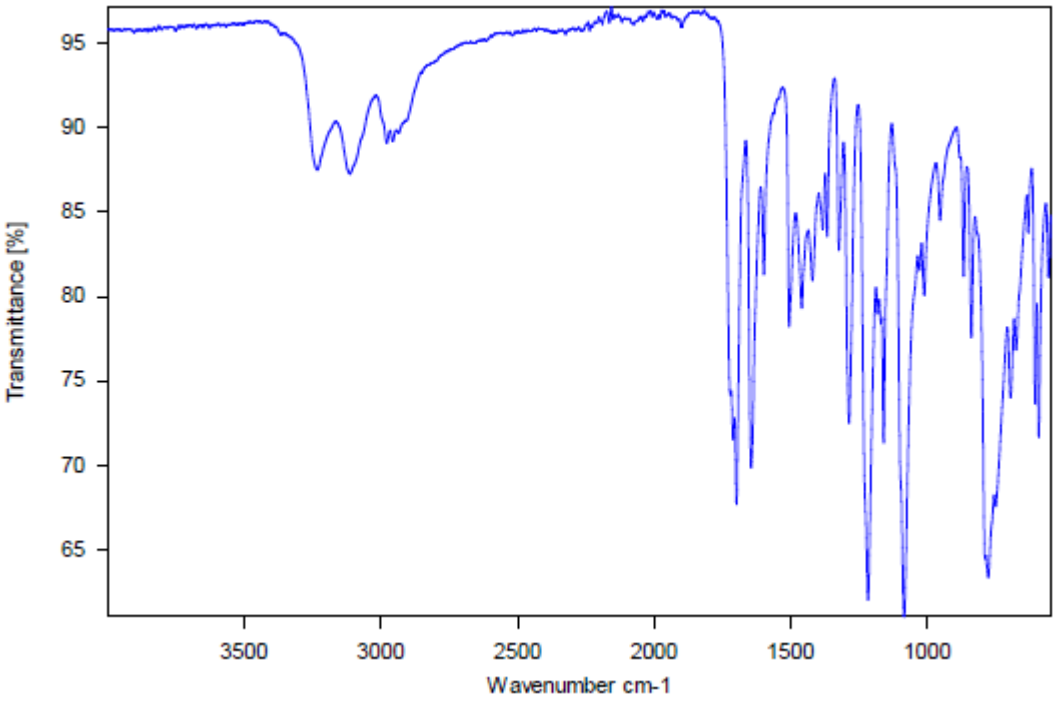
Compound 1f



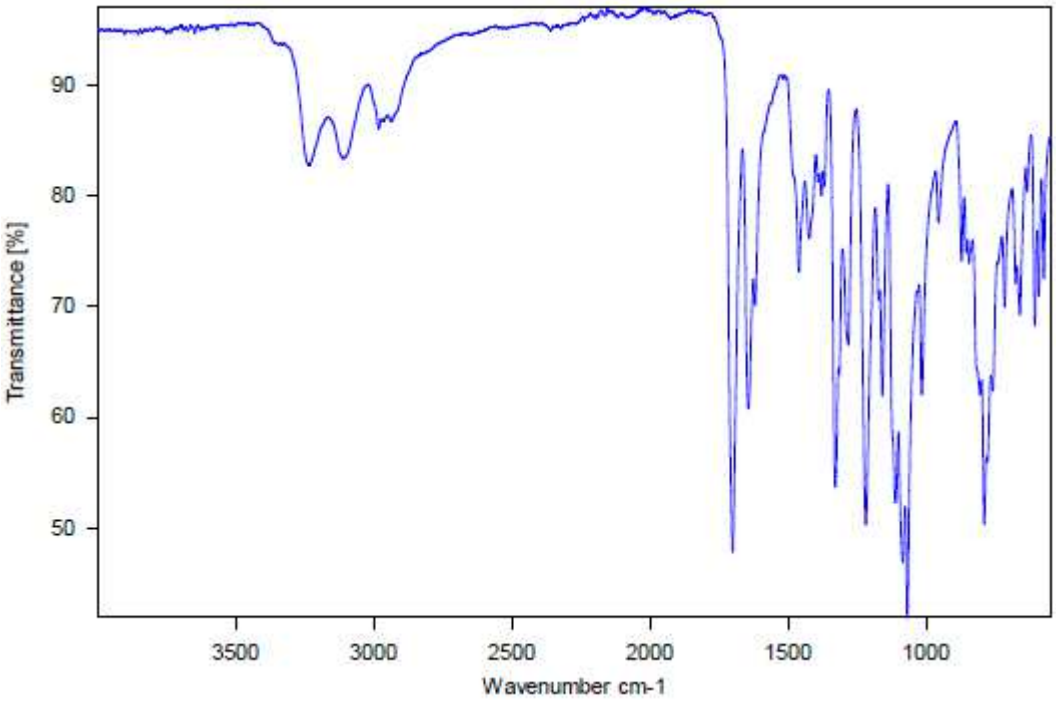
Compound 1g



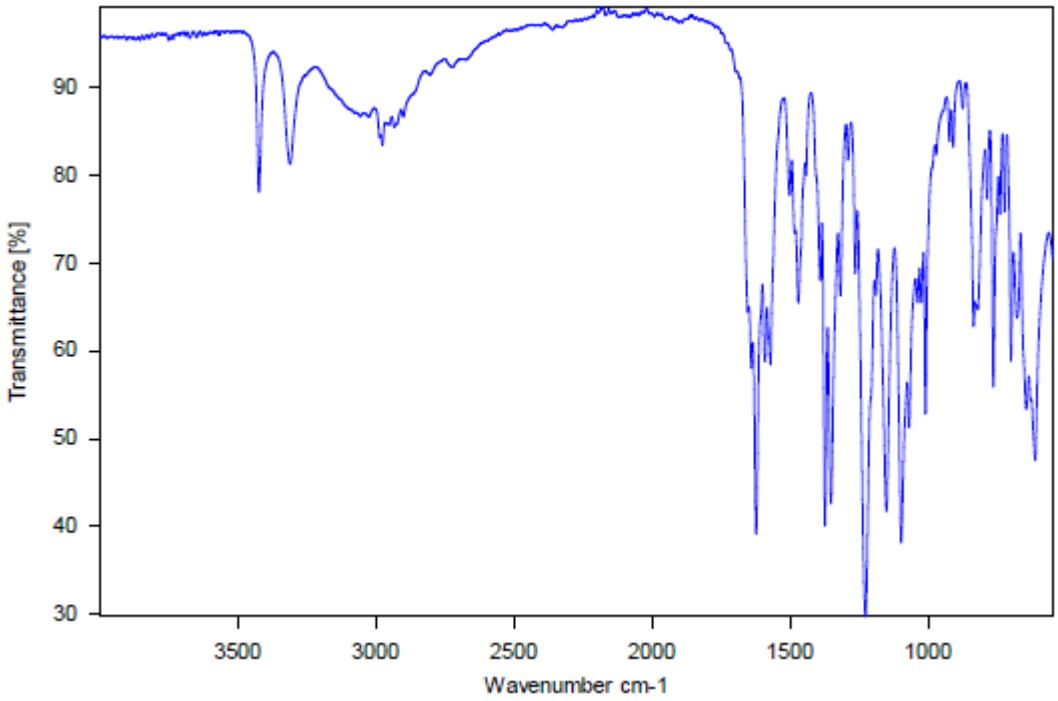
