Design and synthesis of dual acting carbamate inhibitors of acetylcholinesterase and monoamine oxidase

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- My husband, Reinald Landro, for believing in me and supporting me all the way. Thank you for walking this journey with me.

Jeremiah 29:11

‘For I know the plans I have for you,
declares the Lord, plans to prosper
you and not to harm you, plans to
give you hope and a future.’

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ABSTRACT

TITLE

Design and synthesis of dual acting carbamate inhibitors of acetylcholinesterase and monoamine oxidase.

KEY WORDS

Carbamate, Dihydroquinolinone, Acetylcholinesterase, Monoamine Oxidase, Alzheimer's disease, Parkinson's disease, Parkinson's disease dementia.

BACKGROUND AND RATIONALE

Alzheimer's disease (AD), the most common neurodegenerative disorder, affects about 10% of the population over the age of 65 years. The disease is typified by symptoms such as memory loss and impairment in abilities including attention, concentration, orientation and judgment. Two hypotheses exist regarding the pathogenesis of AD: the first proposes that a decrease in the production of acetylcholine (Ach) in the synaptic junction (cholinergic hypothesis) is the main causative factor, while the second suggests that the disease is largely due to the aggregation of toxic amyloid-β (Aβ) peptide in the brain (amyloid hypothesis).

Ach is a neurotransmitter which plays an important role in attention, cognitive processing and other cognitive functions. Consequently, cholinesterases are very important enzymes as they modulate the levels of Ach in the brain. Acetylcholinesterase (AChE) inhibitors including donepezil, rivastigmine and galantamine, inhibits the metabolism of Ach, thus increasing the Ach available for binding. These agents are therefore most commonly used in the symptomatic treatment of AD. Currently, no AD agent is registered as neuroprotective, and this neurodegenerative disorder progresses unhindered with an associated decrease in quality of life. Therefore, the discovery of novel drugs that can slow or stop its progression is of great importance.

Parkinson's disease (PD), on the other hand, is the second most common neurodegenerative disorder and is characterised by the loss of dopamine (DA) in the nigrostriatum. The symptoms of PD can by classified as either motor or non-motor symptoms. Motor symptoms include bradykinesia, muscle rigidity, resting tremor and impaired postural balance. Non-motor symptoms include sleep disturbances, autonomic

dysfunction and sensory abnormalities. Parkinson's disease dementia (PDD), which is typified by cognitive decline, is often experienced during the later stages of the disease. With the increase in life expectancy throughout the Western world, it is expected that PDD will become more prevalent in the future.

The symptomatic treatment of PD usually involves levodopa use. Its continuous use is unfortunately associated with motor complications that impair the quality of life. Additional therapies, which include catechol-O-methyltransferase inhibitors, DA agonists and monoamine oxidase (MAO) B inhibitors are further used in PD treatment. The MAO enzymes (MAO-A and MAO-B), play an important role during the oxidative degradation of amine neurotransmitters including DA, serotonin (5-HT) and epinephrine. The inhibition of these enzymes decreases DA metabolism in the brain, resulting in an increase in the DA concentration. It is also likely to be neuroprotective as the production of harmful metabolic by-products such as hydrogen peroxide is decreased. Since the use of non-selective, irreversible MAO inhibitors are associated with severe side-effects, current efforts are aimed at designing selective, reversible MAO inhibitors. The MAO-B enzyme is of particular significance in PD because it is more active than MAO-A in the basal ganglia which is mainly responsible for the catabolism of DA in the brain. The treatment of the cognitive deficits experienced during the later stages of PD, and in PDD, in particular is not adequate and treatments that address the motor and non-motor aspects simultaneously are urgently required.

It is postulated that a dual MAO-B and AChE inhibitor would improve motor symptoms of PD while improving cognitive deficits at the same time. Such an agent would not only be useful in the treatment of PD, but also in PDD and AD as the MAO inhibition component has the possibility of offering neuroprotection. The dihydroquinolinone scaffold has been shown to be privileged with regards to the inhibition of MAO, while the carbamate moiety often features in the structures of AChE inhibitors.

AIM

The aim of this study was therefore to design, synthesise and evaluate novel carbamates as dual inhibitors of MAO and AChE.

METHODS

Compounds were synthesised using a one step literature procedure. 6-Hydroxy-3,4-dihydro-2(1*H*)-quinolinone, 7-hydroxy-3,4-dihydro-2(1*H*)-quinolinone, 3- and 4-acetamidophenol were reacted with commercially available carbamoyl chlorides under basic conditions to yield

the target compounds. Compounds were characterised by using NMR and IR spectroscopy as well as mass spectrometry. Purity was determined by HPLC and melting points were determined. The potential of the synthesised compounds to inhibit MAO enzymes were expressed as IC_{50} (50% inhibition concentration) values and the SI (selectivity index) was determined. A fluorometric assay, using kynuramine as substrate was employed. AChE inhibitory activity was determined by measuring the rate of thiocholine production, as generated by the hydrolysis of acetylthiocholine, which served as substrate in a spectrophotometric assay.

RESULTS AND DISCUSSION

Twenty eight novel compounds were successfully synthesised, albeit in low yields. Generally, most of the synthesised compounds exhibited weak to no inhibition of both MAO-A and MAO-B. Compound **8g**, 2-oxo-1,2,3,4-tetrahydroquinolin-7-yl methyl(phenyl)carbamate, was the most potent MAO-B inhibitor of the current series with an IC_{50} value of 3.73 µM, and was MAO-B selective. It is postulated that the rigidity of the carbamate side chain is responsible for the loss of activity observed for the compounds of this study, when compared to the highly potent dihydroquinolinone derivatives of a previous study. Disappointingly, none of the synthesised compounds inhibited AChE, possibly due to the replacement of an ionisable amine group with an amide. Although the biological results of this study were disappointing, useful information was obtained regarding structural requirements for binding to both MAO and AChE.

OPSOMMING

TITEL

Ontwerp en sintese van dubbelwerkende karbamaat inhibeerders van asetielcholienesterase en monoamienoksidase.

SLEUTELWOORDE

Karbamaat, Dihidrokinolinoon, Asetielcholienesterase, Monoamienoksidase, Alzheimer se siekte, Parkinson se siekte, Parkinson se siekte demensie.

AGTERGROND EN MOTIVERING

Alzheimer se siekte, die mees algemene neurodegeneratiewe siekte, affekteer ongeveer 10% van die bevolking bo die ouderdom van 65 jaar. Die siekte word gekenmerk deur simptome soos geheueverlies en inkorting van vermoëns soos aandag, konsentrasie, oriëntasie en oordeel. Twee hipoteses bestaan aangaande die patogenese van Alzheimers se siekte: volgens die eerste is 'n afname in die produksie van asetielcholien in die sinaptiese spleet (cholinerge hipotese) die belangrikste veroorsakende faktor, terwyl die tweede hipotese voorstel dat die siekte grootliks te wyte is aan die aggregasie van giftige amiloïed-β peptied (Aβ) in die brein (amiloïede hipotese).

Asetielcholien is 'n neurotransmitter wat 'n belangrike rol in aandag, kognitiewe prosessering en ander kognitiewe funksies speel. Gevolglik is cholienesterases baie belangrike ensieme aangesien hulle die vlakke van asetielcholien in die brein moduleer. Asetielcholienesterase (AChE)-inhibeerders wat donepesil, rivastigmien en galantamien insluit, inhibeer die metabolisme van asetielcholien, en verhoog sodoende die asetielcholien wat beskikbaar is vir binding. Hierdie is dus die mees gebruikte middels in die simptomatiese behandeling van Alzheimers se siekte. Tans is daar geen anti-Alzheimer’s middel wat geregistreer is as neurobeskermend nie, en die verloop van hierdie neurodegeneratiewe siekte gaan dus ongehinderd voort met 'n gepaardgaande afname in lewenskwaliteit. Die ontdekking van nuwe middels wat die siekte kan vertraag of stop, is dus van groot belang.

In teenstelling daarmee is Parkinson se siekte die tweede mees algemene neurodegeneratiewe siekte en word dit gekenmerk deur ’n verlies aan dopamien in die nigrostriatum. Die simptome van Parkinson se siekte kan geklassifiseer word as óf motories of nie-motoriese simptome. Motoriese simptome sluit bradikinesie, spierstyfheid, rustremore en verswakte posturale balans in. Nie-motoriese simptome sluit weer slaapversteurings,

outonome disfunksie asook sensoriese abnormaliteite in. Parkinson-se-siekte-demensie, wat gekenmerk word deur kognitiewe agteruitgang, word dikwels ervaar gedurende die latere stadiums van die siekte. Met die toename in lewens verwagting regdeur die Westerse wêreld, word daar verwag dat Parkinson-se-siekte-demensie meer algemeen in die toekoms sal wees.

Die simptomatiese behandeling van Parkinson se siekte behels gewoonlik die gebruik van levodopa. Ongelukkig lei die langdurige gebruik daarvan tot motoriese komplikasies wat lewensgehalte benadeel. Addisionele terapie, wat die gebruik van katesjol-*O*-metieltransferase-inhibeerders, dopamienagoniste en MAO B inhibeerders insluit, word verder gebruik in die behandeling van die siekte. Die MAO ensieme (MAO-A en MAO-B), speel 'n belangrike rol in die oksidatiewe afbraak van amienneurotransmitters soos dopamien, serotonien en epinefrien. Die inhibisie van hierdie ensieme verminder dopamienmetabolisme in die brein, wat gevolglik lei tot 'n toename in die dopamienkonsentrasie. Dit is waarskynlik ook neurobeskermend aangesien die produksie van skadelike metaboliese byprodukte soos waterstofperoksied verminder word. Aangesien die gebruik van nie-selektiewe, onomkeerbare MAO-inhibeerders geassosieer word met ernstige newe-effekte, is huidige navorsingspogings gemik op die ontwerp van selektiewe, omkeerbare MAO-inhibeerders. Die MAO-B ensiem is van besondere belang in Parkinson se siekte, aangesien dit meer aktief is as MAO-A in die basale ganglia wat hoofsaaklik verantwoordelik is vir die katabolisme van dopamien in die brein. Die behandeling van die kognitiewe defekte wat voorkom gedurende die later stadiums van Parkinson se siekte en in Parkinson-se-siekte-demensie, is veral onvoldoende en behandeling wat die motoriese en nie-motoriese aspekte gelyktydig kan aanspreek is uiters nodig.

Daar word gepostuleer dat 'n tweeledige MAO-B- en AChE-inhibeerder die motoriese simptome van Parkinsons se siekte sal behandel, terwyl verbetering van kognitiewe tekorte terselfdetyd sal plaasvind. So 'n middel sal dus nie net gebruik kan word in die behandeling van Parkinson se siekte nie, maar ook in Parkinson-se-siekte-demensie en Alzheimer se siekte, aangesien die MAO-inhibisie komponent die bykomende voordeel van neurobeskerming kan bied. In vorige studies is aangetoon dat die dihidrokinolonoonkern geassosieer word met die inhibisie van MAO, terwyl die karbamaatgroep dikwels voorkom in die strukture van AChE-inhibeerders.

DOEL

Die doel van hierdie studie was dus om karbamate te ontwerp, te sintetiseer en te evalueer as tweeledige inhibeerders van MAO en AChE.

METODES

Verbindings is gesintetiseer deur gebruik te maak van 'n eenstap literatuurmetode. Die teikenverbindings is verkry deur 6-hidroksi-3,4-dihidro-2(1*H*)-kinolinoon, 7-hidroksi-3,4-dihidro-2(1*H*)-kinolinoon, 3- en 4-asetamidofenol met kommersieel beskikbare karbamoïelchloriede onder basiese toestande te laat reageer. Verbindings is gekarakteriseer deur gebruik te maak van KMR en IR-spektroskopie asook massaspektrometrie. Suiwerheid is bepaal deur HPLC en smeltpunte is bepaal. Die vermoë van die gesintetiseerde verbindings om die MAO ensieme te inhibeer is uitgedruk as IC_{50} (50% inhibisie konsentrasie) waardes terwyl die SE (selektiwiteitsindeks) ook bepaal is. ’n Fluorometriese toets, met kinuramien as substraat, is gebruik. AChE inhiberende aktiwiteit is bepaal deur die tempo van tiocholien produksie te bepaal, wat ontstaan as gevolg van die hidrolise van die asetieltiocholiensubstraat, in 'n spektrofotometriese toets.

RESULTATE EN BESPREKING

Agt-en-twintig verbindings is suksesvol gesintetiseer, alhoewel opbrengste oor die algemeen laag was. In die algemeen het die meeste van die gesintetiseerde verbindings swak of geen inhibisie van beide MAO-A en MAO-B getoon. Verbinding **8g**, 2-okso-1,2,3,4-tetrahidrokinolien-7-iel metiel(feniel)karbamaat, was die mees potente MAO-B inhibeerder van die huidige reeks met 'n IC_{50} waarde van 3.73 µM, en die verbinding is MAO-B selektief. Daar word gepostuleer dat die rigiditeit van die karbamaatsyketting verantwoordelik is vir die verlies aan aktiwiteit wat waargeneem is vir die verbindings van hierdie studie, in vergelyking met die hoogs potente dihidrokinolinone wat gesintetiseer is in ‘n vorige studie. Nie een van die gesintetiseerde verbindings het ongelukkig AChE geïnhibeer nie, moontlik as gevolg van die vervanging van 'n ioniseerbare amiengroep met 'n amied. Hoewel die biologiese resultate van hierdie studie teleurstellend was, is nuttige inligting verkry met betrekking tot strukturele vereistes vir binding aan beide MAO en AChE.

LIST OF ABBREVIATIONS

5-HT	Serotonin
Aβ	Amyloid-β/Aβ
Ach	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer's disease
ADH	Aldehyde dehydrogenase
Amplex Red	N-Acetyl-3,7-dihydroxyphenoxazine
APCI	Atmospheric-pressure chemical ionisation
APOE	Apolipoprotien E
APOEε2	Apolipoprotein ε2
APOEε3	Apolipoprotein ε3
APOEε4	Apolipoprotein ε4
APP	Amyloidprecursor protein
BBB	Blood-brain barrier
BuChE	Butyrylcholinesterase
ChAT	Choline acetyltransferase
ChEI	Cholinesterase inhibitor
COMT	Catechol-*O*-methyltransferase

D_1	DA type 1 receptor
D_2	DA type 2 receptor
D_3	DA type 3 receptor
D_4	DA type 4 receptor
D_5	DA type 5 receptor
DA	Dopamine
DEPT	Distortionless enhancement by polarization transfer
DLB	Dementia with Lewy bodies
DMF	Dimethylformamide
DMSO	Deuterated dimethylsulfoxide
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EtOAc	Ethyl acetate
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced FAD
Fe^{2+}	Ferrous ion
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HMBC	Heteronuclear multiple bond correlation
HPLC	High pressure liquid chromatography

HRMS	High resolution mass spectra
HSQC	Heteronuclear single quantum correlation
IC_{50}	Half maximal inhibitory concentration
IGF	Insulin-like growth factor
IR	Infrared
J	Coupling constant
KCl	Potassium chloride
LRRK2	Leucine-rich-repeatkinase-2
MAO	Monoamine oxidase
MAO-A	Monoamine oxidase isoform A
MAO-B	Monoamine oxidase isoform B
mAChR	Muscarinic acetylcholine receptor
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment
ml	millilitre
mM	Millimolar
mmol	Milimole
MS	Mass spectrometry

N	Equivalence per liter
NA	Noradrenaline
nAChR	Nicotinic acetylcholine receptor
NaH	Sodium hydride
NaOH	Sodium hydroxide
NFT	Neurofibrillary tangles
NH_4^+	Ammonia
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NPS	Neuropsychiatric symptoms
NSP	Neuritic senile plaques
O_2	Oxygen
$O_2^{\bullet-}$	Radical superoxide
$OH^{\bullet}$	Hydroxyl radical
OXPHOS	Oxidative phosphorylation
PD	Parkinson’s disease
PDD	Parkinson’s disease dementia
PI	Phosphoinositol
PIGD	Postural instability gait difficulty
PINK1	PTEN-induced purative kinase 1

PSEN-1	Presenilin 1
PSEN-2	Presenilin 2
ROS	Reactive oxygen species
SD	Standard deviation
SET	Single electron transfer
SI	Selectivity index
SNpc	Substantia nigra pars compacta
SORL1	Sortilin-related receptor gene
TLC	Thin layer chromatography
Tyr	Tyrosyl residues
δ	Chemical shift
λ_{ex}	Excitation wavelength
λ_{em}	Emission wavelength
µl	Microliter
µM	Micromolar

Kinetics

E	Enzyme
[E]	Enzyme concentration
ES	Enzyme-substrate complex

[I]	Inhibitor concentration
[S]	Substrate concentration
K_d	Equilibrium dissociation constant
K_i	Inhibition constant
K_m	Michaelis-Menten constant
v_i	Initial reaction velocity
V_{max}	Maximum velocity

NMR

δ	Delta scale used to indicate chemical shift
J	Coupling constant
br d	Broad doublet
br s	Broad singlet
br t	Broad triplet
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
m	Multiplet
p	Pentet
ppm	Parts per million
q	Quartet
s	Singlet
t	Triplet

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION AND OVERVIEW

Dementia is defined as a progressive deterioration in cognition, function and behaviour. The symptoms of dementia can be ascribed to damaged brain cells or damaged brain cell connections. Without these cells functioning correctly, one's memory, behaviour and ability to reason are therefore changed (Alzheimer's-Association, 2016). The most common neurodegenerative disorder, namely AD, accounts for approximately 70% of all cases of dementia (Reitz & Mayeux, 2014) and affects about 6% of the population over the age of 65 (Guzior *et al*., 2015). PD on the other hand, is classically viewed as a motor-disease, but it is estimated that 40% of PD patients are likely to develop dementia (Emre, 2003). Alongside the worldwide increase in life expectancy, the prevalence of dementia (and age related neurodegenerative disorders), is expected to rise, resulting in an escalating economic and social burden (Dorsey *et al.*, 2007; Muller & Woitalla, 2010; Winter *et al.*, 2010; Tom *et al.*, 2015).

AD is classified as a progressive neurodegenerative disorder characterisedby memory loss and behavioural changes (Carter *et al.*, 2012), the presence of neurofibrillary tangles, senile plaques, cholinergic neuron loss and neuronal atrophy (Balin & Hudson, 2014; Karch & Goate, 2015). There are two hypotheses regarding the pathogenesis of AD. Firstly, it has been suggested that the onset of AD is due to the decrease of ACh production in the synaptic junction (cholinergic hypothesis) (Fisher, 2012), and secondly, that the aggregation of toxic Aβ peptide in the brain results in the development of AD (amyloid hypothesis) (Selkoe, 2001). The cholinergic hypothesis proposes that treatments that prevent cholinergic deficits through inhibition of acetylcholine esterase (AChE) will result in improved cognition (Fisher, 2012).

Cholinergic neurotransmission is affected mainly by the neurotransmitter ACh, which binds to muscarinic and nicotinic receptors (Fisher, 2012). The neurotransmitter is metabolised by both AChE and butyrylcholinesterase (BuChE) (which also hydrolyses other choline esters), resulting in choline and acetate, thus preventing further activation of ACh receptors (Stoddard *et al.*, 2014). Acetylcholinesterase inhibitors (AChEIs), including donepezil,

rivastigmine and galantamine are thus most commonly used in the symptomatic treatment of AD (Burke, 2015).

In addition to decreased cholinergic neurotransmission and the development of senile plaques, the presence of oxidative stress contributes to brain tissue damage and progression of AD (Di Carlo *et al.*, 2012). MAO is an enzyme responsible for the catabolism of monoamines, such as DA, adrenaline and noradrenaline (NA) (Youdim *et al.*, 1988). During the metabolic reaction catalysed by the two isoforms (MAO-A and MAO-B) of this enzyme, hydrogen peroxide (H_2O_2) and subsequently, oxidative free radicals, are produced, which increases oxidative stress. The MAO-B levels in AD patients are up to 3-fold higher in the frontal, temporal and parietal cortex compared to the MAO-B levels in controls (Saura *et al.*, 1994, Nebbioson *et al.*, 2012, Huang *et al.*, 2015). Inhibition of MAO-B will thus potentially result in decreased levels of reactive oxygen species (ROS) and neurotoxic products. The therapeutic potential of MAO-inhibitors in AD treatment is thus due to their potential neuroprotective properties and goes beyond their effect on neurotransmission (Guzior *et al.*, 2015).

Conversely, PD is a neurodegenerative disorder characterised by the loss of DA in the nigrostriatum. The main manifestations associated with PD are motor symptoms, including akinesia or bradykinesia, rigidity and tremor being directly related to dopaminergic striatal loss (Hornykiewicz, 2008). In this case, MAO-B inhibitors, such as rasagiline and selegiline, are used in the symptomatic treatment of PD. Inhibition of MAO-B results in a decrease in the degradation of DA, thereby increasing synaptic DA (Lees, 2005) and improving motor symptoms (Knoll, 2000). As in AD, in addition to their role in the symptomatic treatment of PD, MAO-B inhibitors may also afford neuroprotection (Guzior *et al.*, 2015).

PDD is a complication of PD that arises in the late stages of the disease and is diagnosed by the identification of deficits in recognition memory, attention processes and visual perception as well as visual hallucinations and cognitive fluctuations (Emre *et al.*, 2007). According to Aarsland *et al.* (2001a), 100 out of every 100 000 patients suffering from PD develops PDD. At the time of PD diagnosis, the risk and rate of cognitive decline are associated with the patient's age, for which the risk of cognitive impairment and developing dementia are much greater (Aarsland & Kurz, 2010).

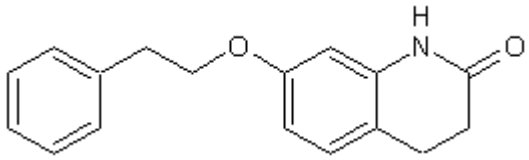
The determination of the pathological mechanisms that contributes to PDD is complicated due to the heterogeneity of the disease and involves limbic and neocortical Lewy body deposition, with neurofibrillary tangles and senile plaques playing a role in some patients. Dysfunction of non-dopaminergic neurotransmitter systemsalso occurs (Williams-Gray *et al.*, 2007). In PDD, as in AD, there is a substantial reduction in cortical cholinergic activity. There

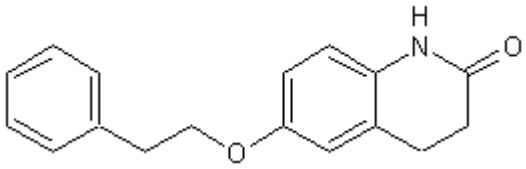
is no cure for PDD, and current symptomatic treatment, which include the use of cholinesterase inhibitors (ChEIs), provides only modest relief (Ballard *et al.*, 2011).

In both AD and PD (particularly in PDD), the combined inhibition of both MAO and AChE will thus be beneficial (Bautista-Aguilera *et al.*, 2014). Inhibition of AChE will result in enhanced cognition, due to an increase in cholinergic neurotransmission (Stoddard *et al.*, 2014), while MAO inhibition will potentially afford neuroprotection and also improve motor symptoms in PD (Guzior *et al.*, 2015).

1.2 RATIONALE

The dihydroquinolinone scaffold has been established as privileged for the inhibition of MAO-B, e.g (**1**, **2**) (Meiring *et al.*, 2013).





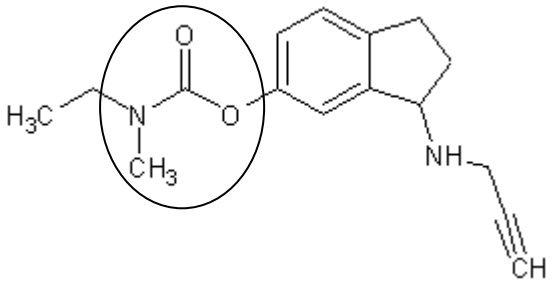
1 IC_{50} MAO-A: 53.7 µM

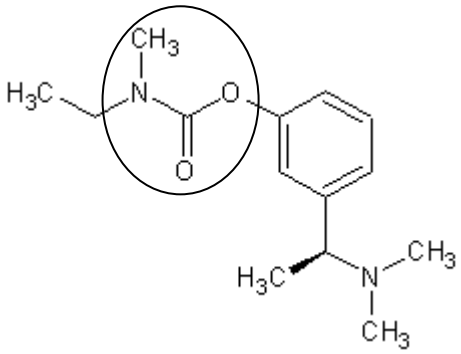
IC_{50} MAO-B: 0.191 µM

2 IC_{50} MAO-A: 22.5 µM

IC_{50} MAO-B: 2.33 µM

The carbamate moiety on the other hand, occurs in the structures of potent inhibitors of AChE e.g. ladostigil (**3**) and rivastigmine (**4**) (Li *et al.*, 2014).





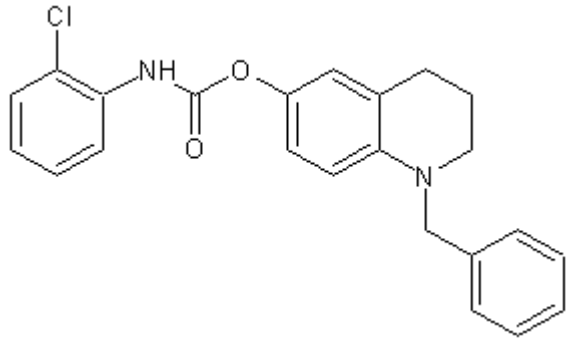
3 Ladostigil

4 Rivastigmine

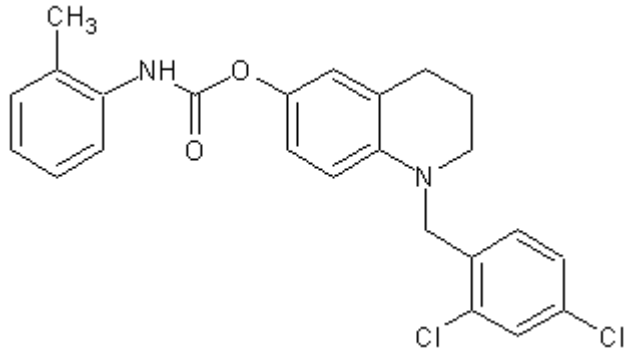
IC_{50} AChE: 0.011 µM

IC_{50} BuChE: 1.28 µM

Of particular interest to this study, was work done by Roy and co-workers (Roy *et al.*, 2012) who synthesised a novel series of tetrahydroquinolinecarbamates with promising AChE inhibitory activities (e.g. **5**, **6**).

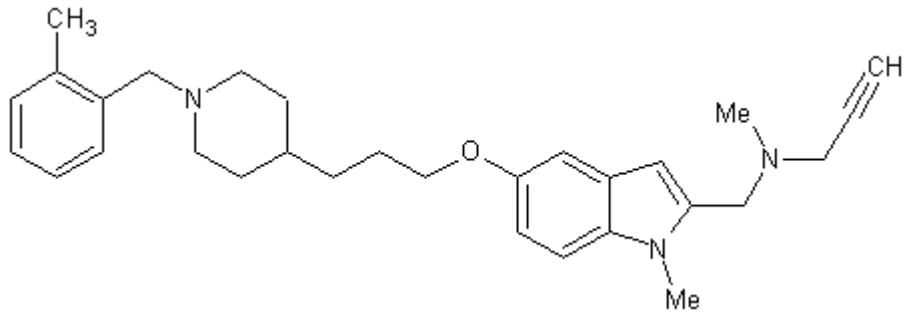


5 IC_{50} AChE: 3.31 µM



6 IC_{50} AChE: 2.57µM

A limited number of dual targeted AChE-MAO-B inhibitors have also been designed and synthesised previously, e.g. N-methyl-N-((1-methyl-5-(3-(1-(2-methylbenzyl)piperidin-4-yl)propoxy)1H-indol-2-yl)methyl)prop-2-yn-1-amine (**7**) with anti-cholinesterase and MAO-A and -B inhibitory activities (Bautista-Aguilera *et al.*, 2014), providing proof of concept. This approach is particularly advantageous because it addresses the multifactorial nature of neurodegenerative diseases such as AD and PD by targeting both the MAO and AChE enzymes.