Compound 1h



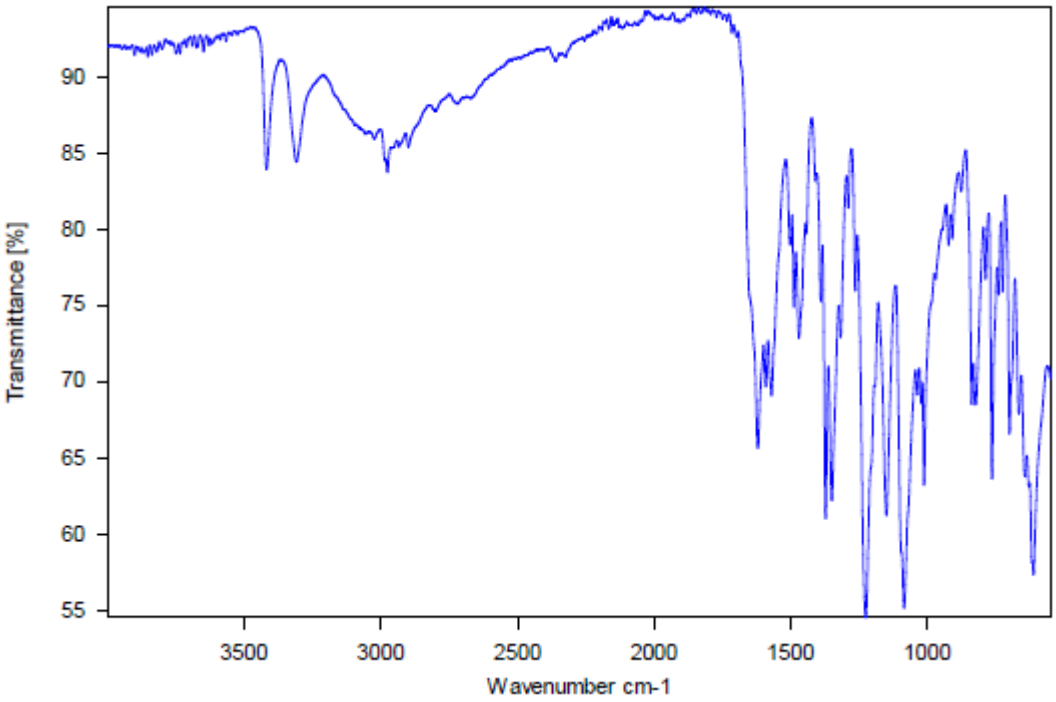
Compound 1i



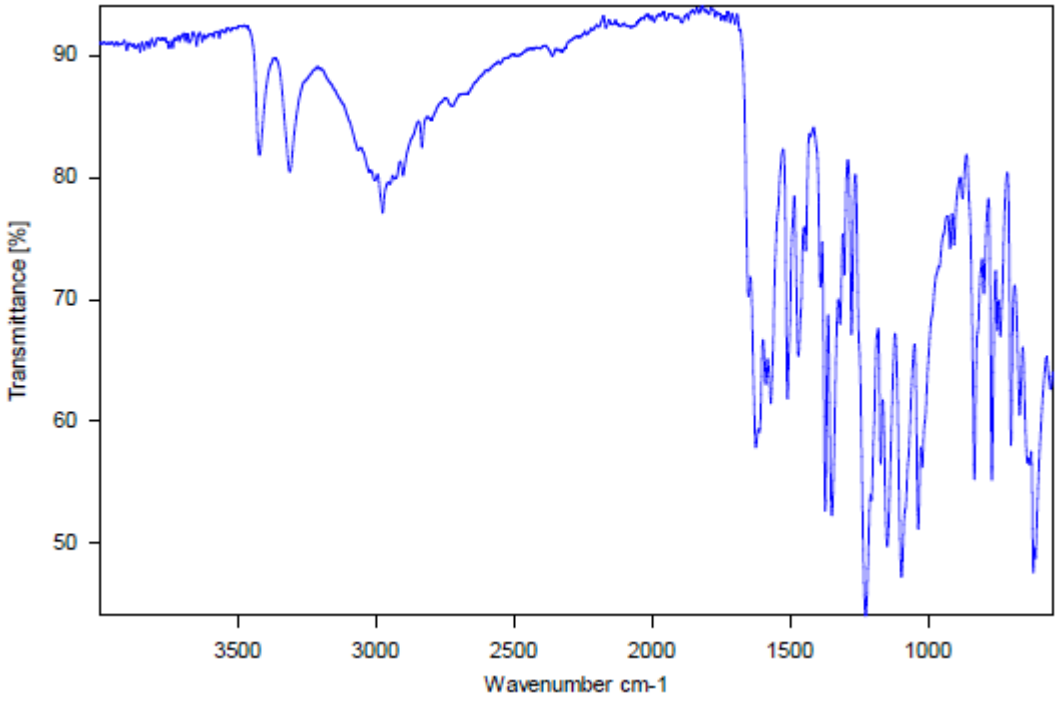
Compound 2b