7 IC_{50} AChE: 2.8 µM

IC_{50} BuChE: 4.9 µM

IC_{50} MAO-A: 6.3 µM

IC_{50} MAO-B: 183.6 µM

As previously stated, the prevalence of AD and PD, which are common, incurable neurodegenerative diseases (Alves *et al.*, 2008) are increasing, indicating the relevance of research in this regard. Although many research efforts have been aimed at relieving the plight of AD sufferers, the only therapies existing are symptomatic (Fereshtehnejad *et al.*, 2014). Similarly, only inadequate symptomatic treatment is available for the treatment of PD or PDD (Ballard *et al.*, 2011). AD is a multifactorial disease (Huang & Mucke, 2012), while PD is likewise characterised by heterogeneous neuropathology (Kehagia *et al.*, 2010). Therefore, compounds that interact with multiple targets, such as AChE and MAO-B, could be particularly effective as they would be complementary.

1.3 HYPOTHESIS OF THIS STUDY

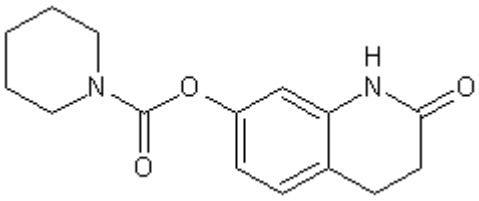
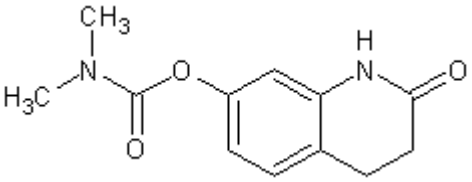
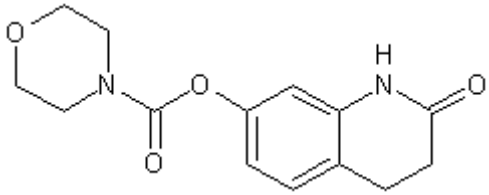
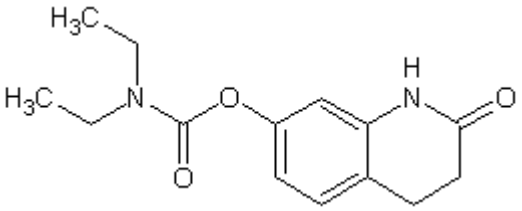
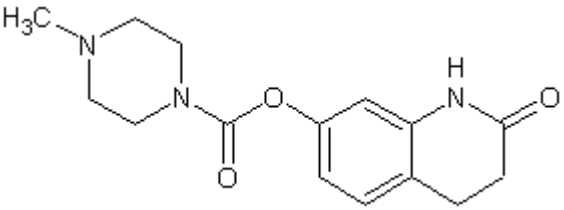
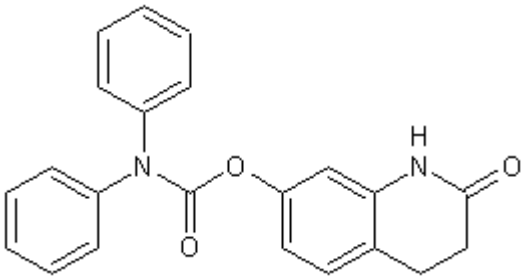
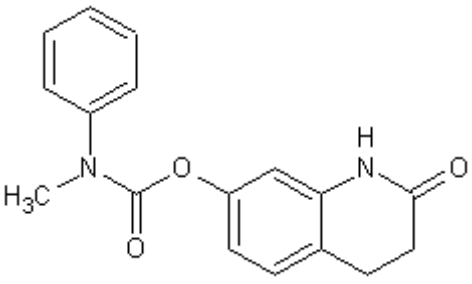
It is postulated that the combination of the dihydroquinolinone and carbamate moieties will result in chemical entities with potent dual MAO-B and AChE inhibitory activities. It is further hypothesised that the acetamidophenol derivatives, as indicated in table 1, will also exhibit dual activity since similar functionalities are present in these molecules. These compounds will find potential application in the treatment of AD and PD while also possibly providing neuroprotection.

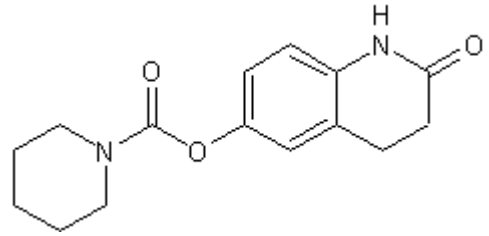
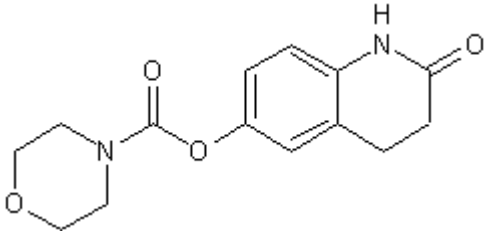
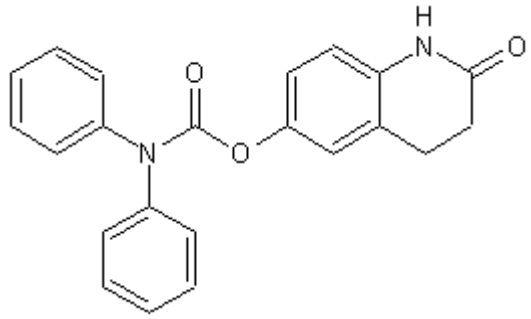
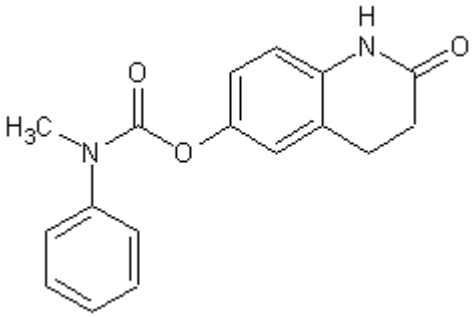
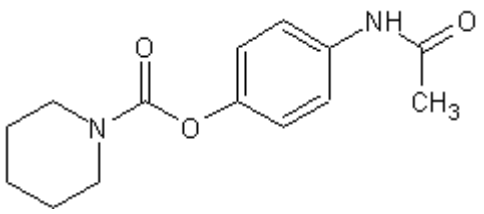
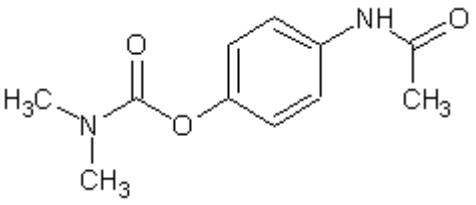
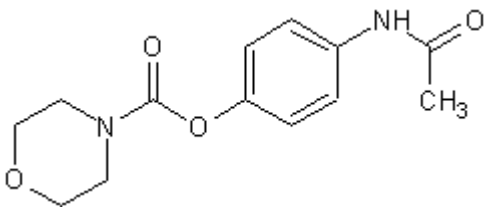
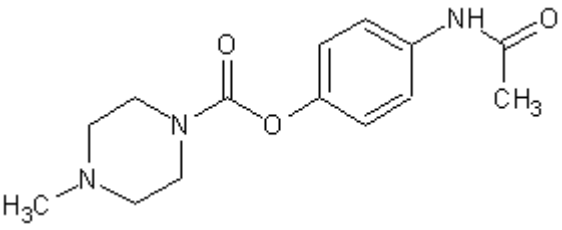
1.4 AIM OF THIS STUDY

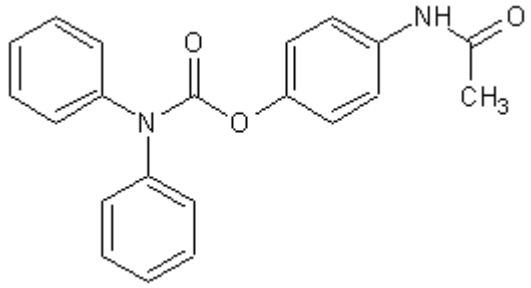
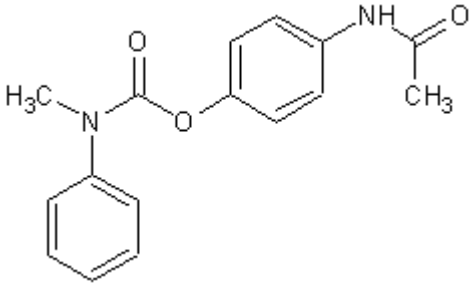
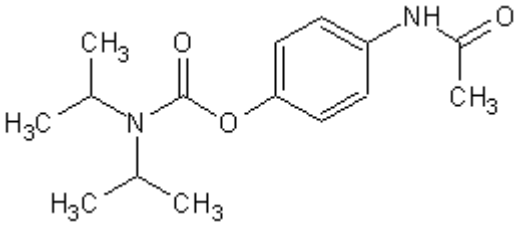
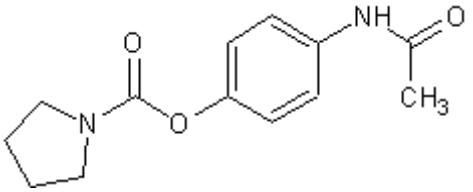
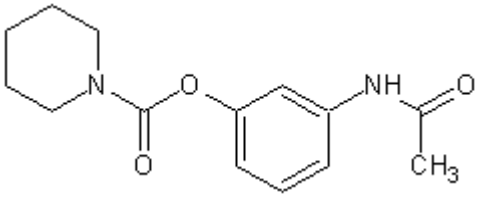
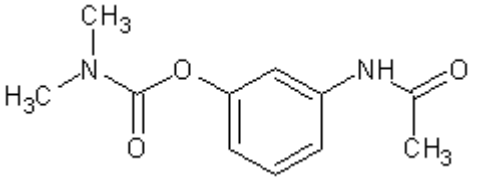
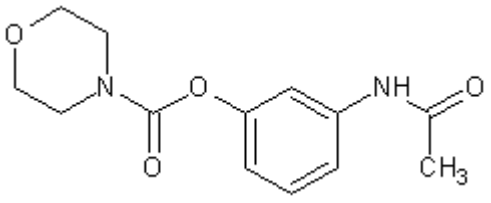
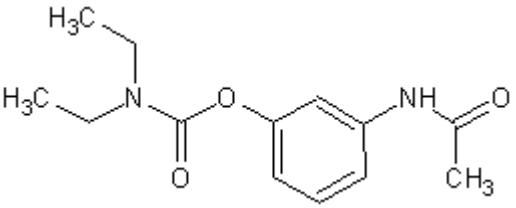
The main aim of this study will be to combine the carbamate and dihydroquinolinone moieties in one molecule in order to obtain a polyfunctional entity with inhibitory activity against both MAO and AChE.

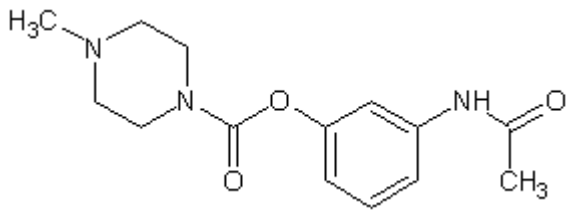
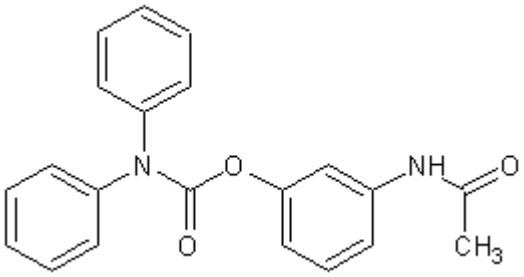
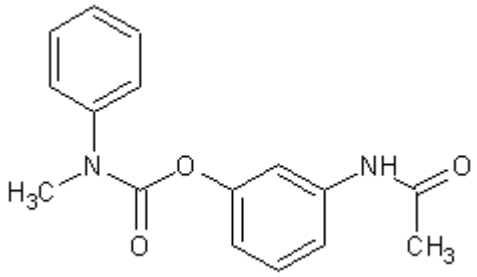
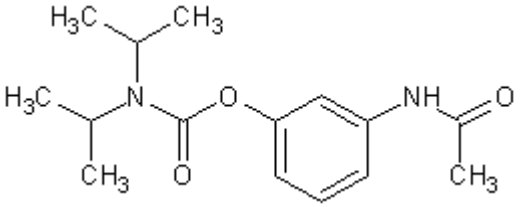
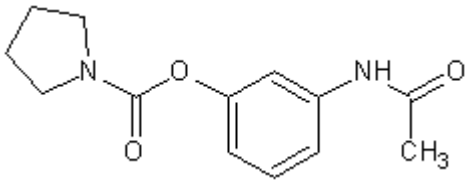
Compounds will be synthesised in one step using literature procedures. The structures of proposed derivatives are indicated in table 1.

Table 1.1 Structures of compounds selected for synthesis

C7 substituted dihydroquinolinone derivatives	
 **8a**	 **8b**
 **8c**	 **8d**
 **8e**	 **8f**
 **8g**	
C6 substituted dihydroquinolinone derivatives	

 **9a**	 **9b**
 **9c**	 **9d**
4-acetamidophenol derivatives	
 **10a**	 **10b**
 **10c**	 **10d**

 **10e**	 **10f**
 **10g**	 **10h**
3-Acetamidophenol derivatives	
 **11a**	 **11b**
 **11c**	 **11d**

 **11e**	 **11f**
 **11g**	 **11h**
 **11i**	

1.5 OBJECTIVES OF THIS STUDY

The objectives of this study are to:

Synthesise dihydroquinolinone carbamates in an attempt to obtain potent dual MAO/AChE inhibitors. Since acetamidophenol is structurally related to dihydroquinolinone, some acetamidophenol derivatives will also be synthesised in order to assess the necessity of the closed dihydroquinolinone ring system. The selection of carbamoyl chlorides will be based on commercial availability.

Evaluate the synthesised compounds *in vitro* as inhibitors of AChE and MAO. Kynuramine and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) will be used as substrates for MAO and AChE, respectively.

CHAPTER 2

LITERATURE REVIEW

2.1 ALZHEIMER'S DISEASE INTRODUCTION

AD was first described by Alois Alzheimer who was a psychiatrist at the Frankfort Psychiatric Hospital. From 1901 until 1906, one of his patients presented with symptoms such as disturbance of memory, aggression, paranoia, crying, sleep disorder and progressive confusion. The symptoms showed fast progression with increased intensity. After the death of the patient he reported "A peculiar severe disease process of the cerebral cortex", now known as Alzheimer's disease (Hippius & Neundorfer, 2003; Ferri *et al.*, 2006; Balin & Hudson, 2014).

AD can be described as a multifactorial (dependant on a number of factors, in particular genetic or environmental factors) (St George-Hyslop & Petit, 2005) and heterogeneous (diverse in character) (Selkoe, 2001) disorder characterised by a decline in cognitive function and a progressive loss of memory (McKhann *et al.*, 1984). AD affects approximately 6 - 10% of people aged 65 or older in the United States (Geldmacher, 2007) and the risk of developing AD is related to age, with a 8% chance of developing the disease for people over the age of 85 (Reitz, 2014). It is the most common neurodegenerative disease, and accounts for more than 70% of all cases involving neurodegeneration (Reitz & Mayeux, 2014).

AD occurs in two forms: an early onset form (genetically determined (Balin & Hudson, 2014), appearing before the age of 65) accounting for 1-5% of AD cases (Reitz & Mayeux, 2014) and a late onset form (more common (Balin & Hudson, 2014) and causes dementia amongst the elderly over the age of 65) responsible for more than 95% of all AD cases (Reitz & Mayeux, 2014). AD can be symptomatically characterised by memory loss and behavioural changes (Balin & Hudson, 2014) and neuropathologically by the presence of neurofibrillary tangles, senile plaques, cholinergic neuron loss (that is irreversible) and neuronal atrophy in specific brain regions (Zhu *et al.*, 2007; Balin & Hudson, 2014; Reitz & Mayeux, 2014).

AD can be classified as mild, moderate or severe and the classification determines the type of treatment. In mild AD, patients have difficulty with cognition and cannot recall short-term memory and new learnings, but can function alone with no caregiver (Forstl & Kurz, 1999; Geldmacher, 2007). Patients with mild AD have however an increased risk for developing

depression. Patients with moderate AD struggle with activities of daily living and require close supervision with noticeable loss in memory. These patients can also experience wandering, insomnia and delusions. In severe AD, a patient has a noticeable loss of activities of daily living with limited language abilities, agitation, incontinence and increased dependence on a caregiver with almost all cognitive function impaired (Geldmacher, 2007).

The average duration of AD is between 4 and 8 years, but the illness can last up to 20 years (Mayeux, 2003; Reitz, 2014). According to the Alzheimer's association (2016), it is essential to determine the precise biological changes that occur during AD and why the progression differs in patients in order to prevent, slow or even stop AD (Alzheimer's-Association, 2016).

2.1.1 Incidence and diagnosis of Alzheimer's disease

The incidence of AD seems to be increasing as the life expectancy of the population increases (Hurd *et al.*, 2013). The worldwide estimation of AD affected individuals is 17 million (Reitz, 2014). The onset of AD can occur as early as the age of 40 years; however AD mainly manifests in patients over the age of 60. In early stages, it is often difficult to distinguish between AD symptoms and normal aging. It is also quite common for the misdiagnosis of AD in its later stages due to the possibility of various different mental illnesses (Khachaturian, 1985).

Sperling *et al.* (2011) suggest that there is an opportunity for disease modification if therapeutic intervention is applied during early onset of AD. However, there is no direct test that can be used for the determination or diagnosis of AD. Instead, various approaches, which include taking a family and medical history, asking family members or other persons of interest if there are any notable behaviour or skill changes and blood tests, to rule out any other potential causes of dementia, are used (Alzheimer's-Association, 2016). Laboratory tests that can be helpful for AD diagnosis in pre-mortem patients, in conjunction with PET, MRI or other imaging methods, include the study of the levels and the presence of Aβ 1-40/1-42 peptides and the total number of modified tau protein in cerebrospinal fluid (Hampel *et al.*, 2004; Risacher *et al.*, 2009; Prvulovic & Hampel, 2011; Teipel *et al.*, 2013; Balin & Hudson, 2014). The diagnosis is also based on the results obtained from general physical and neurological examinations as well as a cognitive test (Alzheimer's-Association, 2016).

2.1.2 Symptoms of Alzheimer's disease

AD symptoms progress gradually from mildly impaired memory functions to severe cognitive loss (Ferris & Farlow, 2013). The disease initially develops after the age of approximately 60 and worsens with an increase in age (Reitz, 2014). Mild AD symptoms include:

forgetfulness, short-term memory loss, a repetitive asking of questions, a loss of interest in hobbies, impaired instrumental functions and anomia, where moderate AD presents with a progression in cognitive deficits, aphasia, dysexecutive syndrome, impairment in basic activities of daily living, which results in a transition into care. Finally, in severe AD, symptoms such as agitation and an altered sleep pattern is present, resulting in a total dependence on care for normal daily activities such as dressing, feeding and bathing (Ferris & Farlow, 2013). According to the Alzheimer's Association (2016), in the severe state, patients may not recognise their own family members, losing their ability to communicate, leaving them bed-bound and completely dependent on full-time care. These patients are also more vulnerable to infections which can be life threatening (Alzheimer's-Association, 2016).

The symptoms of AD differ between patients, with deterioration in the ability to remember new information as the most common first symptom (Alzheimer's-Association, 2016).

A primary feature of AD symptomatology is the progressive decline in cognition (Gelb, 2000). Symptoms of cognitive decline include sporadic episodes of memory loss and impairment in abilities such as concentration and attention, direction, judgement, visuospatial ability (in AD, visuospatial problems suggest that an individual can become disoriented or lost in familiar environments) and language (Vestal *et al.*, 2006). Language impairment or aphasia (the inability to produce or understand speech), is a common issue experienced during the course of AD, as it is passed through from the moderate stage to the severe stage (McKhann *et al.*, 2011).

In 40 to 90% of cases (Hollingworth *et al.*, 2012), AD patients struggle with neuropsychiatric symptoms (NPS), such as depression, anxiety, agitation and delusions, which are significantly more common than in the general population (Steinberg *et al.*, 2008; Steinberg *et al.*, 2014). Patients with AD are more likely to have NPS which makes it difficult for their caretakers, and worsens their quality of life (Craig *et al.*, 2005; Steinberg *et al.*, 2014).

2.1.3 Risk factors for Alzheimer's disease

AD, as previously stated, is a multifactorial disease and develop as a result of not only one cause, but multiple factors (Alzheimer's-Association, 2016).

Age

The main risk factor for AD is aging, but developing AD is not a definite part of aging. AD is most commonly diagnosed in persons over the age of 65 (Alzheimer's-Association, 2016).

Family History

The first-degree relatives (which include parents, siblings or children) of a patient suffering from late-onset AD are at increased risk of developing dementia, with the risk increasing if more than one family member have the disease (Mayeux *et al.*, 1991; Reitz & Mayeux, 2014).

Genetics

Apolipoprotein E (APOE) is a protein that is lipid bound and responsible for the transport of cholesterol into the bloodstream. The APOE gene is localised to the 19q13 chromosome that has been linked to genetic AD in some families with late-onset AD (Struhl & Adachi, 2000; Selkoe, 2001; Reitz & Mayeux, 2014; Alzheimer's-Association, 2016). APOE consists of three isoforms, APOEε2, - ε3 and - ε4 and every individual inherits one isoform from each parent (Raber *et al.*, 2004; Alzheimer's-Association, 2016). The APOEε4 isoform is associated with late-onset AD (Selkoe, 2001). The presence of a single APOEε4 isoform, increases the risk of developing AD 2.3-fold, whereas there is a 5-fold risk increase in individuals who inherit two copies of this genotype (Kuusisto *et al*., 1994; Reitz & Mayeux, 2014; Alzheimer's-Association, 2016). Although the presence of the APOEε4 isoform is one of the 3 most common risk factors for AD (Alzheimer's-Association, 2016). The presence of the APOEε4 in an individual increases the risk for disease development and early onset of symptoms, not all individuals with the isoform will develop late-onset AD (Balin & Hudson, 2014).

Other deterministic gene mutations associated with AD are amyloidprecursor protein (APP), presenilin 1 (PSEN-1), presenilin 2 (PSEN-2) (Bertram & Tanzi, 2005) and genetic variations of the sortilin-related receptor gene (SORL1), which has been identified as a susceptible gene for late-onset AD (Bettens *et al.*, 2008). Mutations in the APP, PSEN-1 and PSEN-2 genes are also implicated in the pathology of early-onset AD (Reitz & Mayeux, 2014). AD caused by these gene mutations are called "familial Alzheimer's disease", where the symptoms may be visible before the age of 60 years. It is possible that the onset of AD symptoms can occur as early as 30 years of age. These deterministic genes will directly cause AD, however, familial AD represents less than 5% of AD cases (Alzheimer's-Association, 2016).

Gender

Based on a study done by Seshadri *et al.* (2006) in the United States, woman are more likely to develop AD than men.

Mild cognitive impairment

Mild cognitive impairment (MCI) is a syndrome that is defined by cognitive decline. For a patient with MCI, the cognitive decline is greater than that of the normal standard of the expected age and education level, but is not severe enough for daily activities to be interrupted. Normally, for people over the age of 65, the risk of developing MCI is 3 to 19% (Gauthier *et al.*, 2006). Patients with single nucleotide polymorphisms of SORL1 and diagnosed with MCI are at higher risk of developing AD. SORL1 is thus recognised as a potential tool for identifying MCI converting into AD (Piscopo *et al.*, 2015).

Non-genetic, modifiable lifestyle risks

The Alzheimer's-Association (2016) states that the health of the brain is directly linked to a well balanced cardiovascular system. The following are lifestyle risks that can lead to AD:

- Cerebrovascular diseases: The direct underlying mechanism which leads to an increase risk of dementia development through hemorrhagic infarcts, small and large ischemic cortical infarcts, vasculopathies and white matter changes remains unclear. It can be said that white matter hyperintensities and infarcts can cause direct damage to the brain regions responsible for memory function (Reitz & Mayeux, 2014).
- Blood pressure: Studies have shown that hypertension (elevated blood levels) between the ages of 40 – 60 years have been associated with an increase in risk for AD, dementia and cognitive impairment in later stages of life (Swan *et al.*, 1998; Launer *et al.*, 2000; Kivipelto *et al.*, 2001; Whitmer *et al.*, 2005; Reitz & Mayeux, 2014). This is due to the effect that hypertension has on the blood-brain barrier (BBB) where it results in protein extravasation (forcing the protein from the vessel) into the brain tissue (Kalaria, 2010; Reitz & Mayeux, 2014). The presence of protein in the brain tissue can, as a result, cause cognitive impairment through brain cell damage, the reduction in neuronal or synaptic function, apotosis and an increase in Aβ accumulation (Deane *et al.*, 2004; Reitz & Mayeux, 2014).
- Type 2 diabetes: It has been found that type two diabetes may double the risk of late-onset AD development, with the mechanism still unclear (Leibson *et al.*, 1997; Luchsinger *et al.*, 2001; Luchsinger, 2008; Reitz & Mayeux, 2014).
- Body weight: Obesity during midlife is associated with the risk of AD development (Reitz & Mayeux, 2014; Alzheimer's-Association, 2016).
- Smoking: Smoking is a risk factor for AD development, but the exact mechanism by which smoking can cause AD is still unclear. It has been suggested that smoking causes an increase in free radical generation, leading to high oxidative stress and

oxidative damage. Smoking can also lead to cerebrovascular diseases which, as previously stated, enhance the risk of AD (Traber *et al.*, 2000; Reitz & Mayeux, 2014). On the other hand, smoking may have a protective effect in AD where the nicotine of smoking induce the increase of nicotinic acetylcholine receptors (nAChR), which is lost in AD pathology leading to cholinergic deficits (Reitz & Mayeux, 2014).

Other risk factors include a history of a traumatic brain injury and low education levels (Reitz & Mayeux, 2014; Alzheimer's-Association, 2016).

2.1.4 Brain structures and pathology in Alzheimer's disease

In the characteristic pathology of AD, the following brain regions are most affected: the neocortex and hippocampus. These two brain regions are associated with higher mental functions (Francis *et al.*, 1999). The destruction of the underlying chemical pathways that lead to dementia symptoms were first described in the mid 1970's, when it was observed that the neurons responsible for Ach synthesis and release underwent degeneration that was uneven and severe. The observation was made after, firstly, the discovery of a decrease in levels and activity of certain enzymes (e.g. choline acetyltransferase (ChAT) and AChE) in the limbic as well as the cerebral cortices. Secondly, AD is also associated with a loss in cholinergic cell bodies in the subcortical nuclei which projects to the spetal nuclei and basal forebrain cholinergic system regions (Selkoe, 2001). The disease is thus characterised by synaptic loss, a cholinergichypofunction and degeneration of cholinergic neurons (Blennow *et al.*, 2006; Fisher, 2012).

The presence of deficits in other neurotransmitter systems was identified in the late 1970s and early 1980s. It was realised that AD is highly heterogeneous and does not involve the degeneration of only a single transmitter class of neurons (Selkoe, 2001), but affects multiple regions of the brain (Zubenko *et al.*, 1991).

The pathological characterisation of AD is further hallmarked by the presence of two abnormal protein aggregates (Hyman *et al.*, 2012). Firstly, Aβ deposits (Johnson *et al.*, 2007), which are extracellular (Braak & Braak, 1991) and accumulate outside neurons (Alzheimer's-Association, 2016), and secondly, aggregated tau protein. These deposits are abnormally hyperphosphorylated tau protein, known as neurofibrillary tangles (NFT), which cause intracellular changes. These NFTs have a specific order of appearance in different brain areas. Their first appearance is in the brainstem and transentorhinal area from where they move to the hippocampus in order to reach the paralimbic and adjacent medial-basal temporal cortex. In the next stage they are found in the cortical association areas before finally reaching the visual and primary sensory-motor areas (Braak & Braak, 1991, Jack &

Holtzman, 2013). The distribution pattern of these NFT's are pathological features of neurodegeneration in AD (Braak & Braak, 1994; Jack & Holtzman, 2013). The progressive accumulation of these proteins outside (Aβ plaques) and inside (tau tangles) the neurons cause changes that can eventually damage and even cause the death of neurons (Alzheimer's-Association, 2016).

AD's neuropathologic changes are progressive with the degree of progression correlating with the severity of the clinical disease (Geldmacher, 2007).

2.1.5 Aetiology and pathogenesis of Alzheimer's disease

In a review by Reitz (2014) it is stated that AD is a complex disease with multifactorial causes and changes. Balin and Hudson (2014) describe AD as a disease with a number of different causal inputs or starting points which result in a definite endpoint: cognitive decline. At the time of death there are several changes present in the brain that is indicative of the disease. These manifestations include the deposits of extracellular Aβ protein in diffuse and neurotic plaques (neurotic plaques contain elements that degenerate neurons). Neurofibrillary tangles are found as extracellular changes where abnormal hyperphosphorylated tau protein and microtubule assembly protein are deposited. There is also widespread neuronal loss and the activation of microglia in the brain (Reitz, 2014). The cause of these manifestations may involve inter alia environmental toxins and infectious agents, which interact in an unknown manner with specific gene aspects of at-risk individuals. For example, an interaction with the APOE gene (previously discussed), may result in neuronal loss, synaptic dysfunction and other characteristics that are experienced during the course of AD (Balin & Hudson, 2014).

Inflammation is considered as an important causative factor for late-onset AD and is associated with neurodegenerative effects (McGeer & McGeer, 2013; Sastra *et al.*, 2011; Balin & Hudson, 2014). Aβ depositions result in the activation of astrocytes and microglia and consequently, in neuroinflammation (Lue *et al.*, 1996, Balin & Hudson, 2014). Microglia are the cells in the brain that are responsible for inflammation and during late-onset AD these cells are activated and often found around amyloid plaques. Activation of microglia cells result in the production of cytokines and reactive oxygen species (Wood, 1998, Balin & Hudson, 2014). Studies have shown that the regular use of non-steroidal anti-inflammatory drugs in the older population may delay the onset of AD; however once cognitive decline has manifested, the use of non-steroidal anti-inflammatory drugs is unsuccessful (Breitner, 1996; Pasinetti, 2002, Balin & Hudson, 2014).

Historically, there are two main hypotheses regarding the aetiology and pathogenesis of AD. The one suggests that the progression of AD is largely influenced by the decrease in ACh production in the synaptic junction (cholinergic hypothesis) (Bartus 2000; Ringman &Cummings, 2006; reviewed in Fisher 2012); while the other suggests that the aggregation of toxic Aβ in the brain contributes to the progression of AD (amyloid hypothesis) (Hardy & Selkoe, 2002). The amyloid hypothesis proposes that treatments that prevent amyloid plaque formation will slow the progress of AD, while the cholinergic hypothesis proposes that treatments that activate the cholinergic system will improve the cholinergic deficits of AD (Stoddard *et al.*, 2014).

2.1.5.1 Amyloid hypothesis

The characteristic neuropathology of AD results from the generation and deposition of Aβ, which arise from a series of cascading events in certain brain areas of an affected individual (Schellenberg, 1995; Claeysen *et al.*, 2012; Balin & Hudson, 2014).

The amyloid plaque is derived from APP, and this plaque consists of deposits of Aβ-peptide, which consists of 39-42 amino acids (Hunt & Turner, 2009, Lichtenthaler, 2011). These peptides are the major protein deposits that are present in AD patients and adult Down syndrome individuals (Masters *et al.*, 1985; Chauhan & Chauhan, 2006). Mutations within the APP are located near and around the APP site which usually promote the proteolytic cleavage of APP by β- and γ- secretase, promoting the generation of Aβ deposits (Citron *et al.*, 1993; Cai *et al.*, 1993; Hardy & Selkoe, 2002). Mutations on the PS1 and PS2 proteins increase the processing of APP which also results in the formation of amyloidogenic Aβ (Scheuner *et al*., 1996; Hardy & Selkoe, 2002).

Aβ can exist as soluble proteins in the cerebrospinal fluid and serum where it is considered a normal metabolic product in both AD patients and healthy individuals (Seubert *et al.*, 1992; Vigo-Pelfrey *et al.*, 1993; Chauhan & Chauhan, 2006). It can also exist as a fibrillar with the ability to aggregate into insoluble fibrils. These fibrils are deposited extracellularly in AD patients' brains (Chauhan & Chauhan, 2006). Aβ-oligomers cause neurotoxicity and neurodegeneration which is responsible for the behavioural and functional shortages present in AD(Hardy & Selkoe, 2002).

The series of pathogenic events that leads to AD as described by the amyloid hypothesis begin with missense mutations in the APP, PS1 or PS2 genes which lead to an increase of Aβ production and accumulation. Oligomerisation of Aβ peptides takes place as well as deposition of Aβ as diffuse plaques. Generated Aβ oligomers have effects on synapses which activate microglial and astrocytic cells resulting in neuroinflammation (as previously

described). Neuroinflammation causes progressive synaptic and neurotic injury, however the Aβ oligomers not only activate the microglia and astrocytes, but it can cause direct injury in the brain through damage to the synapses and neuritis. Injured synapses and neuritis alter kinase and phosphatase activities, producing tangles. These tangles cause widespread neuronal and neurotic dysfunction and cell death, leading to dementia (Hardy & Selkoe, 2002).

2.1.5.2 Cholinergic hypothesis

Working memory, conscious awareness and attention is dependent on the functioning of well balanced cholinergic pathways in the basal and rostral forebrain (Perry *et al.*, 1999; Terry & Buccafusco, 2003). In AD there is an abnormality in these cholinergic pathways which correlates with the level of cognitive decline (Bartus, 2000; Terry & Buccafusco, 2003). In the cholinergic hypothesis, the following is stated: During the course of AD, a loss of cholinergic neurotransmission in the cerebral cortex and other areas occurs as a result of severe damage to the cholinergic neurons in the basal forebrain (this can be detected both histopathologically, by a loss of neurons, and neurochemically by a loss of marker enzymes for ACh synthesis and degradation). The characteristic symptoms of AD, such as memory loss, cognitive and non-cognitive symptoms, are the result of these cerebral cholinergic deficits (Frölich, 2002).

It has been shown that in AD, there is a substantial loss of the enzyme ChAT which is responsible for the synthesis of ACh (Bowen *et al.*, 1976, Davies & Maloney, 1976; Perry *et al.*, 1977; Francis *et al.*, 1999) with a reduction in ACh release (Nilsson *et al.*, 1986; Francis *et al.*, 1999) and choline uptake (Rylett *et al.*, 1983; Francis *et al.*, 1999) as well as the loss in cholinergic perikarya from the nucleus basalis of Meynert, resulting in cholinergic deficits (Whitehouse *et al.*, 1982, Francis *et al.*, 1999). ACh is a neurotransmitter that binds to nicotinic (nAChRs) and muscarinic Ach receptors (mAChRs). Both nicotinic and muscarinic receptors are involved in cognitive processes and in AD, both nAChRs and mAChRs are affected (Blennow *et al.*, 2006; Giacobini & Becker, 2007; Fisher, 2012). For example, a reduced expression of nAChRs occurs in AD (Maelicke *et al.*, 2001).

There are critics of the cholinergic hypothesis which suggest that previous studies focused only on end stage AD while the characteristics of MCI or mild AD were not investigated (Terry & Buccafusco, 2003). For example, a study done by Davis *et al.* (1999) reported that the activity of AChE and ChAT was not reduced in the neocortical tissue of post mortem mild AD patients that was recently diagnosed. Furthermore, a study done by DeKosky *et al.* (2002) on MCI and mild AD patients have failed to identify any reduction in ChAT activity.

The definite aetiology of AD is still uncertain which makes it difficult to find effective means of delaying or halting disease progression (Fisher, 2012).

2.1.5.3 Oxidative stress

Oxygen (O_2) is essential for normal living. It has a high redox potential, allowing it to easily accept electrons from substrates (Gandhi & Abramov, 2012). In a functioning system, electrons that are released from the respiratory chain react with O_2 to produce an oxygen-based free radical anion, better known as superoxide ($O_2^{\bullet-}$). Superoxide dismutase (SOD) converts the $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). H_2O_2 is a potent oxidising agent, reacting with metals and ferrous iron (Fe^{2+}) to produce hydroxal radicals ($OH^{\bullet}$), which are highly reactive. $O_2^{\bullet-}$also cross-reacts with nitric oxide (NO) for the production of $NO_2^{\bullet}$ (Chauhan & Chauhan, 2006). $O_2^{\bullet-}$, $OH^{\bullet}$ and H_2O_2 are examples of different reactive oxygen species (ROS). These species are generally inactivated through glutathione peroxidase which uses glutathione (GSH) as its cofactor. A GSH deficiency can lead to a decrease in the brain's ability to clear ROS, causing oxidative stress and cell death (Chance *et al.*, 1979; Youdim *et al.*, 2006). This is because these unstable radicals (that are constantly in search of electrons) are toxic, reacting with proteins, nucleic acid and lipids, resulting in abnormal cellular function. Oxidative damage to cellular components causes alterations in membrane functions with excessive damage leading to cell death (Bandyopadhyay et al., 1999).

Oxidative stress is thus a state that is induced by the dysfunction of the antioxidant system or the generation of an excess ROS (Andreyev *et al*., 2005). The neuronal degeneration in AD pathology is greatly affected by oxidative stress. Contributing factors that increase oxidative stress in AD include soluble Aβ, Aβ fibrils, mitochondrial abnormalities, aging and NFT. Another contributing factor involved in AD's neuronal degeneration is the imbalance in oxidative homeostasis, as it leads to an increase in lipid peroxidation (Chauhan & Chauhan, 2006). Aging is associated with an increase in ROS production (Chauhan & Chauhan, 2006; Zhu *et al.*, 2007), providing at least in part, some explanation of why aging is such an important risk factor for developing AD.

Metals as contributors to oxidative stress

Metals catalyse redox reactions, inducing oxidative stress (Birben *et al.*, 2012); on the other hand, antioxidant defences consist of the storing and transportation of metals, such as iron, in forms that would not catalyse the formation of reactive radicals. Therefore, with an increase in iron availability, the rate of free radical reactions can accelerate (Valko *et al.*, 2006).

In numerous neurodegenerative diseases, the neuronal death brain sites are the sites where iron accumulates. For example, there is an association between increased iron concentrations and neuronal damage due to hydrogen peroxide formed by MAO (Youdim & Bakhle, 2006). The Fenton reaction between iron in the ferrous ion form (Fe^{2+}) and hydrogen peroxide is a potent source of the highly reactive hydroxyl radical (Scheme 2.1), which reacts with and damages lipids, proteins and DNA (Smith *et al.*, 1997).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

Scheme 2.1 The Fenton reaction (Lloyd *et al.*, 1997).

Numerous studies have further shown that patients suffering from neurodegenerative diseases, such as AD, have increased MAO-B activity (most likely due to transcriptional elevation of MAO-B) (Gulyás *et al.*, 2011) in the brain and blood platelets (Riederer *et al.*, 2004). The MAO-B levels in AD patients are up to 3-fold higher in the frontal, temporal and parietal cortex compared to the MAO-B levels in controls (Huang *et al.*, 2015). This results in an increase in hydrogen peroxide production and, combined with increased iron levels associated with old age (Gandhi & Abramov, 2012), promote the Fenton reaction and the generation of hydroxyl radicals.

In AD there is high affinity binding sites for copper and zinc on the N-terminal metal-binding domains of Aβ and its precursor APP (Miura *et al.*, 2000; Barnham *et al.*, 2003). Copper, which occurs in high concentrations in amyloid plaques (Strozyk *et al.*, 2009), is an effective mediator for the highly reactive hydroxyl radical, contributing to the increase in oxidative stress which is characteristic of an AD brain (Valko *et al.*, 2005).

2.1.6 Alzheimer's disease therapy

Non-Pharmacological treatment

The main objective of non-pharmacological treatment is the maintenance or improvement of the patient's cognitive function (Alzheimer's-Association, 2016).

A study done by Svansdottir and Snaedal (2006) on music therapy in AD showed effective and safe results in patients with moderate and severe AD, specifically for the treatment of agitation and anxiety. Music therapy is based on the use of sounds, tunes and movements. AD patients can easily participate in music therapy, and it results in an increase in well-being (Clair, 1996). During music therapy, patients may interact with other individuals, diminishing their sense of isolation (Pollack & Namazi, 1992).

Other examples of non-pharmacological treatment in AD includes memory-training, art therapy and activity-based therapy (gardening, cooking and word games) (Alzheimer's-Association, 2016).

Pharmacological treatment

AD is an incurable disorder and currently no treatment exists that reverses the pathological processes, despite a considerable research effort. The only available therapies are thus symptomatic, and aim to increase the levels of affected neurotransmitters in the brain (Fereshtehnejad *et al.*, 2014; Alzheimer's-Association, 2016). This is due to the fact that the exact aetiology of AD have not yet been elucidated. However, Fisher (2012) proposes that the pharmacological hallmarks of AD are interconnected, making it possible to at least modulate it.

The discovery of an effective treatment for AD, however remains a priority.

2.1.6.1 Cholinesterase inhibitors

Since the cholinergic system and ChEIs are of particular importance to this study, the role of ACh in neurotransmission, the role of cholinesterases, as well as the inhibitors of the enzyme will be discussed in detail in the following sections.

The motivation for cholinergic treatment for patients suffering from AD is based on the cholinergic hypothesis as discussed before. This hypothesis suggests that cholinergic deficits play a key role in cognitive dysfunction and behavioural impairments such as learning and memory deficiencies as experienced in the disease. The cholinergic deficits of AD can thus be restored by activation of the cholinergic system by, for example, inhibiting cholinesterases (Fisher, 2012; Stoddard *et al.*, 2014).

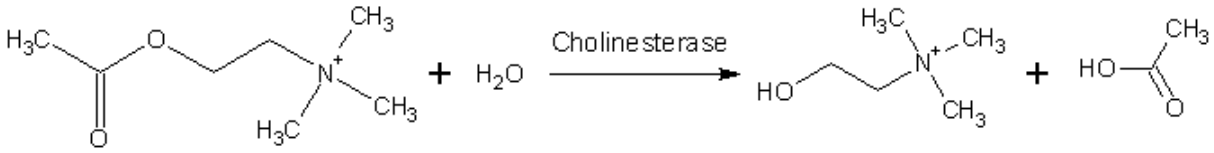
2.1.6.1.1 Acetylcholine and the role of cholinesterase in the human body

ACh is a neurotransmitter that can be found in the central and peripheral nervous systems. ACh is present in the “neuromuscular junction” at the peripheral nervous site where it acts to control muscular contraction. ACh also plays a role in the autonomic nervous system. Normally, there is a cholinergic projection from the nucleus basalis of Meynert, which is in the forebrain, to the forebrain neocortex and associated limbic structures, however this pathway is degenerated in AD (Čolović *et al.*, 2013).

ACh is biosynthesised in a single step, catalysed by the ChAT enzyme. The presence of ChAT indicates that a specific neuron is cholinergic (Čolović *et al.*, 2013). The activation of both nAChRs and mAChRs mediates intracellular effects of ACh (Barnard, 1974). Both

nAChRs and mAChRs play a role in the encoding of new memories (Hasselmo, 2006). nAChRs mediate the transport of potassium and sodium ions across the synaptic membranes and can be divided into two receptor groups namely: neuromuscular nAChRs, mediating signals between the nerve and muscle cell, and neuronal nAChRs, which are present all through the nervous system (Hogg *et al.*, 2003; Voglis & Tavernarakis, 2006). Neuronal nAChRs modulate the effect of cognitive performance (Voglis & Tavernarakis, 2006). mAChR's, on the other hand, are present in the following neurons: central and peripheral nervous system, smooth and cardiac muscle and a variety of exocrinic glands (Hirota, 2001). There are five types of mAChRs, namely M1-M5 (Wess *et al.*, 2007). mAChRs in the central nervous system mediates motor control, cardiovascular and temperature regulation and memory (Gohar, 2006).

Cholinesterases are enzymes that catalyse the hydrolysis of ACh into choline and acetic acid at a very fast rate (Scheme 2.2) (Aldridge, 1953; Čolović *et al.*, 2013). There are two types of ChEs in the human body, namely BuChE and AChE. BuChE occurs in the liver, pancreas, blood plasma, intestinal mucosa and white brain matter of the central nervous system (Massoulié *et al.*, 1993) and of particular importance to this study, can also be found in the plaques and tangles of AD (Geula & Mesulam, 1995). BuChE's activity is increased with aging and its levels are raised in AD (Perry *et al.*, 1978). AChE is found in erythrocytes, the lungs, nerve endings, the spleen and in all the compartments of the brain. AChE is a membrane-bound glycoprotein (Massoulié *et al.*, 1993). The biological role of AChE is the termination of a transmission impulse at the cholinergic synapse throughout the nervous system (Schumacher *et al.*, 1986). It is therefore an enzyme that is fundamental to nerve function and impulse (Barnard, 1974).



Acetylcholine Choline Acetic acid

Scheme 2.2 The metabolism of ACh to choline and acetic acid as catalysed by cholinesterases (Tamulis *et al.*, 2016).

When AChE is inhibited, the ACh concentration is increased and the duration of action of ACh in the synaptic junction is prolonged, which leads to increased binding of ACh to cholinergic receptors (nAChRs and mAChRs) (Čolović *et al.*, 2013).

Of particular importance in AD is the fact that both AChE inhibitors and muscarinic agonists enhance the release of the non-amyloidogenic soluble derivatives of APPs *in vitro* as well as *in vivo*, which could possibly slow the formation of amyloidogenic compounds in the brain (Lahiri *et al.*, 1994; Castro & Martinez, 2006). Furthermore, it has been shown that in AD, the expression of AChE is linked to Aβ depositions in the brain (Giacobini, 2000; Castro & Martinez, 2006). AChE co-localises with Aβ in neurotic plaques and may accelerate the formation of fibrils when Aβ deposits assemble. The expression of AChE as well as the assembly and glycosylation of this enzyme is regulated through Aβ peptides as have been shown in cell cultures, transgenic mice and in the brain of AD patients (Hu *et al.*, 2003; Castro & Martinez, 2006). This leads to the conclusion that AChE inhibition may influence the processing of APP and Aβ, thus leading to the reduction of plaque formation, one of the pathological hallmarks of AD (Rees & Brimijoin 2003; Castro & Martinez, 2006).

2.1.6.1.2 Binding site of AChE

The organisation of the active site of AChE and its catalytic mechanism can be used to differentiate it from other enzymes (Shafferman *et al.*, 1992).

The specific catalytic activity of AChE is extremely high. The rate at which AChE degrades ACh is similar to that of a diffusion-controlled reaction (Quinn, 1987; Taylor & Radic, 1994; Čolović *et al.*, 2013). The AChE enzyme has an ellipsoidal form. At the bottom of its structure is a deep and narrow gorge which penetrates halfway into the enzyme; this gorge widens as it gets closer to the base of the structure (Manavalan *et al.*, 1985; Čolović *et al.*, 2013). The active site of the enzyme is composed of two subsites, the esteratic subsite and the anionic subsite (Nachmansohn & Wilson, 2009; Čolović *et al.*, 2013). The anionic subsite is uncharged and lipophilic, and binds to the positive quaternary amine of the choline moiety of ACh (Wilson & Quan 1958; Mooser & Sigman, 1974;Čolović *et al.*, 2013). Interestingly, the cationic substrate is not bound by a negatively charged amino acid to the active site, but by interacting with 14 highly conserved aromatic residues which line the gorge that leads to the active site (Radić *et al.*, 1992; Ordentlich *et al.*, 1995; Ariel *et al.*, 1998; Čolović *et al.*, 2013). ACh is hydrolysed to acetate and choline inside the esteratic subsite. The esteratic subsite of AChE, contains, like other serine hydrolases, the catalytic triad of three amino acids namely serine 200, histidine 440 and glutamate 327 (Čolović *et al.*, 2013).

2.1.6.1.3 Known inhibitors of AChE

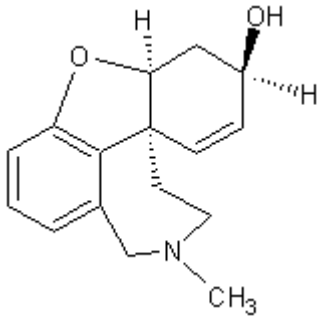
The foremost therapeutic target in AD is the inhibition of AChE in the brain (Giacobini, 2004; Lane *et al.*, 2006). Unfortunately the treatment with AChE inhibitors is not a cure for AD, but only addresses the symptoms related to the cholinergic deficiency. The role of AChE

inhibitors is to maintain the levels of ACh by decreasing its breakdown rate (Stahl, 2000; Čolović *et al.*, 2013).

Unfortunately, oral administration of AChEIs is often associated with adverse events such as cholinergic gastrointestinal adverse events which includes vomiting, nausea, headache, diarrhoea, dizziness and a decreased appetite (Oertel *et al.*, 2007).

Galantamine

Galantamine (**12**), applied in the treatment of mild to moderate AD, is a tertiary plant alkaloid isolated from the bulbs of the Caucasian Snowdrop, *Galanthus woronowii* (Čolović *et al.*, 2013). Galantamine is a selective, reversible, competitive inhibitor of AChE, interacting with the anionic subsite as well as with the acyl-binding pocket in the binding site of AChE (Bartolucci *et al.*, 2001; Pilger *et al.*, 2001; Lane *et al.*, 2006; Kitisripanya *et al.*, 2011). Galantamine is an unique dual cholinergic inhibitor because not only does it cause reversible, competitive inhibition of the enzyme, but it also allosterically modulates nAChRs (Maelicke *et al.*, 2001; Lane *et al.*, 2006). This effect is beneficial in the treatment of AD as a loss of nAChRs correlates with cognitive decline (Bajgar, 2004). The allosteric effect of galantamine affects not only the neurotransmission of ACh but also that of monoamines, GABA and glutamate resulting in more beneficial effects, such as improving cognition and psychiatric illness in schizophrenia, bipolar disorders, alcohol abuse and major depression (Robles 2009; Ago *et al.*, 2011).



12 Galantamine

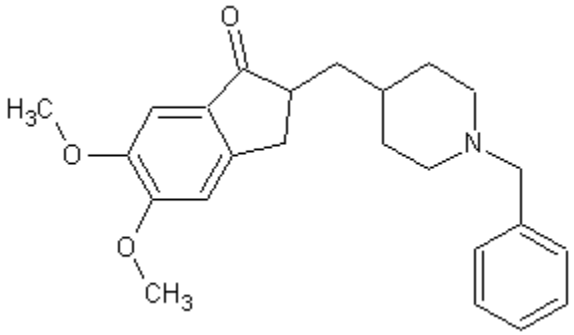
The side-effect profile of galantamine is similar to that of other AChE inhibitors. Side-effects that are commonly associated with the use of galantamine include: vomiting, nausea, headache, diarrhoea, dizziness and a decreased appetite (gastrointestinal adverse events) (Wilkinson & Murray, 2001).

Donepezil

Donepezil (**13**), a piperidine derivative, is a selective, reversible inhibitor of AChE (Lane *et al.*, 2006; Čolović *et al.*, 2013) and binds to the peripheral anionic site (Čolović *et al.*, 2013).

Its use not only results in symptomatic relief in AD, but over time, may also inhibit Aβ aggregation (Castro & Martinez, 2006; Arce *et al.*, 2009; Čolović *et al.*, 2013).

Donepezil is usually used clinically in mild and moderate AD, but recent studies have shown that even in severe AD an improvement in cognitive function is observed (Davidsson *et al.*, 2001).



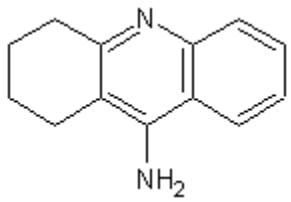
13 Donepezil

The side-effect profile of donepezil is similar to that of other AChE inhibitors, and include gastrointestinal adverse events, such as nausea, anorexia, diarrhoea, abdominal pain and bradycardia (Tayeb *et al.*, 2012).

Interestingly, studies have suggested that the use of donepezil in children with autism may improve their speech (Rojas-Fernandez, 2001; Malouf & Birks, 2004; Čolović *et al.*, 2013; Thakurathi *et al.*, 2013).

Tacrine

Tacrine (tetrahydroaminoacridine) (**14**) is an AChE inhibitor that was approved for the treatment of AD in 1993, but has since been removed from the market. It has a difficult dosing regimen with poor tolerability and significant hepatotoxicity making it undesirable as treatment (Geldmacher, 2007; Robles, 2009).

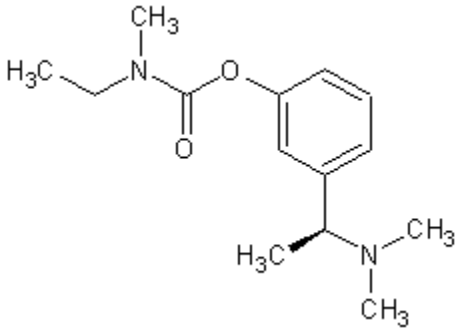


14 Tacrine

Rivastigmine

Rivastigmine (**4**) is a slow-reversible carbamate inhibitor of AChE used in the treatment of mild and moderate AD. It blocks the activity of AChE by binding to the esteratic subsite of the active site. Rivastigmine inhibits both AChE and BuChE (Desai & Grossberg, 2005; Lane

et al., 2006). The early and continuous use of rivastigmine is beneficial in decreasing the rate of cognitive decline, the severity of dementia and the ability to perform daily activities (Birks & Grimley Evans, 2015).



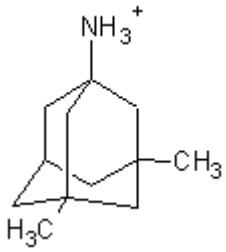
4 Rivastigmine

The adverse events associated with rivastigmine is similar to the side effects caused by other AChE inhibitors, such as gastrointestinal adverse events, nausea, vomiting, anorexia, diarrhoea, abdominal pain, headache, dizziness and syncope (Inglis, 2002; Birks, 2006).

Rivastigmine treatment is also useful in dementia with Lewy Bodies (DLB) and PDD (Desai & Grossberg, 2005; Chitnis & Rao, 2009).

2.1.6.2 N-Methyl-D-aspartate antagonists

Memantine (1-amino-3,5-dimethyladamantane) (**15**) is a neuroprotective, uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist approved for the treatment of moderate and severe AD as well as vascular dementia and PD. Memantine is a derivative of adamantine, which is similar to the anti-viral drug, amantadine (Chen *et al.*, 1992).



15 1-Amino-3,5-dimethyladamantane

Calcium-activated neurotoxicity in the central nervous system is mediated through the excessive stimulation of the NMDA receptor (Levy & Lipton, 1990), while Aβ-induced neuronal death is incited by NMDA-receptor-mediated glutamate release (Sonkusare *et al.*, 2005). Memantine's mechanism of action is to block the over-activated NMDA receptor, normalising the glutamatergic system (Olivares *et al.*, 2012).

The combination of memantine with an AChE inhibitor is the recommended standard treatment in AD (Wilkinson *et al.*, 2004; Rodda & Carter, 2012). This treatment is however,

only a short-term symptomatic intervention, as it only slows the loss in cognition (Van Marum, 2008).