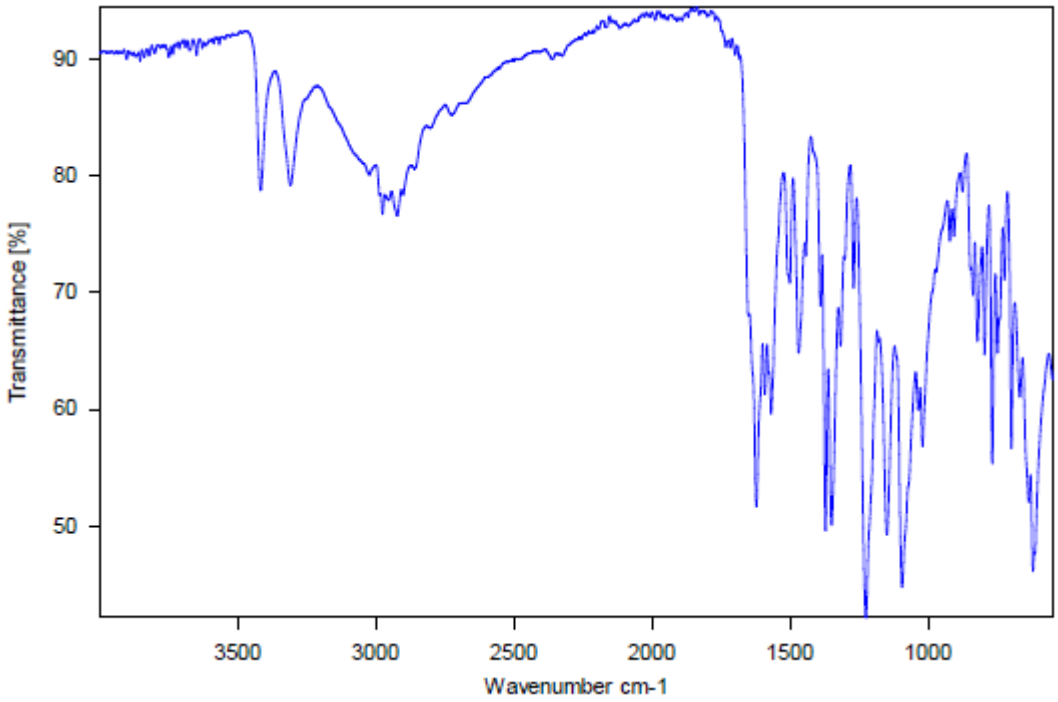
Compound 2c



Compound 2d



Compound 2e


Transmittance [%]
90
80
70
60
50
3500
3000
2500
2000
1500
1000
Wavenumber cm-1