2.1.6.3 MAO-B inhibitors

MAO-B is one of the enzymes available as biological target in AD treatment (Guzior *et al.*, 2015). The therapeutic potential of MAO inhibitors in AD is due to their neuroprotective potential ascribed to their effect on monoaminergic neurotransmission. MAO inhibitors reduce the formation of neurotoxic products, such as ROS. Thus, inhibition of MAO-B is likely to suppress the neurodegenerative processes that occur in AD (Weinreb *et al.*, 2011; Bolea *et al.*, 2013; Song *et al.*, 2013; Zheng *et al.*, 2014).

2.2 INTRODUCTION TO PARKINSON'S DISEASE

PD is the second most common age-related, chronic neurodegenerative disease after AD (Mirza *et al.*, 2014). It is characterised by the neuronal loss of DA in the substantia nigra pars compacta (SNpc) (Dauer & Przedborski, 2003; Bové *et al.*, 2005). In 1817, the core clinical features of PD were first described in James Parkinson's monograph "Essay on the Shaking Palsy", (Dauer & Przedborski, 2003) and more than 50 years later, Jean Martin Charcot proposed that the disease should be called Parkinson's disease (Lees *et al.*, 2009).

Tremor, rigidity and slowness of movement are some of the characteristic features seen in PD patients, with dementia and dysautonomia appearing in advance stages of PD (Tolosa *et al.*, 2006).

The focus of new PD treatments are on the prevention of the degeneration of dopaminergic neurons (Dauer & Przedborski, 2003). This is challenging since there is still inadequate knowledge of how PD begins and progresses (Bové *et al.*, 2005).

2.2.1 Incidence and diagnosis of Parkinson's disease

According to Twelves *et al.* (2003) it is important to study the incidence of PD for future health-care planning and epidemiological research purposes.

PD has an average age of onset of 60 years (DeMaagd & Philip, 2015) and progresses with age (Okereke, 2002), with the mean period from diagnosis to death estimated at 10 years (Elbaz *et al.*, 2003; Alves *et al.*, 2008). There have, however, been cases of people aged 30 to 40 being affected with PD (De Lau & Breteler, 2006; DeMaagd & Philip, 2015). The world wide incidence is estimated to be 20/100 000 at the age of 55-60 and increases to 160/100 000 at the age of 70 (Dauer & Przedborski, 2003), with a 1.6% lifetime risk of disease

development (Elbaz *et al,*. 2002). The increase in age of the population of the western world is predicted to be accompanied by an increase in the incidence of PD (Dorsey *et al.*, 2007).

The diagnosis of PD may be complicated by the fact that the symptoms of PD may overlap with those of other forms of parkinsonism (parkinsonism is a term that is used for any disease that presents with symptoms as a result of a loss in striatal DA) (Hughes *et al.*, 1992).

2.2.2 Aetiology and pathology of Parkinson's disease

The cause of PD is as indefinable as it was in 1817. The main known risk factor for PD is age, but there are susceptible genes and pathologies that are associated with PD (Lees *et al.*, 2009), as well as environmental factors (Huang *et al.*, 2003).

PD is considered to be a multifactorial disease because of the influence of both environmental and genetic factors (Elbaz & Tranchant, 2007).

Genetic factors

Only 10% of PD cases are inherited, thus in 90% of patients there is no definite link to a genetic cause (Klein & Westenberger, 2012). Some evidence has emerged that shows that the close relatives of patients suffering from PD are more likely to be affected by PD when compared to controls (Payami *et al.*, 2002). It has been suggested that in familial PD for example, the first degree relatives have a higher relative risk, up to 2-3 times, of developing PD (Gasser, 1998). The risk for developing familial PD is associated with the mutation of seven genes which includes; PINK1 (PTEN-induced purative kinase 1), Parkin, ATP13A2, DJ-1 (Williams *et al.*, 2005), LRRK2 (leucine-rich-repeatkinase-2) (Paisán-Ruíz *et al.*, 2004; Healy *et al.*, 2008), α-synuclein(Davie, 2008) and glucocerebrosidase(Goker-Alpan *et al.*, 2004).

Mutations in the PINK1, Parkin, ATP13A2 and DJ-1 genes cause early-onset PD, thus onset before the age of 40 years (Williams *et al.*, 2005). The Gly2019Ser mutation of the LRRK2 gene is responsible for 1% of sporadic cases of PD and 4% of heritable cases (Paisán-Ruíz *et al.*, 2004; Healy *et al.*, 2008). The loss of function in glucocerebrosidase results in a 5-fold higher risk of developing PD (Goker-Alpan *et al.*, 2004), while mutations in the α-synuclein gene may lead to early onset PD (Davie, 2008).

The presence of Lewy Bodies in PD is an essential part of PD pathology where it can be found in the substantia nigra's cholinergic neurons (DeMaagd & Philip, 2015). The parkin gene plays a role in the development of Lewy Bodies, as it facilitates the binding of ubiquitin

to other proteins which leads to their degradation rather than the formation of Lewy Bodies (Olanow, 2007; Del Tredici & Braak, 2012; DeMaagd & Philip, 2015).

Environmental factors

The results of chemical epidemiological studies lend support to the environmental hypothesis of PD (Tanner, 1989). Factors associated with an increased risk of developing PD include age, being male (Elbaz &Tranchant, 2007), head-injury (Lai *et al.*, 2002), a lack of exercise (Thacker *et al.*, 2008) and geographical location (rural living) (Tanner *et al.*, 1990; Priyadarshi *et al.*, 2001). Other environmental factors include the exposure to drinking of well water, pesticides (organophosphates, paraquat and rotenone), and farming (Tanner *et al.*, 1990; Priyadarshi *et al.*, 2001; Elbaz &Tranchant, 2007). Exposure to well water is associated, in particular, to early-onset PD (Rajput *et al.*, 1987). Dietary factors have been shown to have an effect on the risk for PD development. For example, diets that depend heavily on animal fat may increase the risk for PD (Anderson *et al.*, 1999; Johnson *et al.*, 1999), whereas the consumption of niacin containing products may reduce the risk (Fall *et al.*, 1999).

Surprisingly, some studies have shown that tobacco smokers are less likely to develop PD than lifelong non-smokers, and smoking is thus considered to be protective (Hernán *et al.*, 2001; Allam *et al.*, 2004). Interestingly, components in tobacco smoke inhibit MAO (Fowler *et al.*, 1996). Men are 1.5–fold more likely to develop PD than women in the western populations when they are over the age of 70 years (Twelves *et al.*, 2003). A study done by Popat *et al.* (2005) revealed that woman who had a hysterectomy and were treated with estrogen-alone hormone replacement therapy were at higher risk of developing PD than women on combined estrogen-progestrin therapy. These results support the hypothesis that endogenous estrogens play a role in the development of PD (Ragonese *et al*., 2004).

Men not taking any or very little caffeine have an increased risk of 25% for disease development compared to woman. The fact that caffeine use has a negligible effect on the risk of women to develop PD may be partly due to the use of postmenopausal hormone replacement therapy (estrogens) and its interaction with caffeine (Ascherio *et al.*, 2004). Caffeine is an adenosine A2 receptor antagonist. Adenosine A2 receptor antagonists are promising anti-parkinsonian agents, however the exact mechanism by which these agents may provide dopaminergic neuron protection are still not completely clear (Schwarzschild *et al.*, 2002).

2.2.2.1 Mechanism of cell death in Parkinson's disease

Abnormal protein processing is a crucial part of cell death in a PD brain (Olanow & Tatton, 1999; Dawson, 2000). Abnormal protein processing may increase the vulnerability to oxidative stress and vice versa. Thus, there are multiple factors which cause cell death during PD (Huang *et al.*, 2003).

Oxidative stress

Oxidative stress plays a pathogenic role in PD, partly due to increased lipid peroxidation (Jenner & Olanow, 1998). In the brain of PD patients, at the site of neuronal death, iron accumulates. This increased iron levels promote the Fenton reaction, as discussed before, leading to oxidative stress (Youdim & Bakhle, 2006).

Mitochondrial dysfunction

Numerous diseases and pathologies result from a reduced capacity for oxidative phosphorylation (OXPHOS) in the mitochondria, which is caused by mitochondrial DNA deletions or point mutations (Leonard & Schapira, 2000). The role of mitochondria in the cell is to provide energy that is critical for the repair, maintenance and the turnover of cellular components. Thus, a decline in mitochondrial function is responsible for aging and neurodegenerative diseases (Melov, 2000; Golden & Melov, 2001). Mutations of LRRK2 and PINK1 in sporadic PD have deleterious effects on mitochondrial function (Schapira 1995; Schapira & Jenner 2011).

Apoptosis

The term apoptosis is defined as programmed cell death. Some studies have reported that there is an increase in apoptotic cell death in the substantia nigrain PD (Agid, 1995; Anglade *et al.*, 1997; Tatton *et al.*, 1998). Conversely, others have reported that there is little or even no evidence linking PD with apoptotic cell death (Kösel *et al.*, 1997; Banati *et al.*, 1998; Jellinger, 2000). The role of apoptosis in PD thus remains unclear.

Excitotoxicity

In the mammalian central nervous system one of the primary excitatory neurotransmitters namely glutamate, is part of the excitotoxic process (Yacoubian & Standaert, 2009). Glutamate receptors are present on the dopaminergic neurons which are situated in the substantia nigra. During cell death, these glutamate receptors receive glutamatergic innervations which overactivates the NMDA receptor, and in turn results in an increase in intracellular calcium levels. This effect of glutamate on the NMDA receptor activates

pathways which is responsible for cell death (Mody & MacDonald, 1995). The calcium influx promotes the production of peroxynitrite (Good *et al.*, 1998).

Inflammatory response

Cell degeneration in PD patients may also be attributed to neuro-inflammation. In PD there is an increase in cytokine levels in the striatum and cerebrospinal fluid which is indicative of a neuro-inflammatory response (Yacoubian & Standaert, 2009).

2.2.3 The role of DA in Parkinson's disease

DA is biosynthesised as follows (Figure 2.1): Firstly, the amino acid tyrosine is converted to levodopa by the enzyme tyrosine hydroxylase. After that, conversion of levodopa to DA is catalysed by the aromatic amino acid decarboxylase enzyme. The DA that is produced by neurons in the basal ganglia plays an important role in managing complex movements (Sun *et al.*, 2004). DA is metabolised and deactivated in the postsynaptic cleft by MAO and catechol-O-methyltransferase (COMT) (Napolitano *et al.*, 1994).

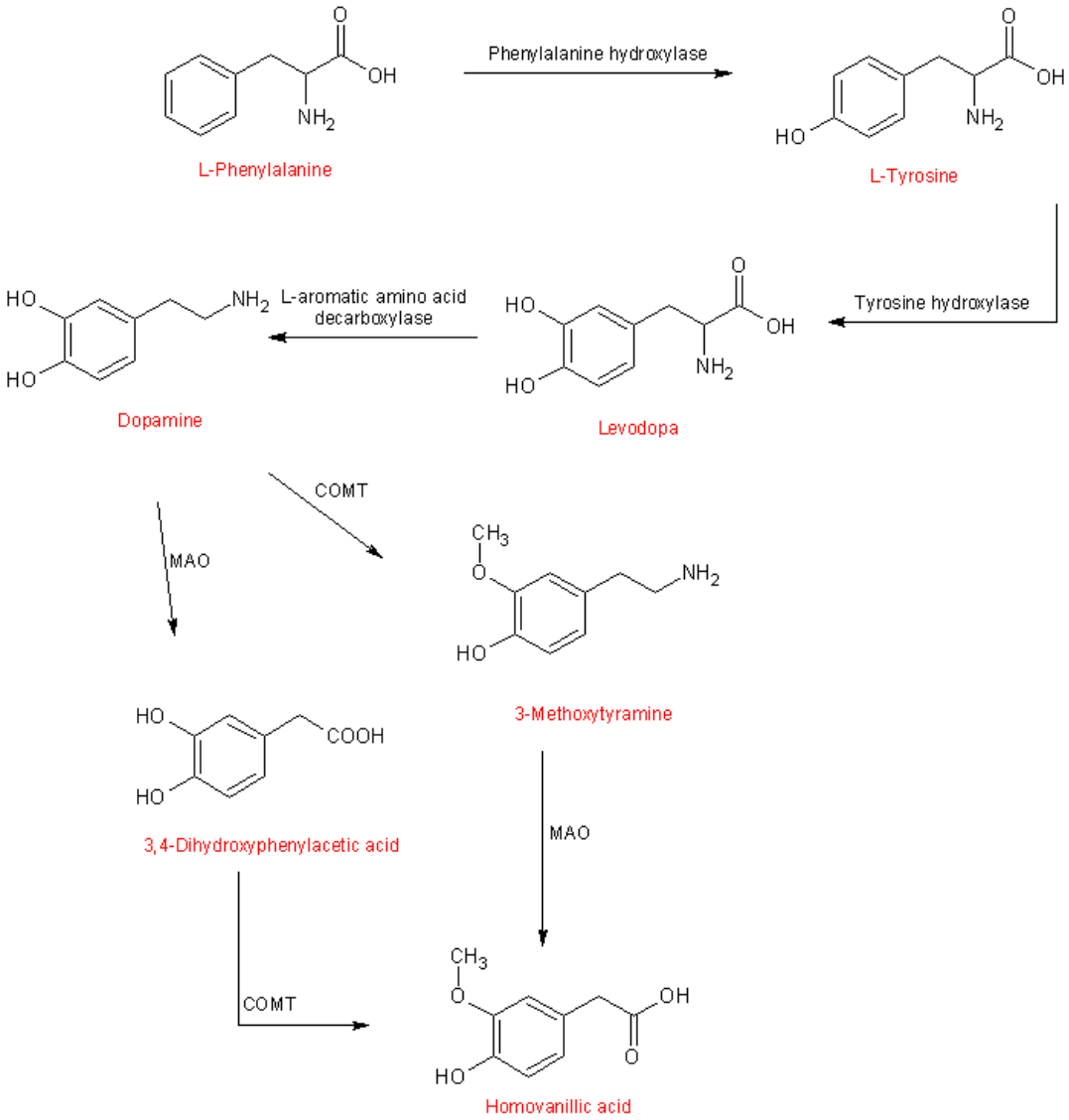


Figure 2.1 The synthesis and metabolism of DA. The enzymes are shown in black letters (Sanders-Bush & Hazelwood, 2006).

As previously explained, degeneration and death of dopaminergic neurons in the SNpc primarily cause depleted DA levels in the striatum, which results in the symptoms of PD (Dauer & Przedborski, 2003; Przedborski, 2005). DA depletion and neurodegeneration continues over time, and the signs and symptoms of PD are directly related to the progressive loss of DA in the basal ganglia (Rodriguez-Oroz *et al.*, 2009).

2.2.4 Symptoms of Parkinson's disease

Parkinsonism is a term that is used for any disease that presents with symptoms as a result of a loss in striatal DA, and is characterised by symptoms such as tremors when at rest, rigidity, slowness or absence of voluntary movement, postural instability and freezing. 80%

of all parkinsonism cases are PD, making PD the most common cause of parkinsonism (Dauer & Przedborski, 2003).

The onset of PD symptoms is very slow and can be misinterpreted or unnoticed at first. PD symptoms usually only appear when the loss of dopaminergic neurons in the SNpc is about 60% (Dauer & Przedborski, 2003; Mirza *et al.*, 2014). A non-specific symptom that some PD patients can experience is fatigue (Friedman *et al.*, 2016). Symptoms usually worsen after 5-10 years, even if the patient is being treated (Dauer & Przedborski, 2003).

PD can symptomatically be characterised by a progressive decline in motor and non-motor functions (Lin & Wu, 2015). Characteristic motor symptoms include bradykinesia (slow and poor movements), rigidity of the muscles, tremors when at rest (these tremors do not impair activities of daily living and decrease when voluntary motion occurs) and impairment in postural balance, disrupting the gait and causes falling (Jellinger, 2012).

PD is a multifaceted illness and, apart from motor symptoms, non-motor symptoms are also present in the disease. According to Poewe (2008) these non-motor symptoms will become the greatest challenge in the treatment of advanced PD and these symptoms include the following:

- Neuropsychiatric dysfunction: Mood disorders, apathy and anhedonia, dementia and psychosis and frontal executive dysfunction.
- Sleep disorders: Sleep fragmentation and insomnia, rapid eye movement sleep behaviour disorder, periodic limb movement in sleep/restless leg syndrome and excessive daytime somnolence.
- Autonomic dysfunctions: Orthostatic hypotension, urogenital dysfunction and constipation.
- Sensory symptoms and pain: Olfactory dysfunction, abnormal sensations and pain.

The non-motor symptoms that are the most important cause of disability in PD, are dementia and cognitive impairment (Hely *et al.*, 2005; Zesiewicz *et al.*, 2006).

2.2.5 Dementia in Parkinson's disease

The non-motor symptoms of PD are usually misdiagnosed during consultations, because they are not well recognised in clinical practice and patients are often unaware that these symptoms are associated with PD (Chaudhuri *et al.*, 2006). Dementia is associated with a reduced quality of life, greater carer burden, increased disability and mortality, nursing home dependency and increased cost of care (Emre, 2003, Docherty & Burn, 2010).

Since PDD is age related, and since there is an increase in life expectancies of PD patients, the prevalence of PDD will become more common in future (Docherty & Burn, 2010). Not only has PDD a negative impact on activities of daily living, but it also increases the morbidity and mortality rate of the population (Emre, 2003) and mortality is thus higher in demented PD patients than in non-demented PD patients (Levy *et al.*, 2002).

Research to better understand the pathology, the treatment and a plan for service provision for PDD is therefore crucial.

2.2.5.1 Risk factors for Parkinson's disease dementia

According to Aarsland *et al.* (2001a), 100 out of every 100 000 patients suffering from PD are at risk of developing PDD, which is a late complication of PD (Buter *et al*., 2008), whereas Emre (2003) suggest that 40% of all PD patients will develop dementia. It has been suggested that the risk of developing dementia in individuals suffering from PD is three- to six fold higher, compared to individuals of the same age without PD (Aarsland *et al.*, 2001b). Furthermore, with an increase in the duration of PD, there is an increased risk of cognitive impairment (Aarsland *et al*., 2010) with an estimated 75% chance of PDD developement if a patient survive more tan 10 years with PD (Aarsland & Kurz, 2010). Neuropsychological deficits which is present in the early stages of PD is often a forecaster for general development of cognitive dysfunction to dementia (Kehagia *et al*., 2010).

The most common risk factors for early dementia in PD are old age, MCI and severe extrapyramidal involvement (Aarsland & Kurz, 2010). PD patients with MCI are more vulnerable to the development of dementia than PD patients with no cholinergic deficiencies (Janvin *et al.*, 2006).

The risk of developing dementia in PD is also higher when postural instability gait difficulty (PIGD) symptoms are present and the conversion of tremor-dominant PD to PIGD occurs (Burn *et al.*, 2006). Interestingly, patients with a non-tremor dominant PD phenotype are 4.1-fold more likely to develop PDD than patients with tremor dominant PD (Williams-Gray *et al.*, 2007).

Visual hallucinations in PD are associated with the presence of Lewy Bodies in the frontal lobe (Harding *et al.*, 2002) as well as cholinergic deficiencies (Perry *et al.*, 1990), and thus indicates a potential risk of dementia in PD (Aarsland *et al.*, 2003). An interesting fact is that patients that cannot copy an intersecting pentagon accurately are 5.2-fold more likely to develop PDD (Williams-Gray *et al*., 2007).

Genetic factors that are associated with an increased risk of developing cognitive decline include triplication of the alpha-synuclein (SNCA) gene which results in PDD in early PD (Spira *et al.*, 2001; Zarranz *et al.*, 2004), mutations in the APOEε4 gene, which is associated with dementia in PD and a risk factor in AD (Huang *et al.*, 2006; Papapetropoulos *et al.*, 2007; Alzheimer's-association, 2016) and mutations in the microtubule-associated protein tau (MAPT) H1 halotype (Healy *et al.*, 2004; Goris *et al.*, 2007; Papapetropoulos *et al.*, 2007).

2.2.5.2 Pathophysiology of dementia in Parkinson's disease

Lewy Bodies, plaques, and vascular changes are present in PDD and DLB and it is a matter of debate whether they are separate non-converging diseases (McKeith, 2009). DLB and PDD thus have clinical and pathological similarities (Emre, 2003). There is an established "one-year relay" used to distinguish between PDD and DLB. If dementia occurs within 12 months of parkinsonism, it is DLB and in the case of PDD, the dementia occurs after 12 months of parkinsonism (McKeith *et al.*, 2004; Frey & Petrou, 2015).

Dementia with Lewy bodies

DLB is one of the neurodegenerative diseases where an abnormal aggregation of α-synuclein protein occurs (McKeith *et al.*, 2004). DLB is diagnosed when symptoms such as REM sleep behaviour disorder, severe neuroleptic sensitivity and reduced striatal DA transporter activity are present, with dementia arising during the first year after diagnosis of parkinsonism (McKeith *et al.*, 2005). The core clinical features of DLB, according to Luis *et al.* (1999), are a fluctuation in the deficit of a patient's memory, recurrent visual hallucinations and parkinsonism.

Parkinson's disease with dementia

PDD occurs when cognitive decline starts more than a year after PD is diagnosed (McKeith *et al.*, 2005; Frey & Petrou, 2015). The PDD patient has some of the characteristic features of cognitive impairment, which is seen in patients without dementia in terms of the administrative and mnemonic features (Dubois & Pillon, 1996). However, PDD differs in complexity, range and severity of cognitive and psychiatric symptoms, which distinguish these patients from one another (Dubois *et al.*, 2007). PDD is typified by visual hallucinations, psychosis, apathy, depression and anxiety (Dubois *et al.*, 2007; Emre *et al.*, 2007) (Table 2.1).

The cognitive profile of dementia in PD exhibits heterogeneous neuropathology (Biundo *et al.*, 2016). The most common cognitive impairments are deficits in the fronto-subcortical and

attentional-executive function associated with visuospatial, social and occupational impairment whereas it is impairment of memory in AD (Kehagia *et al.*, 2010).

Table 2.1 Summary of Parkinson's disease dementia (Kehagia *et al.*, 2010).

Parkinson's disease dementia	
Diagnostic criteria	Diagnosis of PD according to the Queen Square brain bank criteria
	PD precedes dementia onset
	Mini-Mental state examination (MMSE) score of <26
	Severe cognitive dysfunction interfering with daily living
	Impairment on at least two of the following: 1. Three word recall (MMSE) 2. Overlapping pentagons (MMSE) 3. Months reversed or sevens backward (MMSE) 4. Lexical fluency 5. Dock drawing
	Absence of major depression, delirium or other abnormalities
Neuropsychological deficits	Executive: Wisconsin card sorting test, Stroop performance, Odd-Man-Out, verbal fluency
	Working memory: digit and spatial span
	Memory: free and cued recall, auditory verbal learning
	Visuospatial abilities: clock drawing, Benton line orientation, face recognition, fragmented letters
Psychiatric symptoms	Visual hallucinations
	Psychosis
	Apathy
	Depression
	Anxiety

The underlying pathophysiology of PDD involves limbic and neocortical Lewy body deposition with neurofibrillary tangles and senile plaques playing a role in some patients. Dysfunction of non-dopaminergic neurotransmitter systems occur, and in the case of PDD, the cholinergic system in particular, has also been implicated (Williams-Gray *et al.*, 2007).

2.2.5.3 Cholinergic dysfunction in Parkinson's disease dementia

The cholinergic deficits in PDD can be even more pronounced than in patients with PD and AD. These deficits are due to degeneration and Lewy body pathology in the ascending cholinergic pathways as well as in the basal forebrain (Bohnen *et al.*, 2003; Bohnen & Albin, 2011). In the temporal cortex of PDD patients there is a profound reduction in ChAT concentrations which is associated with extended cognitive impairment (Perry *et al.*, 1985). Visual hallucinations in PDD are also mostly due to cholinergic abnormalities (Perry & Perry, 1995; Colloby *et al.*, 2006; Manganelli *et al.*, 2009).

2.2.6 Parkinson's disease therapy

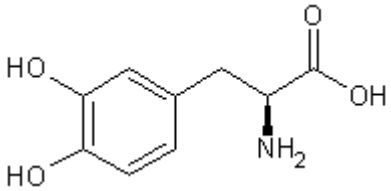
Parkinsonism can temporarily be reversed by pharmacological intervention which restores striatal DA levels (Hornykiewicz, 2002). Compounds that are able to stimulate the postsynaptic striatal DA receptors directly are preferable. Unfortunately, DA itself cannot cross the BBB, which means it will be inactive when administered (LeWitt, 2008).

2.2.6.1 Drugs frequently used in symptomatic treatment of Parkinson's disease

Levodopa

Levodopa (3,4-dihydroxy-*L*-phenylalanine) still provides the most effective treatment for the symptoms experienced in PD (Murer *et al.*, 1999), however, the long-term use of high dosages is associated with the development of motor complications (Fahn, 1999).

Levodopa is a naturally occurring amino acid that is present as an intermediate in the biosynthetic pathway of DA. After oral administration, levodopa is transported from the upper small intestine directly into the circulation. The metabolism and distribution of levodopa occurs throughout the whole body, resulting in only a small fraction that crosses the BBB by active transport (Nutl & Fellman, 1984). DA is then formed in the brain by aromatic L-amino acid decarboxylase (Misu *et al.*, 2003).



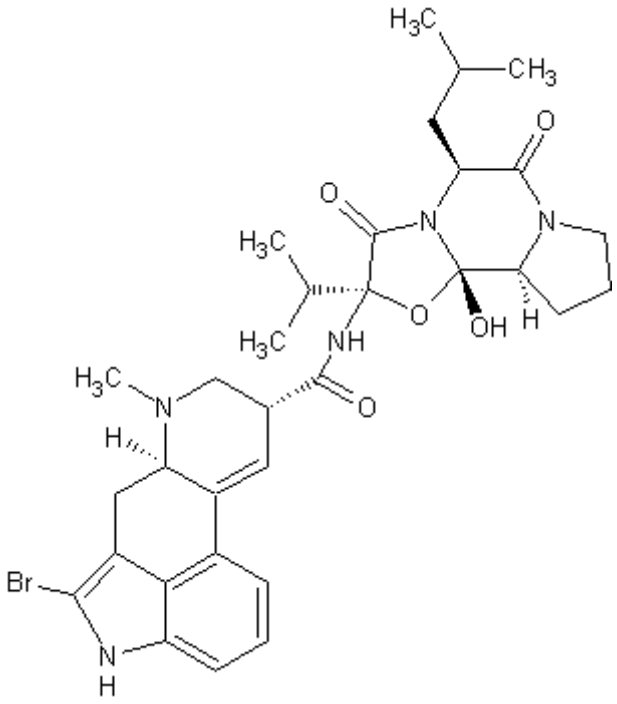
16 Levodopa

Levodopa's efficiency can be improved by the co-administration of other drugs (LeWitt, 2008). To limit the side-effects of the conversion of levodopa to DA outside the central nervous system, carbidopa is usually administered. Carbidopa is a peripheral acting aromatic L-amino acid decarboxylase inhibitor (Nutt *et al.*, 1985).

DA agonists

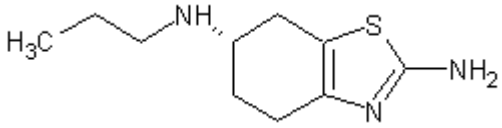
DA agonists are effective drugs used either as monotherapy in early PD (Jenner, 2002), or in combination with levodopa in early or advanced PD (Deleu *et al.*, 2002). DA agonists are drugs that act directly on DA receptors, mimicking the natural effect of DA in the brain, thus requiring no biotransformation or storage by the depleted DA neurons present in PD. Unfortunately, DA agonists are only temporary disease symptom relievers (Pahwa & Lyons, 2007; Mirza *et al.*, 2014). These agonists are divided into three different groups with their own specific pharmacological profile (Deleu *et al.*, 2002).

Group 1: Ergolinederivatives: Bromocriptine (**17**), pergolide, lisuride, cabergoline.



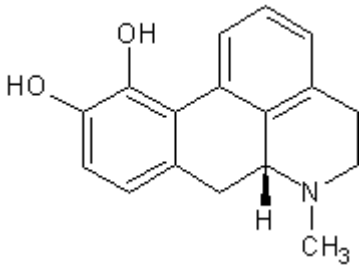
17 Bromocriptine

Group 2: Non-ergolinederivatives: Pramipexole (**18**), ropinirole, piribedil.



18 Pramipexole

Group 3: Aporphines: Apomorphine (**19**).



19 Apomorphine

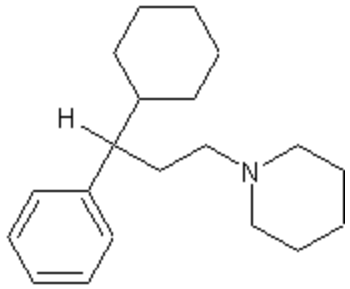
DA agonists directly stimulate DA receptors (D_1 to D_5), but since there often is an absence in agonist selectivity for specific receptors, they are classified as agonists of D_1-like receptors (D_1 and D_5) or agonists of D_2-like receptors (D_2, D_3 and D_4) (Vallone *et al.*, 2000). The stimulation of the postsynaptic D_2 receptor is associated with antiparkinsonian activity, while the stimulation of the presynaptic D_2 receptor is associated with neuroprotective effects (Robertson & Robertson, 1986, Deleu *et al.*, 2002; Lees, 2005). However, the stimulation of both D_1 and D_2 receptors is thought to result in an optimal therapeutic response (Robertson & Robertson, 1986; Deleu *et al.*, 2002). Unfortunately, D_1 receptor stimulation causes dyskinesias (Bedard *et al.*, 1999).

The advantages of DA agonists over the use of levodopa include the fact that DA agonists do not require carrier mediated transport for entering the brain and they stimulate DA receptors directly, thus the bioavailability of DA agonists is not influenced by the presence of food or amino-acids (Deleu *et al.*, 2002; Lees, 2005).

The use of DA agonists is associated with side effects such as agitation, anxiety, depression and insomnia (Mirza *et al.*, 2014). DA agonists are for that reason not recommended for use in elderly patients or elderly patients with cognitive impairment (Lees, 2005).

Centrally-acting antimuscarinic drugs

Centrally-acting antimuscarinic drugs such as trihexyphenidyl (**20**), benztropine, orphenadrine, procyclidine and biperiden are structural analogs of atropine and are used in the early management of PD. Their main pharmacological use is to restore the imbalance between striatal ACh and DA activity (Dewey *et al.*, 1990; Krueger, 1990). Most of these agents also have potent agonist activity on the noradrenergic synapse as well as agonist activity on the NMDA glutamate receptors. This class of central acting antimuscarinic drugs are useful for tremor-predominant PD patients (Kornhuber *et al.*, 1995; McDonough & Shih, 1995; Deleu *et al.*, 2002).



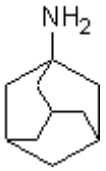
20 Trihexyphenidyl

Amantadine

Amantadine (**21**) is a tricyclic amine and was initially developed for prophylaxis of Influenza A (Schwab *et al.*, 1969; Deleu *et al.*, 2002). It is now used in early and advanced PD as monotherapy or in combination with other DA receptor agonists (Deleu *et al.*, 2002).

The exact mechanism of action of amantadine is still unclear but the following are beneficial during PD treatment:

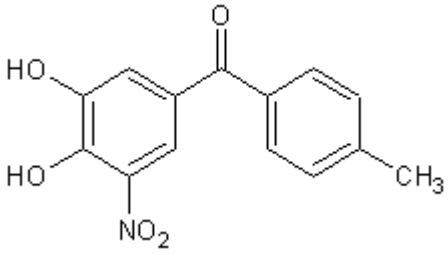
- Amantadine enhances dopaminergic neurotransmission and blocks the reuptake of DA (Kulisevsky & Tolosa, 1990; Deleu *et al.*, 2002; Lees, 2002; Lees, 2005);
- Amantadine offers mild antimuscarinic effects (Lees, 2005);
- At low micromolar concentrations, amantadine inhibits NMDA glutamate receptors in a non-competitive way (offering the possibility of neuroprotection) (Stoof *et al.*, 1992; Deep *et al.*, 1999; Deleu *et al.*, 2002; Lees, 2002; Lees, 2005);
- Amantadine mediates the stimulation of cerebral dopa decarboxylase activity (Deep *et al.*, 1999; Deleu *et al.*, 2002).



21 Amantadine

Catechol-O-methyltransferase inhibitors

COMT inhibitors are commonly used as adjunct to levodopa. The therapeutic effect of COMT inhibitors are thus mostly seen in conjunction with levodopa administration, where concomitant administration of these agents results in a more continuous delivery of levodopa to the brain (Müller, 2015). COMT inhibitors [tolcapone (**22**) and entacapone] cause a selective, reversible, dose-dependent inhibition of COMT. This action takes place in the liver, kidney, red blood cells and small intestines (Kaakkola, 2000). Tolcapone also inhibits *O*-methylation in the brain while entacapone is a peripherally-acting COMT inhibitor (Nissinen *et al.*, 1992).



22 Tolcapone

2.2.6.2 Monoamine oxidase (MAO) inhibitors

Since MAO and its inhibitors are of particular importance to this study, this enzyme and its inhibitors will be discussed in detail in the following sections.

2.2.6.2.1 MAO

In 1928 Mary Hare-Bernheim described an enzyme, tyramine oxidase, responsible for the oxidative deamination of tyramine. It was a few years later when it was discovered that tyramine oxidase, NA oxidase and aliphatic amine oxidase all were the same enzyme. This enzyme is capable of metabolising primary, secondary and tertiary amines, thus the name monoamine oxidase was given (Youdim *et al.*, 1988).

MAO is a flavoprotein that is located in the outer mitochondrial membrane and uses flavin adenine dinucleotide (FAD) as cofactor. MAO exists as two isoforms, MAO-A and MAO-B (Youdim *et al.*, 2005). The two isoforms have different pH optima and different sensitivities to heat inactivation. They also differ in substrate and inhibitor specificities, which is of great pharmacological significance. MAO-A metabolises NA and 5-HT (Johnston, 1968), while tyramine and DA are metabolised by both MAO-A and MAO-B (Youdim *et al.*, 2005). MAO-B is the isoform that has the greater activity in the basal ganglia (Collins & Youdim, 1970) and is thus ideally located for the treatment of movement disorders such as PD. One of the main functions of MAO-B is its protective role, especially where present in the microvessels of the BBB (Youdim *et al.*, 2006).

Inhibition of MAO-B has a therapeutic role in the treatment of PD. MAO-A inhibitors on the other hand, have therapeutic value in the treatment of depression, which is associated with a decrease in NA and 5-HT. Thus, MAO-A inhibitors are mostly used as antidepressants to increase central nervous system DA, NA and 5-HT (Youdim & Bakhle, 2006; Youdim *et al.*, 2006).

The cheese reaction

MAO-A is predominant in the periphery where it catalyses the oxidation of dietary amines, thus preventing their entry into the systemic circulation (Youdim & Bakhle, 2006; Youdim *et*

al., 2006). Tyramine, for example, is a dietary amine that is metabolised extensively in the liver and gut by MAO-A (Mahmood, 1997; Deleu *et al.*, 2002). When a MAO-A inhibitor is used, the protective function of MAO metabolism is inactivated, allowing tyramine and other monoamines present in ingested food (for example cheese and fermented drinks), to enter the circulation. These amines induce a significant release of NA from the peripheral adrenergic neurons causing a hypertensive response which can be fatal. This reaction is called the "cheese reaction" (Finberg *et al.*, 1981, Finberg & Tenne, 1982; Youdim & Bakhle, 2006).

Serotonin syndrome

MAO-A is also responsible for the catabolism of 5-HT. When combining a MAO-A inhibitor with a 5-HT enhancing drug (for example a selective 5-HT reuptake inhibitor), it can cause serotonin syndrome due to the overactivity of 5-HT in the central nervous system (Fernandez & Chen, 2007). This syndrome results in central nervous system toxicity and includes symptoms such as hyperthermia (increased body temperature) (Zornberg *et al.*, 1991; Chen & Swope, 2005), loss of coordination, hallucinations, rapid heartbeat, nausea and vomiting, diarrhoea, overactive reflexes and sudden changes in blood pressure (Fernandez & Chen, 2007).

2.2.6.2.2 MAO mechanism of action

The mechanism of action of MAO involves the oxidative deamination of, as mentioned before, primary, secondary and tertiary amines (which include dietary amines and neurotransmitters). During this reaction the amines are oxidised to the corresponding aldehydes and free amines while generating hydrogen peroxide (Figure 2.2). Aldehyde dehydrogenase (ADH) then rapidly metabolises these aldehydes to acidic metabolites (Grünblatt *et al.*, 2004; Youdim & Bakhle, 2006). The hydrogen peroxide generated through the FAD-$FADH_2$ cycle requires inactivation by glutathione peroxidase in the brain (Youdim & Bakhle, 2006).

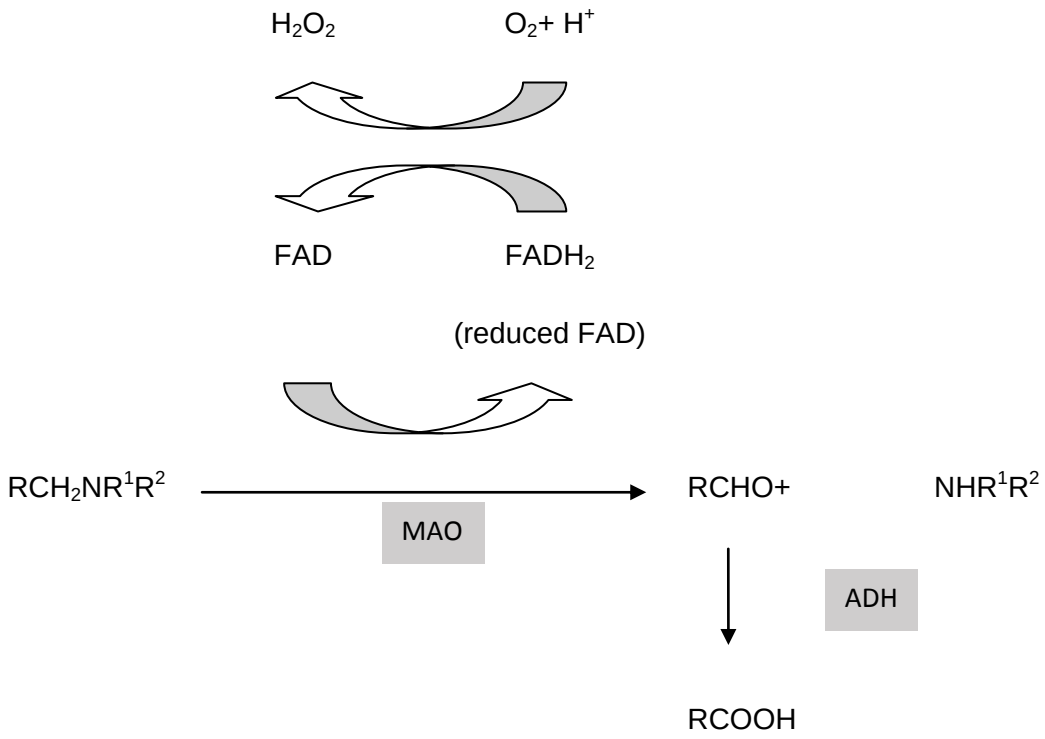


Figure 2.2 Oxidative deamination of monoamines by mitochondrial MAO (Youdim & Bakhle, 2006).

The identity of the generated aldehyde depends on the identity of the substrate that is being metabolised, conversely, the substrate's identity has no effect on the use of oxygen and the formation of hydrogen peroxide (Holt *et al.*, 1997).

2.2.6.2.3 Reaction mechanism

MAO enzymes are flavoproteins, and the redox cofactor essential for the catalysis of the reaction, is a covalently bound FAD. During the oxidative deamination of neurotransmitters, the MAO isoforms use O_2 as electron acceptor. Firstly, the deprotonated amine group of the substrate is oxidised to a protonated imine when bound to the MAO enzyme's active site, while the FAD cofactor is reduced to the hydroquinone form. H_2O_2 and the oxidised flavin are generated through the reaction of the reduced FAD cofactor with O_2. The protonated imine dissociates from the enzyme, it then undergoes a non-catalysed hydrolysis to form the corresponding amine or ammonia (NH_4^+) and an aldehyde (Edmondson *et al.*, 2007).

Two reaction mechanisms have been proposed for MAO catalysis (Binda *et al*., 2002).

Single electron transfer (SET) mechanism

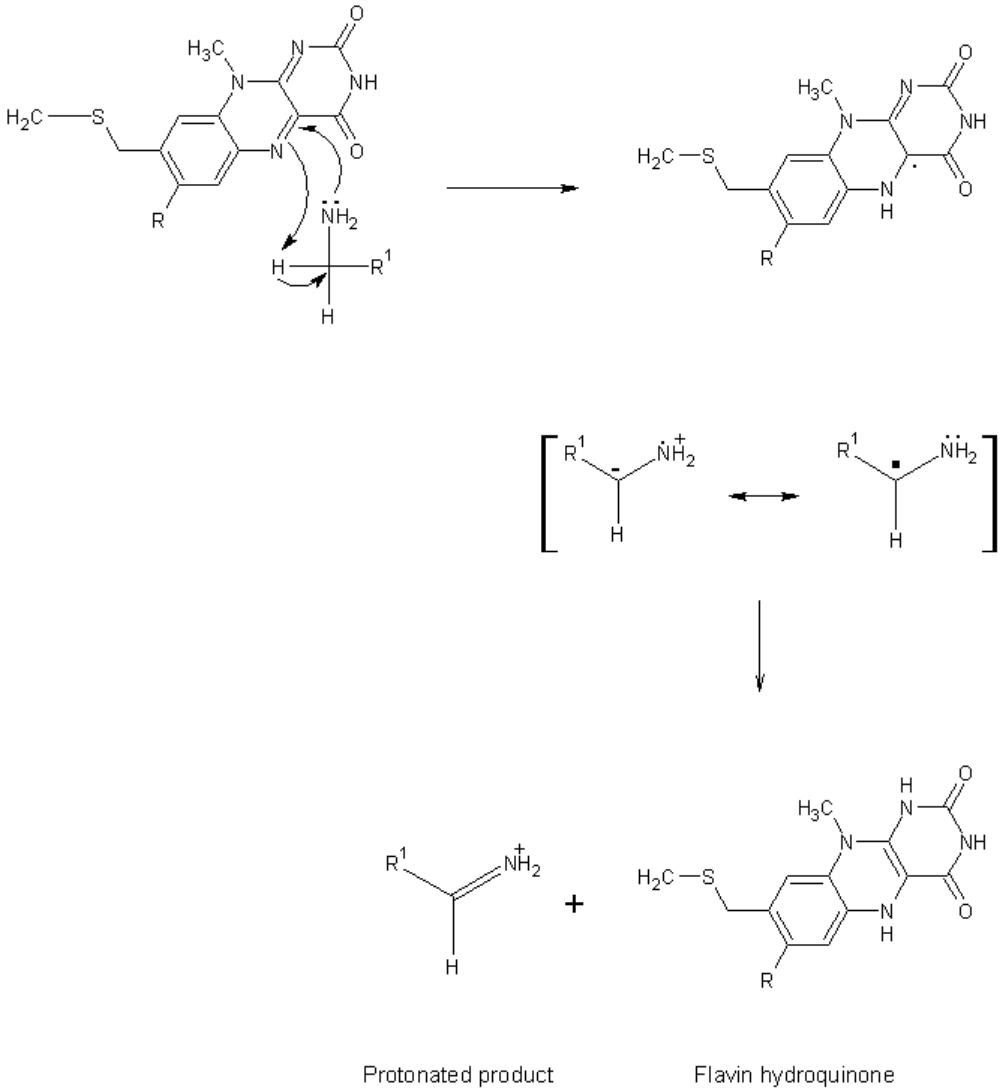


Figure 2.3 Single electron transfer (SET) mechanism as proposed by Lu *et al.*, 2002.

Firstly, the lone pair of electrons of the amine nitrogen is oxidised by the flavin, which acts as a one electron oxidant, resulting in an aminium cation radical and a flavin radical. The α-proton (α-C-H) of the aminium radical is now acidic enough to be abstracted by a basic amino acid residue in the active site. A second electron transfer occurs and an imine and reduced flavin form as products (Binda e*t al*., 2002).

Although the SET mechanism (Figure 2.3) provides an explanation for the ring-opened products of cyclopropylamine substrates (Lu *et al*., 2002), thermodynamically, this mechanism is highly unlikely. There is also no evidence for the formation of flavin radical intermediates during substrate oxidation (Binda *et al.*, 2002; Edmondson *et al*., 2007).

Polar nucleophilic mechanism

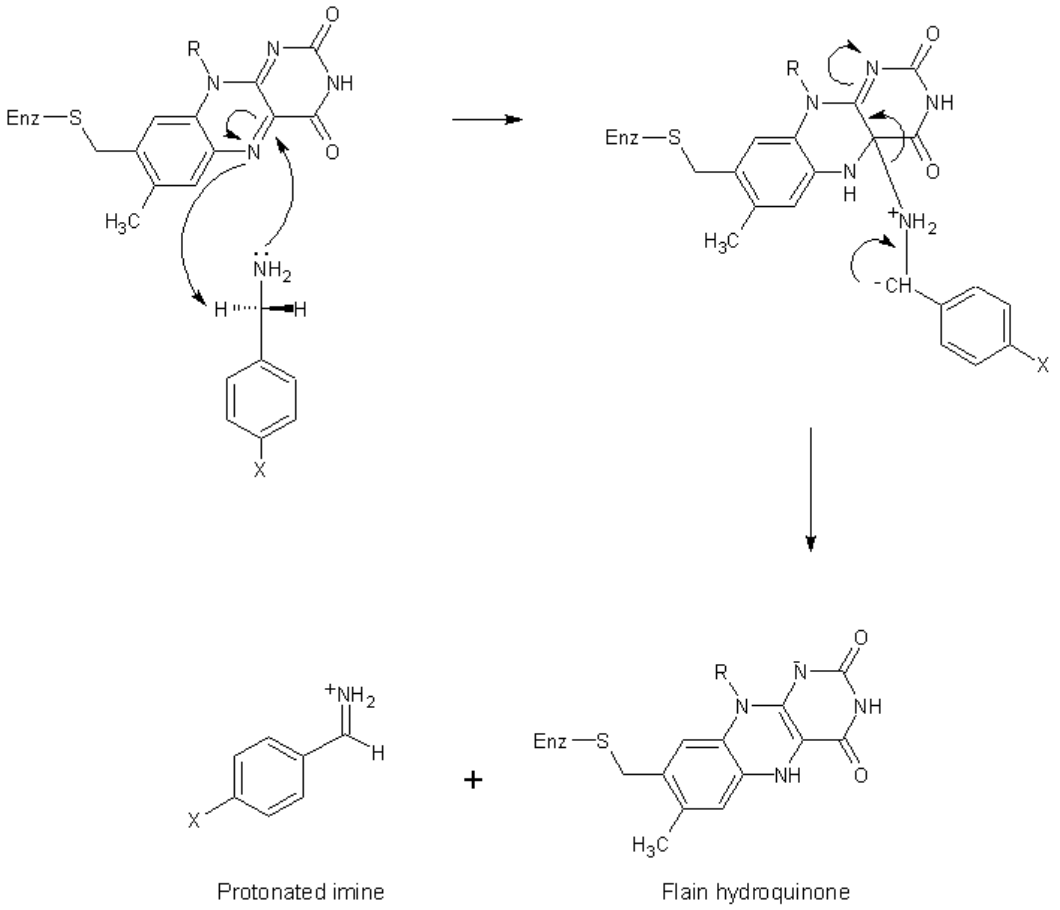


Figure 2.4 Polar nucleophilic mechanism as proposed by Edmondson *et al.*, 2007.

The polar nucleophilic mechanism, as illustrated in figure 2.4 is more likely, according to available structural and mechanistic data (Binda *et al*., 2002). In this mechanism, the substrate amine launches a nucleophilic attack on the flavin at the C-4a position, which activates the flavin at the N-5 position. The flavin N-5 is now a strong enough base to abstract a α-proton from the substrate (Edmondson *et al*., 2007).

2.2.6.2.4 The role of MAO inhibitors in PD

The inhibition of MAO-B is advantageous in the symptomatic treatment of PD because it increases synaptic DA (Schapira, 2011).

2.2.6.2.5 Known inhibitors of MAO

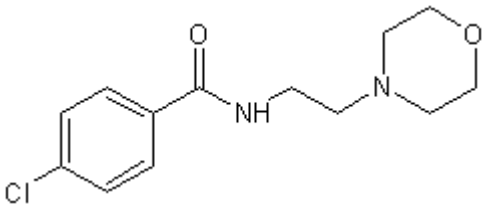
Table 2.2 Summary of MAO inhibitors (Bortolato *et al.*, 2008).

Group	Class	Drug	MAO selectivity
Non-selective	Hydrazines	Isocarboxazid	A&B
		Pheneline	A&B
		Nialamide	
		Iproniazid	A&B
	Amphetamine derivatives	Iproclozide	A&B
		Tranylcypromine	
Selectiveirreversible	Propargylamines	Clorgyline	A
		Selegiline	B
		Rasagiline	B
Selectivereversible	Piperidylbenzofurans	Brofaromine	A
	Morpholinobenzamides	Moclobemide	A
	Oxazolidinones	Toloxatone	A
		Linezolid	A
		Befloxatone	A
		Cimoxatone	A
Mixed MAO-cholinesterase inhibitors	Propylamines	Ladostigil	A&B

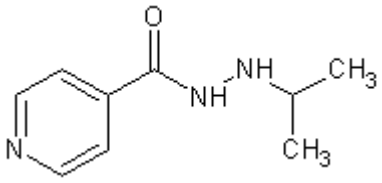
MAO-A inhibitors

Moclobemide (**23**) is a reversible MAO-A inhibitor and does not cause the “cheese reaction”, because reversible MAO-A inhibitors can be displaced by dietary amines and these amines can then undergo normal metabolism by MAO (Haefely *et al.*, 1992; Youdim & Bakhle, 2006).

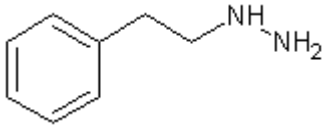
Iproniazid (**24**) was the first drug available that exhibited MAO inhibitory effects and was initially developed for the treatment of tuberculosis, but was later used in the treatment of depression. Derivatisation of iproniazid led to the development of more hydrazine derivatives i.e. phenelzine (**25**), but due to their serious liver toxicity, non-hydrazine derivatives, such as tranylcypromine (**26)** and pargyline (**27**), were developed. However, these non-hydrazine derivatives still have the potential to cause the “cheese reaction” (Youdim *et al.*, 1988; Youdim & Bakhle, 2006).



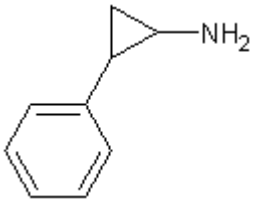
Moclobemide (**23**)



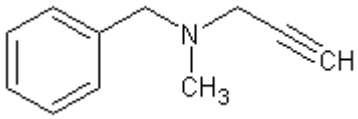
Iproniazid (**24**)



Phenelzine (**25**)



Tranylcypromine (**26**)

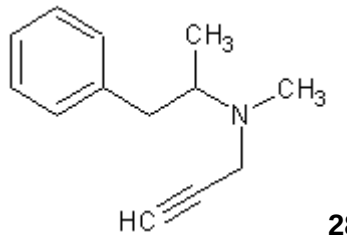


Pargyline (**27**)

Figure 2.5 The chemical structures of some MAO-A inhibitors.

MAO-B inhibitors

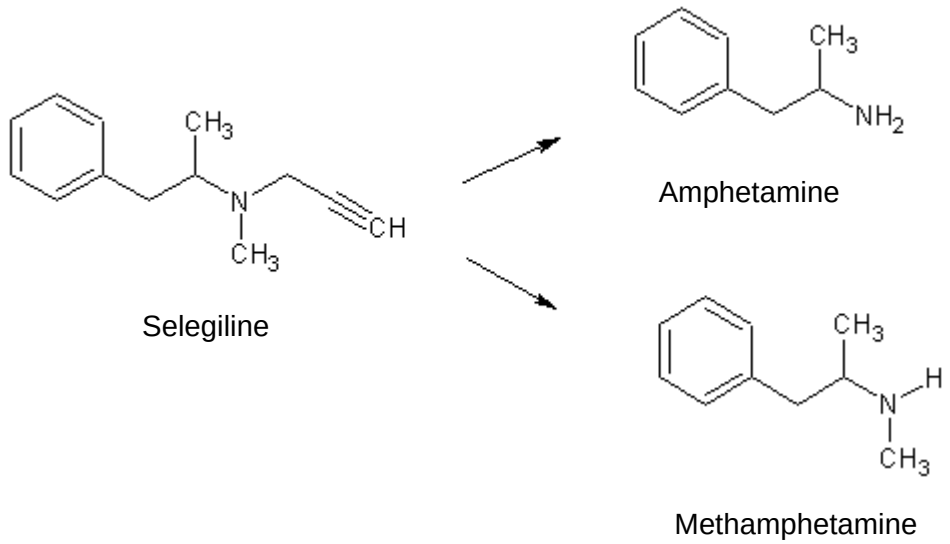
Selegeline



28 Selegiline

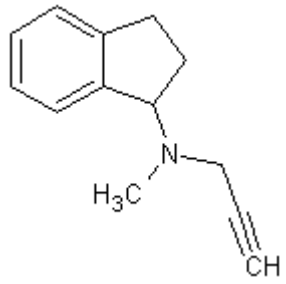
Selegiline (**28**) is a propargylamine derivative of 1-amphetamine and an irreversible, selective MAO-B inhibitor which do not cause the cheese reaction (Knoll & Magyar, 1971; Youdim & Bakhle, 2006). Selegiline is useful in PD as monotherapy and as combined therapy with levodopa, where selegiline has levodopa sparing actions (Birkmayer *et al.*, 1977; Youdim & Bakhle, 2006).

After ingestion, selegiline is metabolised to methamphetamine- and amphetamine-like metabolites (Scheme 2.3) (Reynolds *et al.*, 1978) which increase the synaptic release of catecholamines and reduce catecholamine stores in nerve terminals (Gill *et al.*, 1967; Cavanaugh *et al.*, 1970). This may result in adverse effects such as dizziness, a dry mouth, abdominal pain, stomach upset, nausea, sleeplessness and headache (Shoulson *et al.*, 2002).



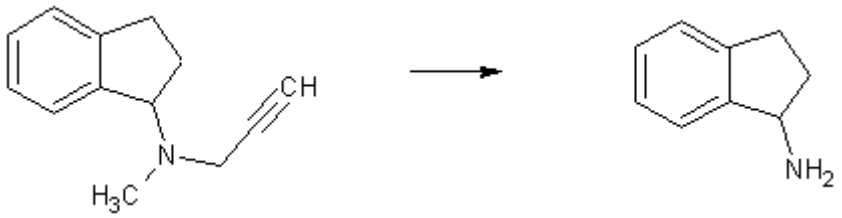
Scheme 2.3 Selegiline and its metabolites(Youdim *et al.*, 2001).

Rasagiline



29 Rasagiline

Rasagiline (**29**) is an analogue of selegiline with potent, irreversible MAO-B inhibitory action, and contrary to selegiline, is metabolised to an inactive aminoindane metabolite (Scheme 2.4) (Sabbagh & Youdim, 1978; Kalir *et al.*, 1981). Rasagiline is ten fold more potent than selegiline as a MAO-B inhibitor and is less toxic, because it has no amphetamine-like adverse effects (Finberg *et al.*, 1999). Rasagiline does not produce the "cheese reaction" (Kalir *et al.*, 1981), exhibits no sympathomimetic effect and is potentially more neuroprotective than selegiline (Mandel *et al.*, 2005). Rasagiline is effective as monotherapy in early PD (Rascol *et al.*, 2005; Siderowf *et al.*, 2002).



Rasagiline Aminoindane

Scheme 2.4 Rasagiline and its metabolite (Youdim *et al.*, 2001).

2.2.7 Parkinson's disease dementia treatment

The therapeutic intervention in patients suffering from PDD is structured around the treatment of non-motor symptoms as well as motor impairment. These non-motor symptoms have the greatest impact on the patients' quality of life, therefore improving symptoms and promotion of functional independence is of great importance (Janvin *et al.*, 2006).

Cholinesterase inhibitors (CHEIs)

In some cases, ChEIs provide moderate benefits early in the course of PDD (Emre *et al.*, 2007). Drugs that stimulate the cholinergic system help to improve cognition in PD. However, cholinergic drugs may worsen motor symptoms in PD patients because

normalising motor symptoms in PD is in part, dependent on restoring the cholinergic-dopaminergic balance in the striatum (Oertel *et al.*, 2008).

Rivastigmine is a dual inhibitor of AChE and BuChE which improves dementia symptoms in PDD primarily by improving the fluctuations in attention that interfere with the most common tasks in PDD patients (Lachman *et al.*, 1996; Gurevich *et al.*, 2006).

Dopamine agonists

Dopaminergic treatment is used to treat cognitive deficits in PDD. The benefits of dopaminergic treatment in patients with advanced disease and neuropsychiatric symptoms often outweighs the side effects it causes (Fénelon *et al.*, 2000).

Treatment of PDD only provides symptomatic relief and does not address disease progression. The development of new treatments is hindered by the fact that the mechanism that underlies the neuronal dementia process and the associated cognitive deficits is poorly understood.

2.3 CONCLUSION

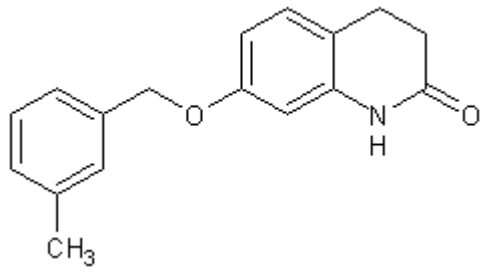
In this chapter, background regarding AD, PD and the late complication of PD, namely PDD, was provided. This chapter showed that AD, PD and PDD are neurodegenerative diseases with complex pathogeneses, which is not fully understood. Although these are different diseases, there are similarities in their pathogeneses and pathology. For example, oxidative stress plays a key role in disease progression, while protein deposits are present in both AD and PD. The MAO enzyme was discussed in the chapter and it was shown that inhibition of MAO may lead to neuroprotection in these three diseases. Although therapies such as AChE inhibitors and levodopa exist for the treatment of AD and PD, respectively, their effectiveness declines over time and the therapies for PDD in particular are inadequate. Most of the treatments available only address the disease symptoms while the underlying cause of neorodegeneration and disease progression is left untreated. The multifactorial nature of these disorders are further not addressed satisfactorily by current therapies and neuroprotection is not obtained. It is thus clear that there is an urgent need for alternative, disease modifying agents.

CHAPTER 3

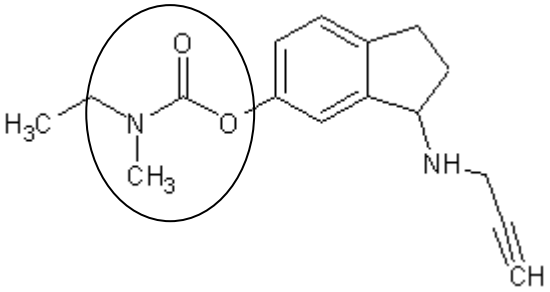
CHEMISTRY

3.1 INTRODUCTION

It is postulated that the combination of the dihydroquinolinone and carbamate moieties will result in chemical entities with potent dual MAO-B and AChE inhibitory activities. In this study, the dihydroquinolinone and carbamate scaffolds will thus be combined to obtain a synthesised product which would potentially inhibit both MAO-B and AChE. In previous studies dihydroquinolinones showed inhibition of MAO-B (**30**) (Meiring *et al.*, 2013), while the carbamate moiety occurs in the structures of potent inhibitors of AChE e.g. ladostigil (**3**) (Li *et al.*, 2014). Since phenylacetamide derivatives such as paracetamol (**31**) are structurally similar to dihydroquinolinones, some of these derivatives will also be synthesised to investigate the importance of the closed dihydroquinolinone ring system.



30



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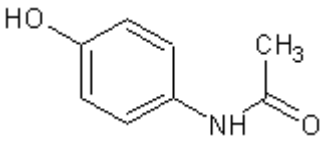


31

Figure 3.1 The chemical structures of a dihydroquinolinone (**30**), ladostigil (**3**) and paracetamol (**31**).

During the design of compounds for this study, the following were taken into consideration:

- Both 6- and 7-hydroxy-3,4-dihydro-2(1*H*)-quinolinone would be used as starting materials in order to obtain 6- and 7-substituted carbamate derivatives. By doing this, the effect of the position of the carbamate substitution on MAO and AChE inhibitory activity could be assessed. Similarly, 3- and 4-acetamidophenol derivatives would be synthesised.
- Synthesis of the acetamidophenol derivatives would further provide insights into the importance of the rigid, closed dihydroquinilinone ring system.
- Selection of carbamate substituents was based on commercial availability in order to facilitate ease of synthesis.

3.1.1 Materials and instrumentation

Materials

All reagents were obtained from Sigma-Aldrich, and were used without further purification. Solvents for nuclear magnetic resonance (NMR) spectroscopy were purchased from Merck and solvents for reactions and chromatography were obtained from Rochelle.

Thin layer chromatography (TLC)

Precoated Kieselgel 60 F254 plates (Merck) were used for TLC analysis and detection was done by UV light at a wavelength of 254 nm. The mobile phases that were used were dichloromethane: methanol (9:1) (for compounds **8a**, **b**, **d**, **e**; **9a**, **b**; **10g** and **11h**) and dichloromethane: ethyl acetate (6:4) (for compounds **8c**, **f**, **g**; **9c**, **d**; **10a**, **b**, **c**, **d**, **e**, **f**, **h** and **11a**, **b**, **c**, **d**, **e**, **f**, **g**, **i**)

Melting points

All melting points were determined using a Buchi B-545 apparatus and are uncorrected.

Mass spectrometry

High resolution mass spectra (HRMS) were recorded with a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionization (APCI) mode.

Nuclear magnetic resonance spectroscopy

Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz for ^{1}H and 150 MHz for ^{13}C spectra, respectively. All samples were dissolved in deuterodimethylsulfoxide (DMSO-*d6*). Data were analysed and

processed with the program MestReNova2. ^{1}H NMR data is reported indicating the chemical shift (δ) in ppm, the integration, the multiplicity and the coupling constant (J) in Hz. The following abbreviations are used:

- s (singlet)
- br s (broad singlet)
- d (doublet)
- br d (broad doublet)
- dd (doublet of doublets)
- t (triplet)
- br t (broad triplet)
- q (quartet)
- p (pentet/quintet)
- m (multiplet)

Chemical shifts are referenced to the residual solvent signal: DMSO-*d*6: 2.5 ppm for ^{1}H and 39.5 ppm for ^{13}C spectra.

High pressure liquid chromatography (HPLC)

HPLC analyses were conducted with an Agilent 1100 HPLC system equipped with a quaternary pump and an Agilent 1100 series diode array detector for determination of the purity of the compounds synthesised in this study. HPLC grade ethanol (Merck) and Milli-Q water (Millipore) were used for the chromatography. A Venusil XBP C18 column (4.60 × 150 mm, 5 µm) was used with 30% ethanol and 70% MilliQ water as the initial mobile phase at a flow rate of 1 ml/min. A solvent gradient program was initiated at the start of each HPLC run; the concentration of acetonitrile in the mobile phase was linearly increased up to 85% over a period of 5 min. Each HPLC run lasted 15 minutes and a time period of 5 min was allowed for equilibration between runs. The test compound was injected (20 µl, 1 mM) into the HPLC system and the eluent was monitored at wavelengths of 210, 254 and 300 nm.

Infrared (IR) spectroscopy

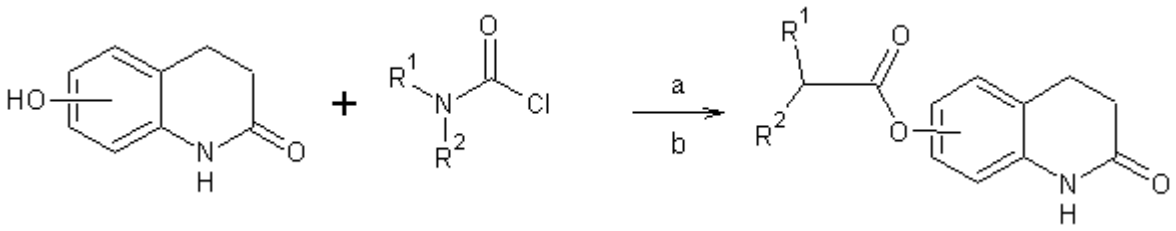
An Alpha FT-IR spectrometer (Bruker®), platinum ATR was used for further characterisation of the synthesised compounds in this study. OPUS/Mentor software interface was used to process the spectrometer readings.

3.2 SYNTHESIS OF TARGET COMPOUNDS

During this study, compounds were synthesised in one step, using general synthetic methods, obtained from literature. Coupling of the phenol and carbamoyl chloride was first attempted in the presence of potassium carbonate (John & Nicholas, 2012) and when that failed, harsher conditions, utilising sodium hydride were used (Griffen *et al.*, 1995). Characterisation of the synthesised compounds was primarily done by NMR, involving the analysis of 1D (^{1}H, ^{13}C, DEPT) and 2D (HSQC, HMBC, COSY) spectra. Mass spectrometry and infrared spectroscopy were further used for structural confirmation and melting points were also determined. The purity of the compounds was assessed by HPLC.

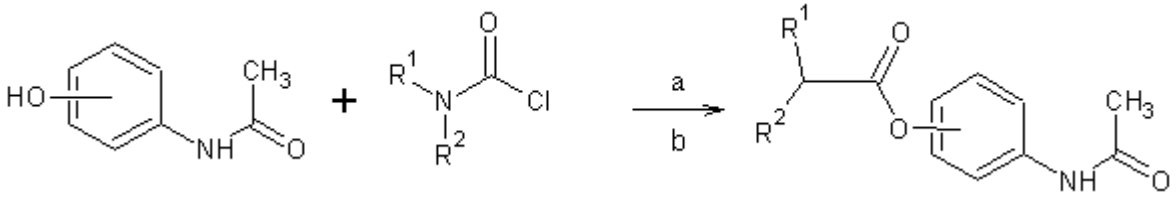
3.2.1 General synthetic procedures

Procedure for the synthesis of dihydroquinolinone-carbamates (8c,f,g; 9c,d) and acetoamidophenol-carbamate derivatives (10a-f,h; 11a-g,i) (John & Nicholas, 2012).



32 **33** **8,9**

Or



34 **33** **10, 11**

Scheme 3.1 The general synthetic route for quinolinone-carbamates and acetamidophenol derivatives (John & Nicholas, 2012). (a) K_2CO_3; (b) CH_3CN.

A mixture of the phenol (**27** or **30**, 3.06 mmol) carbamoyl chloride (**28**, 4.60 mmol), and K_2CO_3 (4.60 mmol) was refluxed in CH_3CN (8 ml) for approximately five hours while stirring. After TLC analysis indicated that the reaction was complete, it was quenched by the addition of 30 ml water. The precipitate that formed upon the addition of the water was filtered, the solvent evaporated under vacuum and the residue air dried. The product was recrystallised from ethanol.

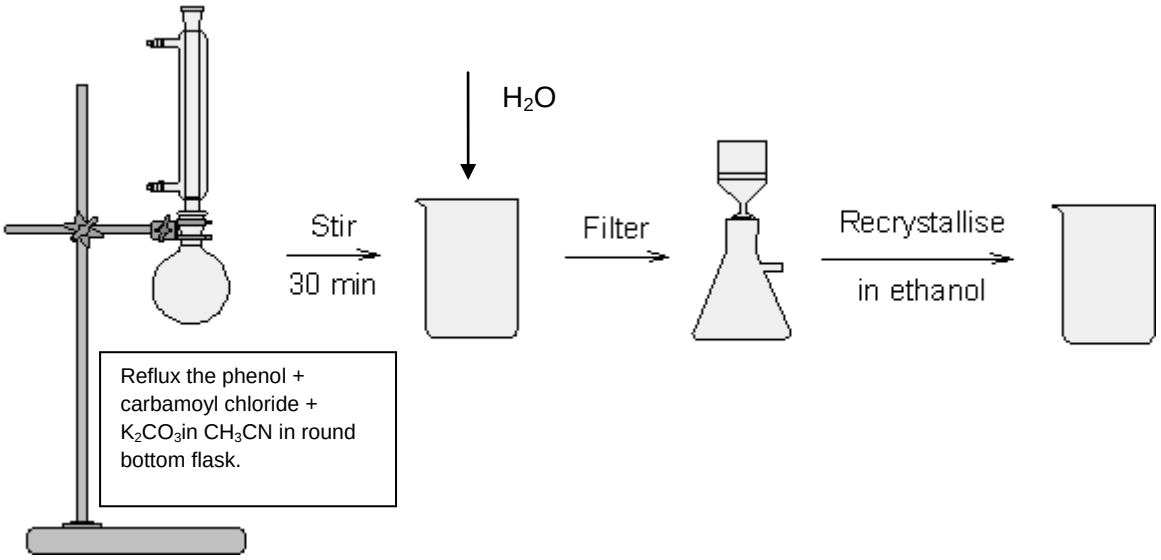
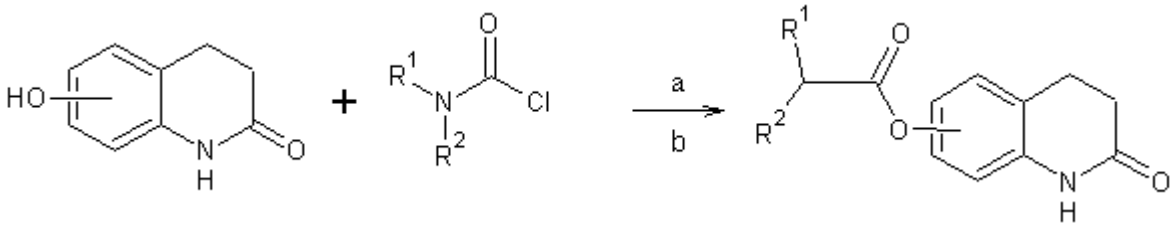


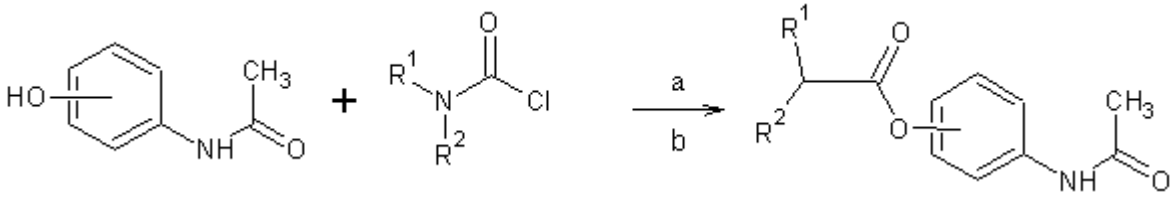
Figure 3.2 Experimental setup for the synthesis of dihydroquinolinone-carbamates (**8c**, **f**, **g**; **9c**, **d)** and acetamidophenol-carbamate derivatives (**10a-f**, **h**; **11a-g**, **i**) (*John & Nicholas, 2012*).

Procedure for the synthesis of dihydroquinolinone-carbamates (8a-b, d-e; 9a-b) and acetoamidophenol-carbamate derivatives (10g; 11h) (Griffen et al., 1995).



32 **33** **8, 9**

Or



34 **33** **10, 11**

Scheme 3.2 The general synthetic route for dihydroquinolinone-carbamates and acetamidophenol derivatives (Griffen *et al.*, 1995). (a) NaH; (b) DMF.

The phenol (**32** or **34**, 4.96 mmol) was dissolved in dry DMF (20 ml) under inert conditions (nitrogen gas), while being cooled in an ice bath. NaH (5.95 mmol) was added portion-wise. When all evolution of hydrogen gas (H_2) ceased, the suspension was stirred for a further 30 minutes after which the carbamoyl chloride (**33**, 5.95 mmol) was added. The mixture was heated to 80°C and stirred for a further 75 minutes. After it was established by TLC analysis that the reaction was complete it was allowed to cool to room temperature and then quenched by the addition of water. The mixture was extracted three times with ethyl acetate, where after the ethyl acetate fractions were combined and washed with brine (3x) to remove all traces of DMF. The combined organic fractions were dried using $MgSO_4$, filtered and the filtrate dried under vacuum. The product was recrystallised from ethanol.

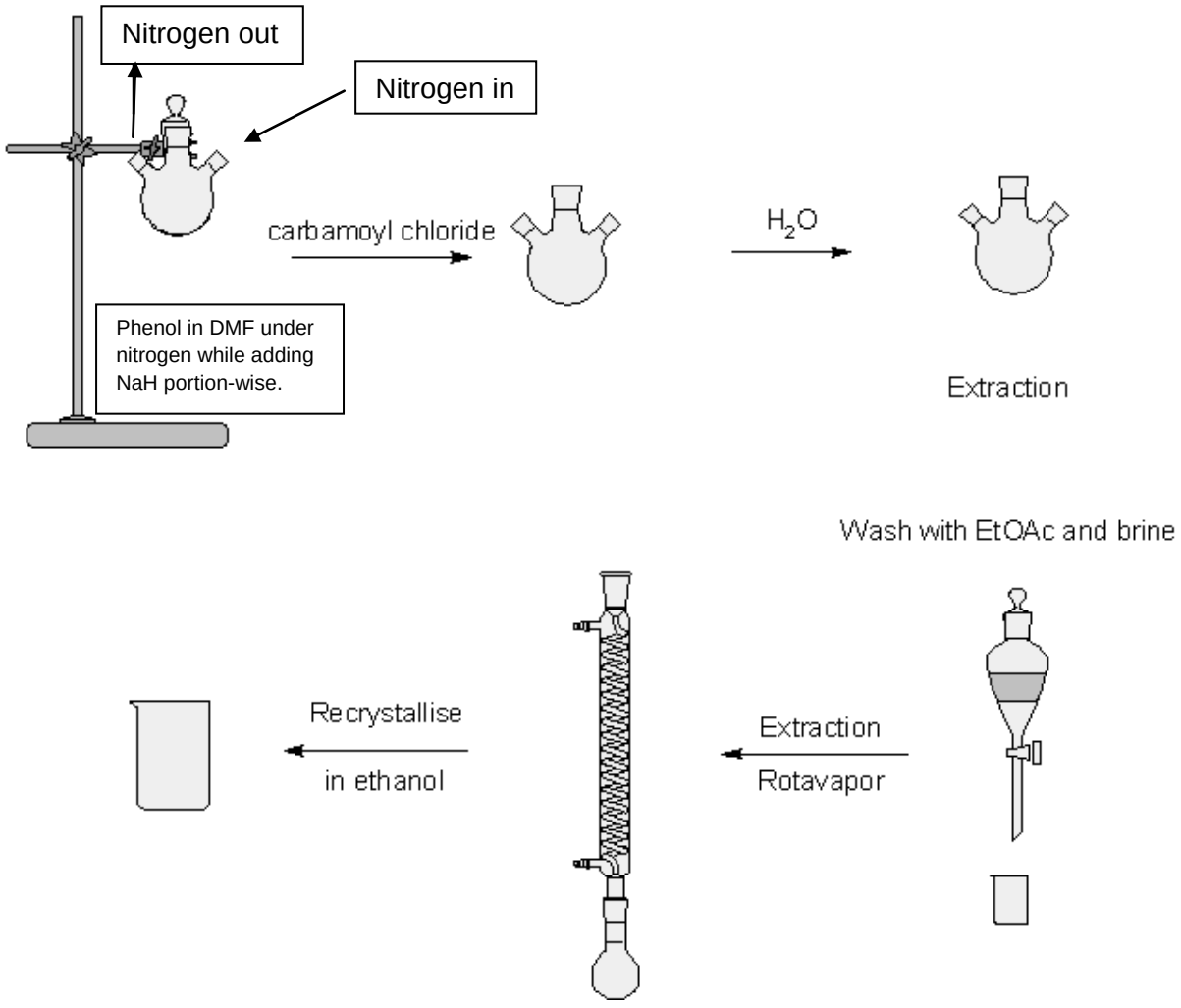
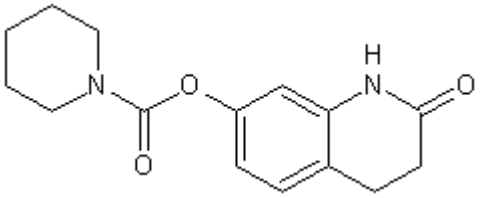
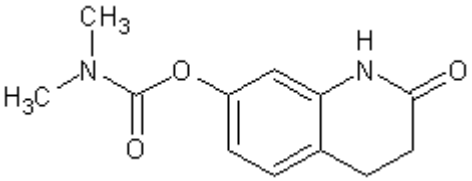
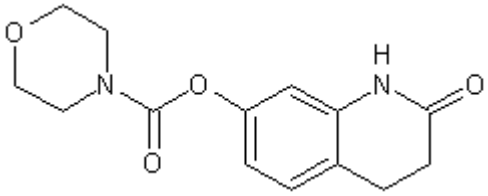


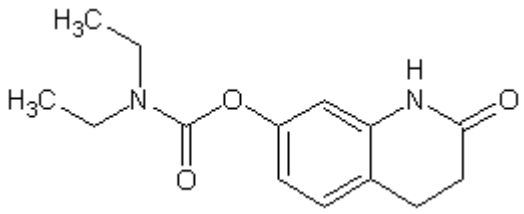
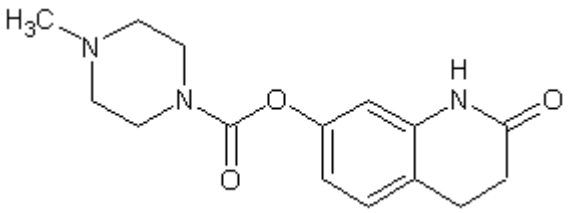
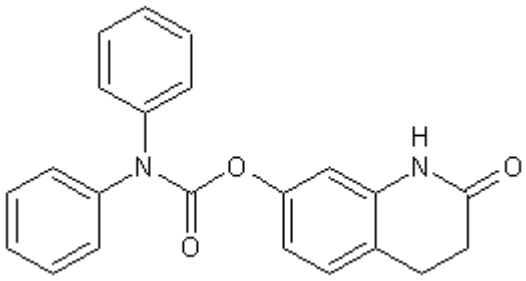
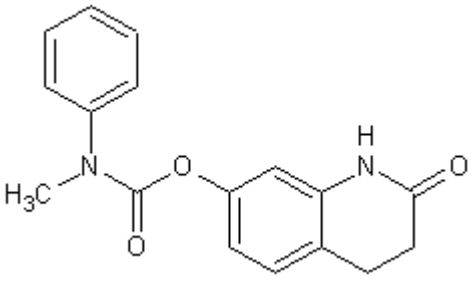
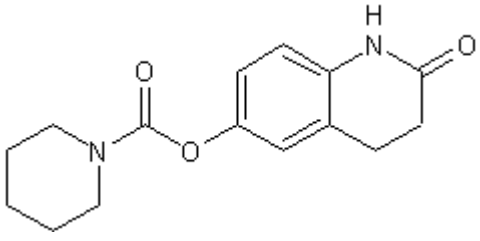
Figure 3.3 Experimental setup for the synthesis of dihydroquinolinone-carbamates (**8a-b**, **d-e**; **9a-b**) and acetamidophenol-carbamate derivatives (**10g**; **11h**) (Griffen *et al.*, 1995).

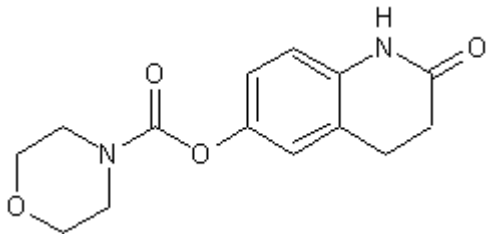
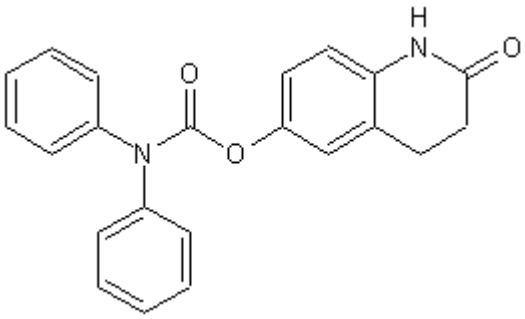
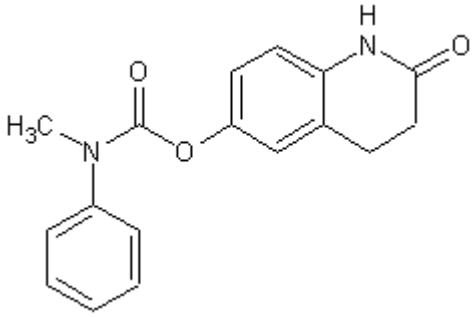
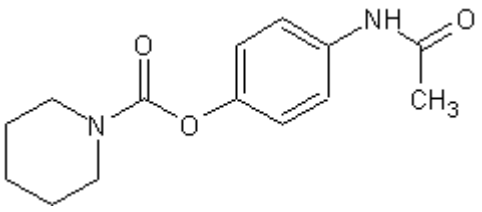
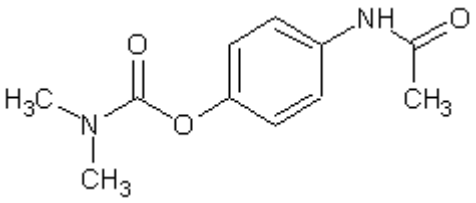
3.2.2 Results and discussion

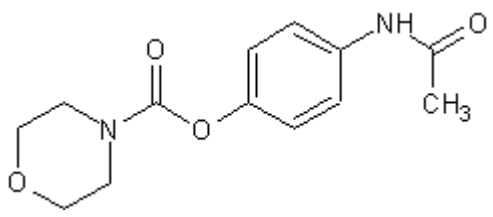
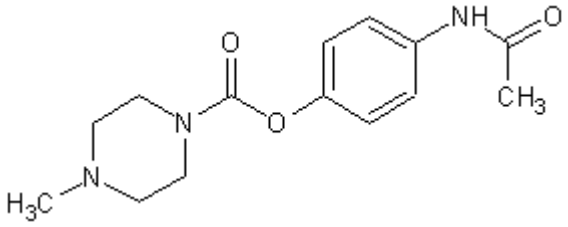
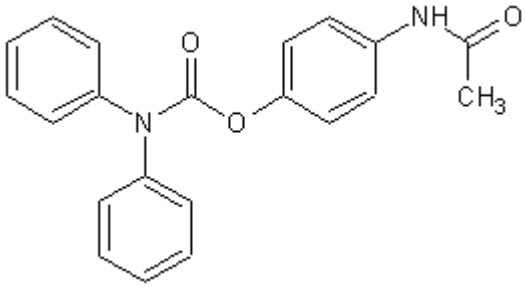
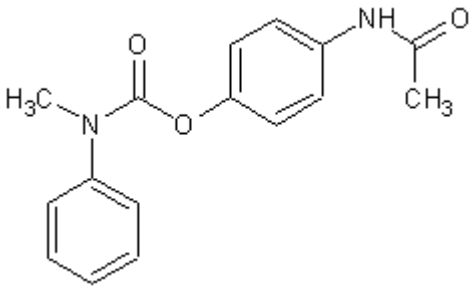
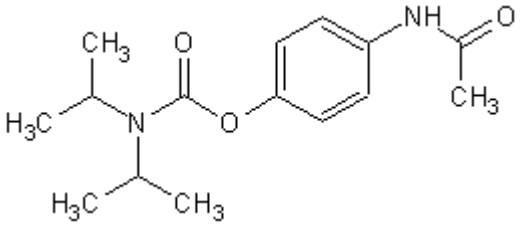
Twenty eight compounds (Table 3.1) were synthesised successfully in one step using literature procedures, generally in acceptable yields. Purity, as determined by HPLC, was generally above 80%. The synthesised compounds were characterised by NMR and infrared spectroscopy and mass spectrometry. For all these compounds the mass data correlated well with the calculated mass values. For both the dihydroquinolinone-carbamates and acetamidophenol-carbamate derivatives, characteristic peaks were present in the IR spectra. These include those for the secondary amide NH, the carbamate and amide carbonyls and the C-O-C group in the regions of 3500 - 3100 cm^{-1}, 1700 cm^{-1}, 1500 - 1900 cm^{-1} and 1170 cm^{-1}, respectively (Bellamy, 2013).

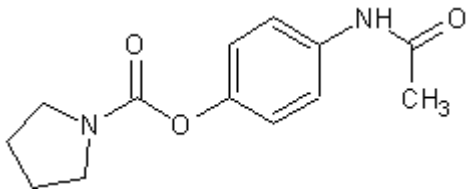
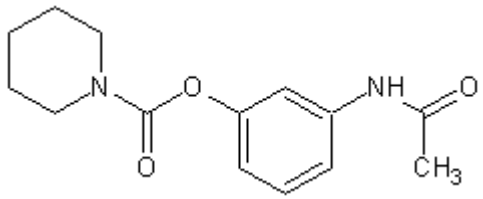
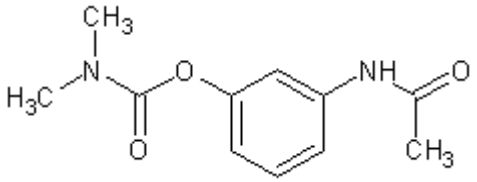
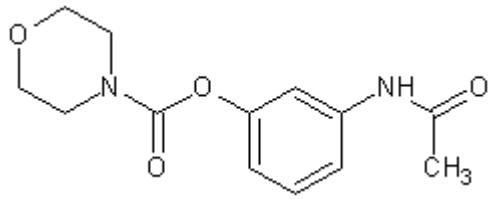
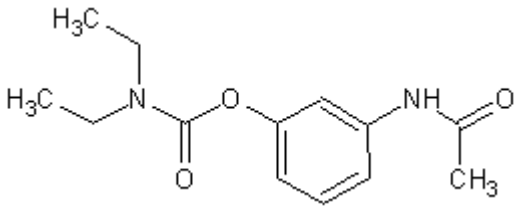
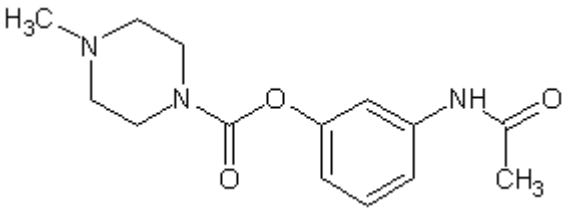
Table 3.1 Chemical structures of dihydroquinolinone-carbamates (**8a – g**, **9a – d**) and acetamidophenol-carbamate derivatives (**10a – h**, **11a** - **i**) that were successfully synthesised in this study.

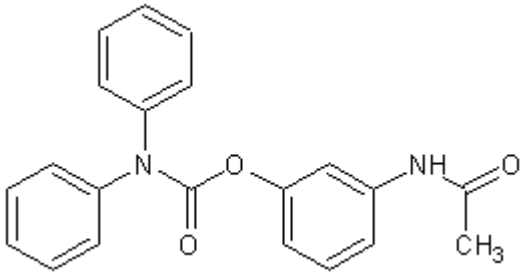
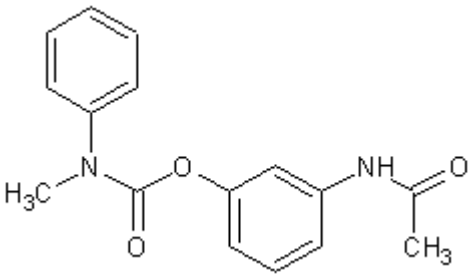
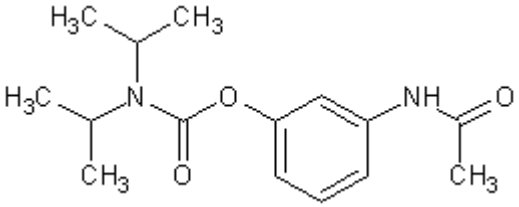
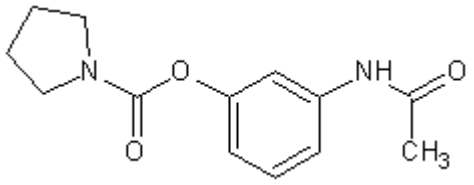
7-hydroxy-3,4-dihydro-2(1*H*)-quinolinone derivatives	
8a	
8b	
8c	

8d	
8e	
8f	
8g	
6-hydroxy-3,4-dihydro-2(1*H*)-quinolinone derivatives	
9a	

9b	
9c	
9d	
4-Acetamidophenol derivatives	
10a	
10b	

10c	
10d	
10e	
10f	
10g	

10h	
3-Acetamidophenol derivatives	
11a	
11b	
11c	
11d	
11e	

11f	
11g	
11h	
11i	

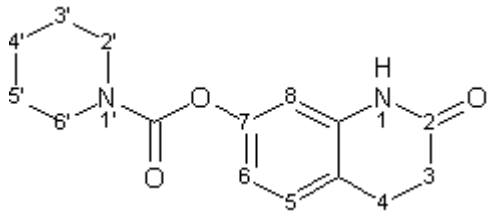
Characterisation of the synthesised compounds was primarily done by NMR. NMR assignments were based on analysis of 1D (^{1}H, ^{13}C, DEPT) and 2D (HSQC, HMBC, COSY) spectra, while NMR data of known, related compounds were also taken into consideration. The NMR-spectra of the synthesised compounds are given in the appendix. The approach followed during the assignment of NMR signals will be discussed for 4 compounds namely **8a**, **9c**, **10b** and **11h** as examples.

For compound **8a**, in the ^{1}H NMR spectrum, eight signals, integrating for 18 protons were observed. The most downfield signal, which did not couple to a carbon in the HSQC

spectrum, was assigned as the NH of the dihydroquinilinone ring. The signals at 7.13, 6.63 and 6.57 ppm were assigned as the aromatic protons H-5, H-6 and H-8, respectively, primarily based on the observed coupling constants and COSY couplings. In the aliphatic region, four signals were observed, with the triplets at 2.84 and 2.44 ppm assigned as H-4 and H-3, respectively. HMBC couplings were observed between H-4 and C-3 (30.4 ppm), C-4a (120.3 ppm), C-5 (128.2 ppm), C-8a (138.9 ppm) and C-2 (170.2 ppm) and between H-3 and C-4 (24.2 ppm), C-4a (120.3 ppm), and C-2 (170.2 ppm) aiding in this assignment. The multiplet at 3.63 – 3.23 ppm was assigned as the protons adjacent to the nitrogen in the piperidinering, while the multiplet at 1.63 – 1.46 ppm accounted for the six remaining protons of the piperidine ring.

In the ^{13}C spectrum 15 signals were observed, accounting for the 15 carbons present in this compound. After considering the DEPT data in conjunction with the ^{13}C NMR data, the five signals at 170. 2 (C-2), 152.8 (carbamate carbonyl), 150.2 (C-7), 138.9 (C-8a), 120.3 (C-4a) were identified as quaternary carbons. These assignments were based on the respective shifts observed as well as HMBC couplings. For the signal at 138.9 (C-8a), for example, HMBC couplings were observed with H-4 (2.84 ppm), H-8 (6.57 ppm), H-5 (7.13 ppm) and the NH (10.11 ppm). On the other hand, for the signal at 120.3 (C-4a), HMBC couplings were observed with H-3 (2.44 ppm), H-4 (2.84 ppm), H-8 (6.57 ppm), H-6 (6.63 ppm) and the NH (10.11 ppm). Observed HSQC couplings between H-3, H-4, H-5, H-6, H-8 and the carbons of the piperidine ring led to the assignments of the respective carbons at 30.3 (C-3), 24.5 (C-4), 128.2 (C-5), 115.0 (C-6) and 108.7 (C-8). The dihidroquinilinone part of the spectra was very similar for all the **8** derivatives, and the differences observed in spectroscopic data were mostly observed with regards to the carbamate side chains.

Table 3.2 NMR data and HMBC correlations of 2-oxo-1,2,3,4-tetrahydroquinolin-7-yl piperidine-1-carboxylate (**8a**).



Atom number	^{1}H-NMR (multiplicity, *J* in Hz)	^{13}C-NMR (Type)	HMBC (δ_H to δ_c)

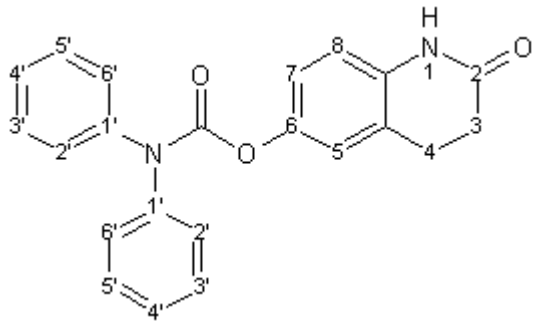
1	10.11 (s)		2, 3, 4a, 8, 8a
2		170.2 (C=0)	
3	2.44 (t, 7.5)	30.4 (CH_2)	4, 4a, 2
4	2.84 (t, 7.5)	24.2 (CH_2)	2, 3, 4a, 5, 8a
4a		120.3 (C)	
5	7.13 (d, 8.1)	128.2 (CH)	4, 7, 8a
6	6.63 (dd, 8.1, 2.4)	115.0 (CH)	4a, 8, 7
7		150.2 (C)	
8	6.57 (d, 2.3)	108.7 (CH)	4a, 7
8a		138.9 (C)	
1′			
2′	3.63 – 3.23 (m)	45.0 or 44.5 (CH_2)	
3′	1.63 – 1.46 (m)	25.5 or 25.1 (CH_2)	
4′	1.63 – 1.46 (m)	23.7 (CH_2)	
5′	1.63 – 1.46 (m)	25.5 or 25.1 (CH_2)	
6′	3.63 – 3.23 (m	45.0 or 44.5 (CH_2)	
C=O		152.8 (C=0)	

Similarly, for compound **9c**, in the ^{1}H NMR spectrum, eight signals, integrating for 18 protons were observed. The most downfield signal, for which no coupling was observed to a carbon in the HSQC spectrum, was again assigned as the NH of the dihydroquinilinone ring. The signals at 7.05, 6.96 and 6.84 ppm were assigned as the aromatic protons H-5, H-7 and H-8, respectively, primarily based on the observed coupling constants and COSY couplings. In the aliphatic region, only two triplets were observed, at 2.85 and 2.42 ppm, assigned as H-4 and H-3 respectively. HMBC couplings were observed between H-4 and C-3 (30.1 ppm), C-5 (121.1 ppm), C-4a (124.6 ppm), C-8a (135.9 ppm) and C-2 (170.0 ppm). H-3 on the other

hand, coupled to C-4 (24.6 ppm), C-4a (124.6 ppm) and C-2 (170.0 ppm), confirming this assignment. The multiplet at 7.30 - 7.24 ppm was assigned as the two H-4′ protons, while the multiplet at 7.43 – 7.37 ppm accounted for the eight remaining protons of the diphenylrings.

In the ^{13}C spectrum 14 signals were observed, accounting for the 22 carbons present in this compound. After considering the DEPT data in conjunction with the ^{13}C NMR data, the six signals at 170.0 (C-2), 152.8 (carbamate carbonyl), 145.3 (C-6), 142.2 (C-1'), 135.9 (C-8a) and 124.6 (C-4a) were identified as quaternary carbons. These assignments were based on the respective shifts observed as well as HMBC couplings. For the signal at 135.9 (C-8a), for example, HMBC couplings were observed with H-4 (2.85 ppm), H-7 (6.96 ppm), H-5 (7.05 ppm) and the NH (10.15 ppm). For the signal at 124.6 (C-4a), HMBC couplings were observed with H-3 (2.42 ppm), H-4 (2.85 ppm), H-8 (6.86 ppm), and the NH (10.15 ppm). Observed HSQC couplings between H-3, H-4, H-5, H-7, H-8 and the carbons of the diphenyl rings led to the assignments of the respective carbons at 30.1 (C-3), 24.6 (C-4), 121.1 (C-5), 120.2 (C-7) and 115.3 (C-8). The dihidroquinilinone part of the spectra was very similar for all the **9** derivatives, and the differences observed in spectroscopic data were mostly observed with regards to the carbamate side chains.

Table 3.3 NMR data and HMBC correlations of 2-oxo-1,2,3,4-tetrahydroquinolin-6-yl diphenylcarbamate (**9c**).



Atom number	^{1}H-NMR (multiplicity, *J* in Hz)	^{13}C-NMR (Type)	HMBC (δ_H to δ_c)
1	10.15 (s)		2, 3, 4a, 8a
2		170.0 (C=0)	

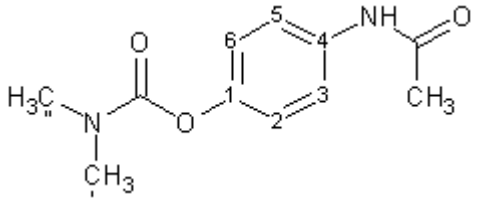
3	2.42 (t, 7.6)	30.1 (CH_2)	2, 4, 4a
4	2.85 (t, 7.6)	24.6 (CH_2)	2, 3, 4a, 5, 8a
4a		124.6 (C)	
5	7.05 (d, 2.6)	121.1 (CH)	4, 6, 7, 8a
6		145.3 (C)	
7	6.96 (dd, 8.6)	120.2 (CH)	6, 8a
8	6.84 (d, 8.6)	115.3 (CH)	5, 6, 7
8a		135.9 (C)	
1′		142.2 (C)	
2′	7.43 – 7.37 (m)	129.1 or 127.2 (CH)	1′, 2′/6′, 3′/5′
3′	7.43 – 7.37 (m)	129.1 or 127.2 (CH)	1′, 2′/6′, 3′/5′
4′	7.30 – 7.24 (m)	126.6 (CH)	2′/6′, 3′/5′
5′	7.43 – 7.37 (m)	129.1 or 127.2 (CH)	1′, 2′/6′, 3′/5′
6′	7.43 – 7.37 (m)	129.1 or 127.2 (CH)	1′, 2′/6′, 3′/5′
C=O		152.8 (C=0)	

For compound **10b**, in the ^{1}H NMR spectrum, six signals, integrating for 14 protons were observed. The most downfield signal was assigned as the NH of the acetamidophenol side chain. The signals at 7.55 and 7.02 ppm, which integrated for 2 protons each, were assigned as the aromatic protons H-3/ H-5 and H-2/H-6, respectively, primarily based on the observed shifts and coupling constants. In the aliphatic region, three signals were observed, with the singlets at 3.02 and 2.89 ppm identified as the two N$C\underline{H}_3$'s, and the singlet at 2.03 ppm assigned as the CO$C\underline{H}_3$.

In the ^{13}C spectrum 9 signals were observed, accounting for the 11 carbons present in this compound. After considering the DEPT data in conjunction with the ^{13}C NMR data, the four signals at 168.1 ($\underline{C}OCH_3$), 154.2 (carbamate carbonyl), 146.5 (C-1) and 136.4 (C-4) were identified as quaternary carbons. Assignments were based on the respective shifts observed

as well as HMBC couplings. For the signal at 122.0 (C-2/6), for example, HMBC couplings were observed with H-2/6 (7.02 ppm) while for the signal at 119.6 (C-3/5), HMBC couplings were observed with H-3/5 (7.55 ppm), and the NH (9.96 ppm). Observed HSQC couplings between H-3/5, H-2/6, the $COCH_3$ protons and the carbons of the acetamidophenol ring led to the assignments of the respective carbons at 23.9 ($COCH_3$), 119.6 (C-3/5), 122.0 (C-2/6), 136.4 (C-4) and 146.5 (C-1). The acetamidophenol part of the spectra was very similar for all the **10** derivatives, and the differences observed in spectroscopic data were mostly observed with regards to the carbamate side chains.

Table 3.4 NMR data and HMBC correlations of 4-(acetylamino)phenyldimethylcarbamate (**10b**).

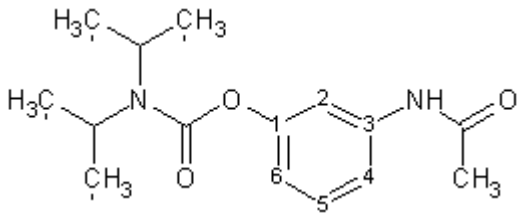


Atom number	^{1}H-NMR (multiplicity, *J* in Hz)	^{13}C-NMR (Type)	HMBC (δ_H to δ_c)
1		146.5 (C)	
2 and 6	7.02 (d, 8.9)	122.0(CH)	1, 2, 4, 6
3 and 5	7.55 (d, 8.9)	119.6(CH)	1, 3, 4, 5
4		136.4(C)	
NH	9.96 (br s)		3, 4, 5, $COCH_3$
$COCH_3$		168.1 (C)	
$COCH_3$	2.03 (s)	23.9 (CH_3)	$COCH_3$
C=O		154.2 (C)	
NCH_3'	3.02 (s)	36.0 (CH_3)	C=O, NCH_3
NCH_3''	2.89 (s)	36.3 (CH_3)	C=O, NCH_3

For compound **11h**, in the ^{1}H NMR spectrum, eight signals, integrating for 22 protons were observed. Similar to **10b**, the most downfield signal, which did not couple to a carbon in the HSQC spectrum, was assigned as the NH of the acetamidophenol side chain. The signals at 7.43, 7.34, 7.27 and 6.75 ppm were assigned as the aromatic protons H-2, H-4 or H-6, H-5 and H-4 or H-6, respectively, primarily based on the observed coupling constants and COSY couplings. In the aliphatic region, three signals were observed, with the singlet at 2.03 ppm identified as the COC$\underline{H}_3$. The signals for the remaining isopropyl CH and CH_3 groups were present at 3.97 and 1.28 – 1.21 ppm respectively.

In the ^{13}C spectrum 12 signals were observed, accounting for the 15 carbons present in this compound. After considering the DEPT data in conjunction with the ^{13}C NMR data, the four signals at 168.4 ($\underline{C}OCH_3$), 152.7 (carbamate carbonyl), 151.3 (C-1), 140.1 (C-3) were identified as quaternary carbons. These assignments were based on the respective shifts observed as well as HMBC couplings. For the signal at 140.1 (C-3), for example, HMBC couplings were observed with H-5 (7.27 ppm) and H-2 (7.43 ppm), while for the signal at 151.3 (C-1), HMBC couplings were observed with H-5 (7.27 ppm), H-2 (7.43 ppm), and H-6 (6.75 ppm). Observed HSQC couplings between H-2, H-4, H-6, H-5, the COC$\underline{H}_3$ protons and their respective carbons at, 112.4 (C-2), 115.3 (C-4), 116.3 (C-6), 129.2 (C-5) and 45.6 (isopropyl CH) ppm. The acetamidophenol part of the spectra was very similar for all the **11** derivatives, and the differences observed in spectroscopic data were mostly observed with regards to the carbamate side chains.

Table 3.5 NMR data and HMBC correlations of 3-(acetylamino)phenyl dipropan-2-ylcarbamate (**11h**).



Atom number	^{1}H-NMR (multiplicity, *J* in Hz)	^{13}C-NMR (Type)	HMBC (δ_H to δ_c)
1		151.3 (C)	
2	7.43 (t, 2.2)	112.4(CH)	1, 3, 4/6, 5

3		140.1(C)	
4	7.34 (ddd, 8.2, 2.0, 1.0)	115.3(CH)	6
6	6.75 (ddd, 8.0, 2.4, 1.1)	116.3 (CH)	1, 4
5	7.27 (t, 8.1)	129.2 (CH)	1, 3
NH	10.03 (s)		4, 6, $\underline{C}OCH_3$
$\underline{C}OCH_3$		168.4 (C)	
$COCH_{\underline{3}}$	2.03 (s)	21.3 (CH_3)	$\underline{C}OCH_3$
C=O		152.7 (C)	
isopropyl CH	3.97 (brs)	45.6(CH)	
isopropyl CH_3	1.28 – 1.21 (m)	20.3, 20.1(CH_3)	

The quinolinone-carbamate and acetoamidophenol-carbamate derivatives that were synthesised in this study were also characterised by mass spectrometry. Generally, the obtained mass data correlated well with the theoretical masses calculated, and the differences between these values were generally less than 5 ppm. The calculated and experimentally determined high resolution masses are noted in table 3.6 and 3.7.

Table 3.6 The experimentally determined and calculated high resolution masses of the various synthesised quinolinone-carbamatederivates (**8a**-**g**, **9a**-**d**).

Compound	Calculated mass	Experimental mass	Formula [M+H]	Difference between calculated and experimental masses
8a	275.1390	275.1413	$C_{15}H_{19}N_2O_3$	-8.1
8b	235.1077	235.1077	$C_{12}H_{15}N_2O_3$	0.2
8c	277.1183	277.1174	$C_{14}H_{17}N_2O_4$	3.1
8d	263.1390	263.1389	$C_{14}H_{19}N_2O_3$	0.4

8e	290.1499	290.1496	$C_{15}H_{20}N_3O_3$	1.2
8f	359.1390	359.1410	$C_{22}H_{19}N_2O_3$	-5.5
8g	297.1234	297.1222	$C_{17}H_{17}N_2O_3$	4.3
9a	275.1390	275.1396	$C_{15}H_{19}N_2O_3$	-2.0
9b	277.1183	277.1185	$C_{14}H_{17}N_2O_4$	-0.7
9c	359.1390	359.1377	$C_{22}H_{19}N_2O_3$	3.8
9d	297.1234	297.1233	$C_{17}H_{17}N_2O_3$	0.2

Table 3.7 The experimentally determined and calculated high resolution masses of the various synthesised acetomidophenol-carbamatederivates (**10a**-**h**, **11a**-**i**).

Compound	Calculated mass	Experimental mass	Formula [M+H]	Difference between calculated and experimental masses
10a	263.1390	263.1391	$C_{14}H_{19}N_2O_3$	-0.5
10b	223.1077	223.1078	$C_{11}H_{15}N_2O_3$	-0.4
10c	265.1183	265.1186	$C_{13}H_{17}N_2O_4$	-1.2
10d	278.1499	278.1487	$C_{14}H_{20}N_3O_3$	4.3
10e	347.1390	347.1387	$C_{21}H_{19}N_2O_3$	1.0
10f	285.1234	285.1225	$C_{16}H_{17}N_2O_3$	3.0
10g	279.1703	279.1713	$C_{15}H_{23}N_2O_3$	-3.4
10h	249.1234	249.1228	$C_{13}H_{17}N_2O_3$	2.4
11a	263.1390	263.1384	$C_{14}H_{19}N_2O_3$	2.2
11b	223.1077	223.1066	$C_{11}H_{15}N_2O_3$	5.2
11c	265.1183	265.1185	$C_{13}H_{17}N_2O_4$	-0.7

11d	521.1390	251.1401	$C_{13}H_{19}N_2O_3$	-4.1
11e	278.1499	278.1499	$C_{14}H_{20}N_3O_3$	0.0
11f	347.1390	347.1382	$C_{21}H_{19}N_2O_3$	2.5
11g	285.1234	285.1224	$C_{16}H_{17}N_2O_3$	3.4
11h	279.1703	279.1706	$C_{15}H_{23}N_2O_3$	-1.0
11i	249.1234	249.1229	$C_{13}H_{17}N_2O_3$	1.8

HPLC analyses were carried out on the synthesised compounds to calculate their approximate purities. The conditions were selected based on the expected chromatographic properties of these compounds. The chromatograms obtained during the analyses are given in the appendix. For most compounds a sharp, single peak was observed, indicating a high degree of purity. The purities of the derivatives were calculated based on the integrated areas of the analyte and impurity peaks (if present) as shown by the equation below.

$$\%\ purity = \frac{integrated\ area\ of\ the\ analyte}{(integrated\ area\ of\ the\ analyte + integrated\ area\ of\ the\ impurity)}$$

For the purpose of this study, the chromatograms that were used were recorded 210, 254 and 300 nm. The purities of all compounds were above 80%, except for compound **8a**, which had a purity of 72%. It was decided to continue with biological screening and to resynthesise this compound if it showed promising activity to eliminate the contribution of impurities to activity.

Table 3.8 HPLC analysis results of the quinolinone-carbamatederivates (**8a**-**g**, **9a**-**d**).

Compound	Retention time (min)	Area (mAUxs)	Height (mAU)	Purity (%)
8a	5.357	1.98926e4	2440.09644	72.6
8b	3.930	2.27990e4	2447.30762	99.5
8c	3.709	1.84406e4	2442.27686	97.4
8d	5.135	1.62465e4	2432.03271	83.4

8e	1.322	4.66057e4	2366.56372	83.6
8f	6.605	2.25559e4	2436.00928	99.5
8g	5.517	2.25441e4	2440.97705	97.7
9a	5.208	1.98619e4	2446.73682	98.5
9b	3.469	1.90350e4	2449.76147	80.6
9c	6.490	1.38851e4	2418.61401	99.4
9d	5.452	1.04174e4	2189.31763	97.6

Table 3.9 HPLC analysis results of the acetomidophenol-carbamatederivates (**10a**-**h**, **11a**-**i**).

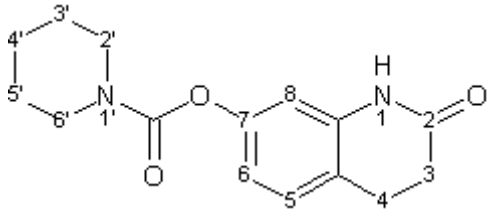
Compound	**Retention time (min)**	**Area (mAUxs)**	**Height (mAU)**	**Purity (%)**
10a	5.162	1.79402e4	2438.52100	96.6
10b	3.676	1.92927e4	2451.89038	99.3
10c	3.429	1.74671e4	2429.82471	99.6
10d	1.281	4.27598e4	2375.29883	87.6
10e	6.509	2.11784e4	2434.93799	97.4
10f	5.379	1.93828e4	2439.63208	99.7
10g	6.082	1.65390e4	2439.04077	96.0
10h	4.330	1.57546e4	2445.40381	99.6
11a	5.360	2.29883e4	2435.64600	97.4
11b	3.895	1.15148e4	2440.67798	83.5
11c	3.673	1.94825e4	2438.66235	99.3
11d	5.133	1.79718e4	2429.84961	97.4

11e	1.303	3.03190e4	1830.78015	85.1
11f	6.642	1.90475e4	2410.22314	98.1
11g	5.559	2.10161e4	2435.46191	99.3
11h	6.251	2.06981e4	2418.32349	99.1
11i	4.536	1.85016e4	2438.31616	98.9

3.3 PHYSICAL DATA

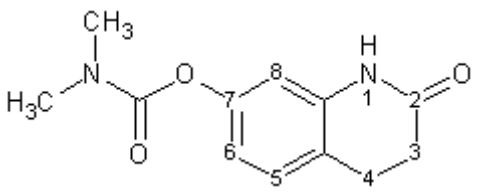
Dihydroquinolinone-carbamate derivatives

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl piperidine-1-carboxylate (**8a**)



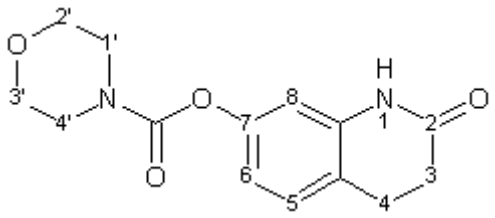
The title compound was prepared from 7-hydroxy-3,4-dihydro-2(1H)-quinolinone and 1-piperidinecarbonyl chloride in a yield of 73%: mp. 149.6-151.3 °C (ethanol), white powder.^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.11 (s, 1H, NH), 7.13 (d, *J* = 8.1 Hz, 1H, H-5), 6.63 (dd, *J* = 8.1, 2.4 Hz, 1H, H-6), 6.57 (d, *J* = 2.3 Hz, 1H, H-8), 3.63 – 3.23 (m, 4H, N-C$\underline{H}_2$), 2.84 (t, *J* = 7.5 Hz, 2H, H-4), 2.44 (t, *J* = 7.5 Hz , 2H, H-3), 1.63 – 1.46 (m, 6H, remaining CH_2's of piperidine ring).^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.2 (C-2), 152.8 (C=O, carbamate), 150.2 (C-7), 138.9 (C-8a), 128.2 (C-5), 120.3 (C-4a), 115.0 (C-6), 108.7 (C-8), 45.0, 44.5 (N-$\underline{C}H_2$), 30.4 (C-3), 25.5, 25.1 (CH_2's of piperidine ring), 24.2 (C-4), 23.7 (CH_2 of piperidine ring). IR ν_{max} (cm^{-1}) 2944, 1691, 1654, 1155, 747. APCI-HRMS m/z: calculated for $C_{15}H_{19}N_2O_3$, 275.1390, *[M+H]*$^{+}$ found275.1413. Purity (HPLC): 73%.

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl dimethylcarbamate (**8b**)



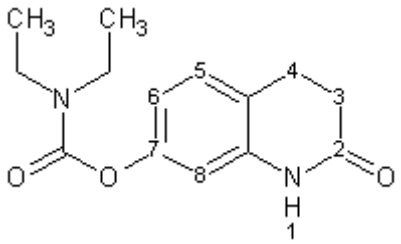
The title compound was prepared from 7-hydroxy-3,4-dihydro-2(1H)-quinolinone and dimethylcarbamoyl chloride in a yield of 5%: mp. 216.1-218.3 °C (ethanol), white powder.[1]H NMR (600 MHz, DMSO-*d6*) δ 10.13 (s, 1H, NH), 7.13 (d, *J* = 8.1 Hz, 1H, H-5), 6.63 (dd, *J* = 8.1, 2.4 Hz, 1H, H-6), 6.58 (d, *J* = 2.3 Hz, 1H, H-8), 3.01 (s, 3H, N-C$\underline{H}_3$), 2.89 (s, 3H, N-C$\underline{H}_3$), 2.84 (t, *J* = 7.6 Hz, 2H, H-4), 2.45 (t, *J* = 7.6 Hz, 2H, H-3). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.2 (C-2), 153.9 (C=O, carbamate), 150.2 (C-7), 138.9 (C-8a), 128.1 (C-5), 120.3 (C-4a), 115.0 (C-6), 108.7 (C-8), 36.3, 36.1 (N-$\underline{C}H_3$), 30.4 (C-3), 24.2 (C-4). IR ν_{max} (cm^{-1}) 2978, 1726, 1677, 1167, 753. APCI-HRMS m/z: calculated for $C_{12}H_{15}N_2O_3$, 235.1077, *[M+H]*$^+$ found 235.1077. Purity (HPLC): 99%.

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**8c**)



The title compound was prepared from 7-hydroxy-3,4-dihydro-2(1H)-quinolinone and 4-morpholinecarbonyl chloride in a yield of 18%: mp. 190.3-191.5 °C (ethanol), white powder.[1]H NMR (600 MHz, DMSO-*d6*) δ 10.14 (s, 1H, NH), 7.15 (d, *J* = 8.1 Hz, 1H, H-5), 6.66 (dd, *J* = 8.1, 2.4 Hz, 1H, H-6), 6.60 (d, *J* = 2.3 Hz, 1H, H-8), 3.70 – 3.58 (m, 4H, O-C$\underline{H}_2$), 3.55 (br s, 2H, N-C$\underline{H}_2$), 3.40 (s, 2H, N-C$\underline{H}_2$), 2.85 (t, *J* = 7.5 Hz, 2H, H-4), 2.45 (t, *J* = 7.6 Hz, 2H, H-3). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.2 (C-2), 152.9 (C=O, carbamate), 150.0 (C-7), 139.0 (C-8a), 128.2 (C-5), 120.5 (C-4a), 115.0 (C-6), 108.6 (C-8), 65.8 (2 x O-$\underline{C}H_2$), 44.5, 43.7 (N-$\underline{C}H_2$), 30.3 (C-3), 24.2 (C-4). IR ν_{max} (cm^{-1}) 2992, 1718, 1680, 1170, 750. APCI-HRMS m/z: calculated for $C_{14}H_{17}N_2O_4$, 277.1183, *[M+H]*$^+$ found 277.1174. Purity (HPLC): 97%.

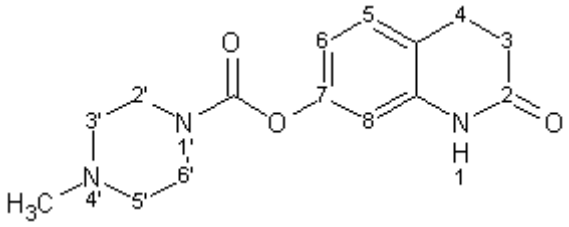
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diethylcarbamate (**8d**)



The title compound was prepared from 7-hydroxy-3,4-dihydro-2(1H)-quinolinone and diethylcarbamoyl chloride in a yield of 7%: mp.135.6-138.7 °C (ethanol), white powder.[1]H

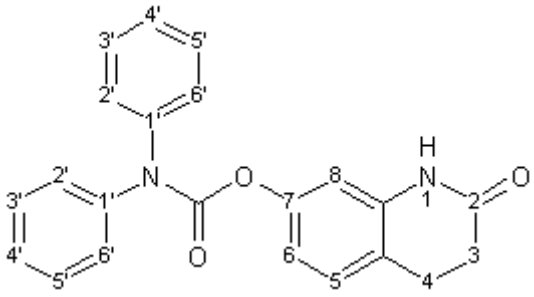
NMR (600 MHz, DMSO-*d6*) δ 10.09 (s, 1H, NH), 7.14 (d, *J* = 8.1 Hz, 1H, H-5), 6.64 (dd, *J* = 8.1, 2.4 Hz, 1H, H-6), 6.59 (d, *J* = 2.3 Hz, 1H, H-8), 3.43 – 3.18 (m, 4H, N-C$\underline{H}_2$), 2.84 (t, *J* = 7.6 Hz, 2H, H-4), 2.44 (t, *J* = 7.4 Hz, 2H, H-3),1.23 – 1.02 (m, 6H, $CH_2C\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.2 (C-2), 153.2 (C=O, carbamate), 150.2 (C-7), 138.9 (C-8a), 128.2 (C-5), 120.3 (C-4a), 115.0 (C-6), 108.7 (C-8), 41.7, 41.5 (N-$\underline{C}H_2$), 30.4 (C-3), 24.2 (C-4), 14.2, 13.3 ($CH_2\underline{C}H_3$). IR ν_{max} (cm^{-1}) 2975, 1709, 1675, 1160, 754. APCI-HRMS m/z: calculated for $C_{14}H_{19}N_2O_3$, 263.1390, *[M+H]*$^+$ found 263.1389. Purity (HPLC): 83%.

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl 4-methylpiperazine-1-carboxylate (**8e**)



The title compound was prepared from 7-hydroxy-3,4-dihydro-2(1H)-quinolinone and 4-methyl-1-piperazinecarbonyl chloride hydrochloride in a yield of 6%: mp. 172.6 -174.9 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.12 (s, 1H, NH), 7.14 (d, *J* = 8.1 Hz, 1H, H-5), 6.64 (dd, *J* = 8.1, 2.4 Hz, 1H, H-6), 6.58 (d, *J* = 2.4 Hz, 1H, H-8), 3.54 (br s, 2H, CONC$\underline{H}_2$), 3.40 (br s, 2H, CONC$\underline{H}_2$), 2.85 (t, *J* = 7.5 Hz, 2H, H-4), 2.45 (t, *J* = 7.5 Hz,2H, H-3), 2.33 (br s, 4H, C$\underline{H}_2$NCH$_3$), 2.21 (s, 3H, N-C$\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.2 (C-2), 152.8 (C=O, carbamate), 150.1 (C-7), 138.9 (C-8a), 128.2 (C-5), 120.4 (C-4a), 115.0 (C-6), 108.6 (C-8), 54.3, 54.1 ($\underline{C}H_2$NCH$_3$), 45.7 (N-$\underline{C}H_3$), 44.0, 43.5 (CON$\underline{C}H_2$), 30.3 (C-3), 24.2 (C-4). IR ν_{max} (cm^{-1}) 2992, 1720, 1670, 1153, 746. APCI-HRMS m/z: calculated for $C_{15}H_{20}N_3O_3$, 290.1499, *[M+H]*$^+$ found 290.1496. Purity (HPLC): 84%.

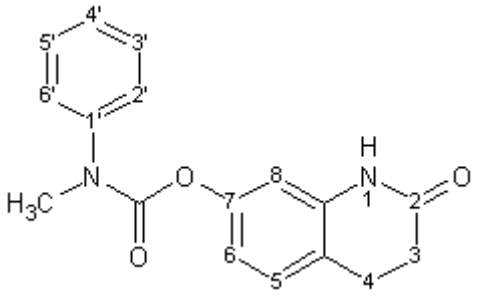
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diphenylcarbamate (**8f**)



The title compound was prepared from 7-hydroxy-3,4-dihydro-2(1H)-quinolinone and diphenylcarbamoyl chloride in a yield of 53%: mp. 152.4-154.3 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.13 (s, 1H, NH), 7.45 – 7.38 (m, 8H, Ph-H, H-2',H-3',H-

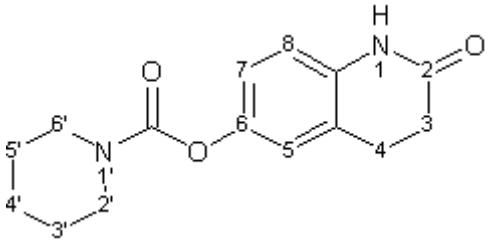
5',H-6'), 7.32 – 7.25 (m, 2H, Ph-H, H-4'), 7.16 (d, *J* = 8.2 Hz, 1H, H-5), 6.74 (dd, *J* = 8.2, 2.4 Hz, 1H, H-6), 6.64 (d, *J* = 2.3 Hz, 1H, H-8), 2.84 (t, *J* = 7.5 Hz, 2H, H-4), 2.46 – 2.41 (m, 2H, H-3).^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.2 (C-2), 152.4 (C=O, carbamate), 149.7 (C-7), 142.2 (2C, C-1'), 139.0 (C-8a), 129.2 (4C, C-2'/6' or C-3'/5'), 128.3 (C-5), 127.2 (4C, C-2'/6' or C-3'/5') 126.8 (2C, C-4'), 120.9 (C-4a), 114.9 (C-6), 108.4 (C-8), 30.3 (C-3), 24.2 (C-4). IR ν_{max} (cm^{-1}) 2977, 1729, 1675, 1168, 755. APCI-HRMS m/z: calculated for $C_{22}H_{19}N_2O_3$, 359.1390, *[M+H]*$^+$ found 359.1410. Purity (HPLC): 99%.

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl methyl(phenyl)carbamate (**8g**)



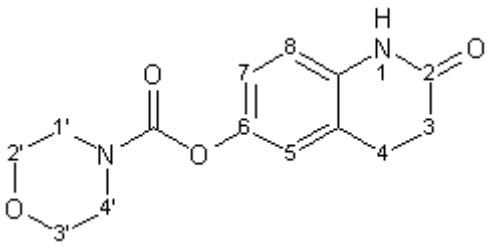
The title compound was prepared from 7-hydroxy-3,4-dihydro-2(1H)-quinolinone and N-methyl-N-phenylcarbamoyl chloride in a yield of 42%: mp. 140.0-142.9 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.13 (s, 1H NH), 7.46 – 7.39 (m, 4H, H-2', H-6', H-3', H-5'), 7.31 – 7.24 (m, 1H, H-4'), 7.14 (d, *J* = 8.1 Hz, 1H, H-5), 6.68 (br d, *J* = 8.1, 2.3 Hz, 1H, H-6), 6.60 (br s, 1H, H-8), 3.32 (s, 3H, CH_3), 2.84 (t, *J* = 7.5 Hz, 2H, H-4), 2.44 (t, *J* = 7.5 Hz, 2H, H-3). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.2 (C-2), 153.0 (C=O, carbamate), 150.0 (C-7),142.8 (C-1′), 139.0 (C-8a), 129.0 (2C, C-2′/6′ or C-3′/5′), 128.2 (C-5), 126.4 (C-4'), 125.9 (2C, C-2′/6′ or C-3′/5′), 120.6 (C-4a), 114.9 (C-6), 108.5 (C-8), 37.9 (CH_3), 30.3 (C-3), 24.2 (C-4). IR ν_{max} (cm^{-1}) 2970, 1716, 1677, 1154, 750. APCI-HRMS m/z: calculated for $C_{17}H_{17}N_2O_3$, 297.1234, *[M+H]*$^+$ found 297.1222. Purity (HPLC): 98%.

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl piperidine-1-carboxylate (**9a**)



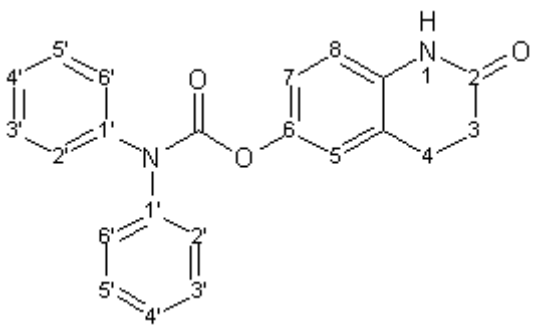
The title compound was prepared from 6-hydroxy-3,4-dihydro-2(1H)-quinolinone and 1-piperidinecarbonyl chloride in a yield of 11%: mp. 177.3 - 179. °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.09 (s, 1H, NH), 6.93 (d, *J* = 2.5 Hz,1H, H-5), 6.86 (dd, *J* = 8.5, 2.6 Hz, 1H, H-7), 6.81 (d, *J* = 8.5 Hz, 1H, H-8), 3.61 – 3.36 (m, 4H, N-C$\underline{H}_2$), 2.84 (t, *J* = 7.6 Hz, 2H, H-4), 2.42 (t, *J* = 7.6 Hz, 2H, H-3), 1.72 – 1.35 (m, 6H, remaining CH_2's of piperidine ring). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.0 (C-2), 153.1 (C=O, carbamate), 145.8 (C-6), 135.4 (C-8a), 124.4 (C-4a), 121.3 (C-5), 120.3 (C-7), 115.2 (C-8), 45.0,44.5 (N-$\underline{C}H_2$), 30.1 (C-3), 25.5, 25.1 (CH_2's of piperidine ring), 24.7 (C-4), 23.7 (CH_2 of piperidine ring). IR ν_{max} (cm^{-1}) 2923, 1702, 1669, 1152, 753. APCI-HRMS m/z: calculated for $C_{15}H_{19}N_2O_3$, 275.1390, *[M+H]*$^{+}$ found 275.1396. Purity (HPLC): 99%.

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**9b**)



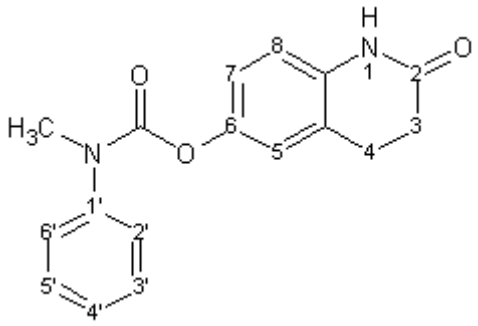
The title compound was prepared from 6-hydroxy-3,4-dihydro-2(1H)-quinolinone and 4-morpholinecarbonyl chloride in a yield of 6%: mp. 219.4-220.9 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.10 (s, 1H, NH), 6.96 (d, *J* = 2.6 Hz, 1H, H-5), 6.89 (dd, *J* = 8.5, 2.6 Hz, 1H, H-7), 6.82 (d, *J* = 8.5 Hz, 1H, H-8), 3.63 (t, *J* = 4.8 Hz, 4H, O-C$\underline{H}_2$), 3.58 – 3.38 (m, 4H, N-C$\underline{H}_2$), 2.85 (t, *J* = 7.6 Hz, 2H, H-4), 2.46 – 2.40 (m, 2H, H-3). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.0 (C-2), 153.3 (C=O, carbamate), 145.6(C-6), 135.6 (C-8a), 124.5 (C-4a), 121.3 (C-5), 120.3 (C-7), 115.3 (C-8), 65.8 (2 x O-$\underline{C}H_2$), 44.5, 43.8 (N-$\underline{C}H_2$), 30.1 (C-3), 24.7 (C-4). IR ν_{max} (cm^{-1}) 2907, 1715, 1672, 1156, 751. APCI-HRMS m/z: calculated for $C_{14}H_{17}N_2O_4$, 277.1183, *[M+H]*$^{+}$ found 277.1185. Purity (HPLC): 80.6%.

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl diphenylcarbamate (**9c**)



The title compound was prepared from 6-hydroxy-3,4-dihydro-2(1H)-quinolinone and diphenylcarbamoyl chloride in a yield of 52%: mp. It is higher than 410 °C and couldn't be measured with the available equipment (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.15 (s, 1H, NH), 7.43 – 7.37 (m, 8H, H-2′, H-6′, H-3′, H-5′), 7.30 – 7.24 (m, 2H, H-4′), 7.05 (d, *J* = 2.6 Hz, 1H, H-5), 6.96 (dd, *J* = 8.6, 2.6 Hz, 1H, H-7), 6.84 (d, *J* = 8.6 Hz, 1H, H-8), 2.85 (t, *J* = 7.6 Hz, 2H, H-4), 2.42 (t, *J* = 7.6 Hz, 2H, H-3). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.0 (C-2), 152.8 (C=O, carbamate), 145.3 (C-6), 142.2 (C-1′), 135.9 (C-8a), 129.1 (4C, C-2′/6′ or C-3′/5′), 127.2 (4C, C-2′/6′ or C-3′/5′), 126.6 (2C, C-4′), 124.6 (C-4a), 121.1 (C-5), 120.2 (C-7), 115.3 (C-8), 30.1 (C-3), 24.6 (C-4). IR ν_{max} (cm^{-1}) 2977, 1716, 1669, 1143, 760. APCI-HRMS m/z: calculated for $C_{22}H_{19}N_2O_3$, 359.1390, *[M+H]*$^{+}$ found 359.1377. Purity (HPLC): 99%.

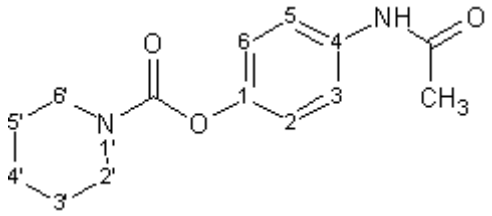
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl methyl(phenyl)carbamate (**9d**)



The title compound was prepared from 6-hydroxy-3,4-dihydro-2(1H)-quinolinone and N-methyl-N-phenylcarbamoyl chloride in a yield of 43%: mp. 168.4-170.1 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.11 (s, 1H, NH), 7.46 – 7.38 (m, 4H, H-2′/6′, H-3′/H-5′), 7.27 (t, *J* = 7.0 Hz, 1H, H-4′), 6.98 (br s, 1H, H-5), 6.92 – 6.88 (m, 1H, H-7), 6.81 (d, *J* = 8.5 Hz, 1H, H-8), 3.32 (s, 3H, C$\underline{H}_3$), 2.84 (t, *J* = 7.6 Hz, 2H, H-4), 2.42 (t, *J* = 7.6 Hz, 2H, H-3). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 170.0 (C-2), 153.4 (C=O, carbamate), 145.6 (C-6), 142.8 (C-1′), 135.7 (C-8a), 129.0 (2C, C-2′/6′ or C-3′/5′), 126.4 (C-4′), 125.9 (2C, C-2′/6′ or C-3′/5′), 124.5 (C-4a), 121.2 (C-5), 120.3 (C-7), 115.3 (C-8), 37.9 ($\underline{C}H_3$), 30.1 (C-3), 24.7 (C-4). IR ν_{max} (cm^{-1}) 2973, 1702, 1647, 1156, 751. APCI-HRMS m/z: calculated for $C_{17}H_{17}N_2O_3$, 297.1234, *[M+H]*$^{+}$ found 297.1233. Purity (HPLC): 98%.

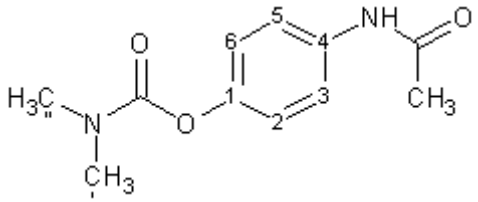
Acetoamidophenol-carbamatederivates

4-(acetylamino)phenyl piperidine-1-carboxylate (**10a**)



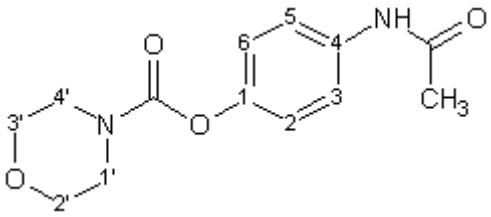
The title compound was prepared from 4-acetamidophenol and 1-piperidinecarbonyl chloride in a yield of12%: mp. 177.8 - 180.0 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.96 (br s, 1H, NH), 7.57 – 7.52 (m, 2H, H-3/5), 7.04 – 6.99 (m, 2H, H-2/6), 3.45 (m, *J* = 87.2 Hz, 4H, N-C$\underline{H}_2$), 2.03 (d, *J* = 1.9 Hz, 3H, CH_3), 1.62 – 1.49 (m, 6H, remaining CH_2's of piperidine ring). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.1 ($\underline{C}OCH_3$), 153.0 (C=O, carbamate), 146.5 (C-1), 136.4 (C-4), 122.0 (2C, C-2/6), 119.6 (2C, C3/5), 45.0, 44.5 (N-$\underline{C}H_2$), 25.5, 25.1(CH_2's of piperidine ring), 23.9 (CH_3), 23.7 (CH_2 of piperidine ring). IR ν_{max} (cm^{-1}) 3249, 1708, 1660, 1197, 748. APCI-HRMS m/z: calculated for $C_{14}H_{19}N_2O_3$, 263.1390, *[M+H]*$^{+}$ found 263.1391. Purity (HPLC): 97%.

4-(acetylamino)phenyldimethylcarbamate (**10b**)



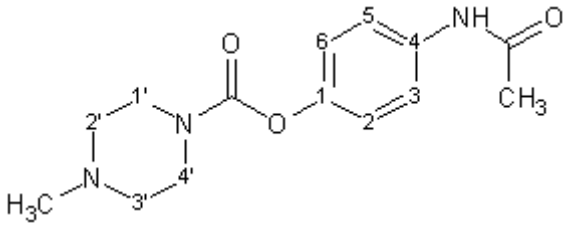
The title compound was prepared from 4-acetamidophenol and dimethylcarbamoyl chloride in a yield of 30%: mp. 143.9-144.7 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.96 (br s, 1H, NH), 7.55 (d, *J* = 8.9 Hz, 2H, H-3/5), 7.02 (d, *J* = 8.9 Hz, 2H, H-2/6), 3.02 (s, 3H, N$\underline{C}H_3$'), 2.89 (s, 3H, N$\underline{C}H_3$''), 2.03 (s, 3H, CO$\underline{C}H_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.1 ($\underline{C}OCH_3$), 154.2 (C=O, carbamate), 146.5 (C-1), 136.4 (C-5), 122.0 (2C, C-2/6), 119.6 (2C, C-3/5), 36.3 (N$\underline{C}H_3$''), 36.0 (N$\underline{C}H_3$'), 23.9 (CO$\underline{C}H_3$). IR ν_{max} (cm^{-1}) 3321, 1706, 1683, 1178, 753. APCI-HRMS m/z: calculated for $C_{11}H_{15}N_2O_3$, 223.1077, *[M+H]*$^{+}$ found 223.1078. Purity (HPLC): 99%.

4-(acetylamino)phenyl morpholine-4-carboxylate (**10c**)



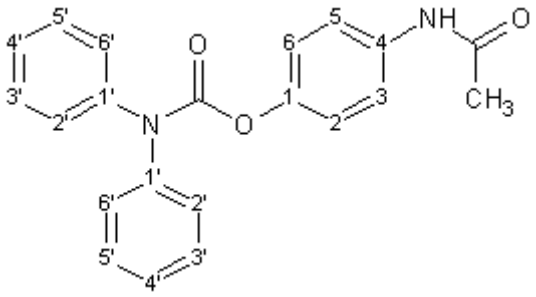
The title compound was prepared from 4-acetamidophenol and 4-morpholinecarbonyl chloride in a yield of 61%: mp. 182.8-184.0 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.98 (s, 1H, NH), 7.56 (d, *J* = 8.9 Hz, 2H, H-2/6 or H-3/5), 7.05 (d, *J* = 8.9 Hz, 2H, H-2/6), 3.66 – 3.61 (m, 4H, O-C$\underline{H}_2$), 3.56 (br s, 2H, N-C$\underline{H}_2$), 3.40 (br s, 2H, N-C$\underline{H}_2$), 2.03 (s, 3H, C$\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.2 (C$\underline{O}$CH$_3$), 153.2 (carbamate, C=O), 146.3 (C-1), 136.5 (C-4), 122.0 (2C, C-2/6), 119.6 (2C, C-3/5), 65.8 (2 x O-C$\underline{H}_2$), 44.5, 43.8 (N-C$\underline{H}_2$), 23.9 (C$\underline{H}_3$). IR ν_{max} (cm^{-1}) 3293, 1707, 1661, 1191, 762. APCI-HRMS m/z: calculated for $C_{13}H_{17}N_2O_4$, 265.1183, *[M+H]*$^+$ found 265.1186. Purity (HPLC): 100%.

4-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**10d**)



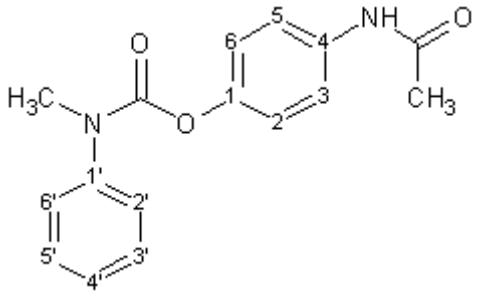
The title compound was prepared from 4-acetamidophenol and 4-methyl-1-piperazinecarbonyl chloride hydrochloride in a yield of 10%: mp. 199.7-201.7 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.97 (s, 1H, NH), 7.55 (d, *J* = 8.9 Hz, 2H, H-2/6 or H-3/5), 7.03 (d, *J* = 8.9 Hz, 2H, H-2/6), 3.55 (br s, 2H, CONC$\underline{H}_2$), 3.38 (br s, 2H, CONC$\underline{H}_2$), 2.33 (brs, 4H, CH$_2$NCH$_3$), 2.20 (s, 3H, NC$\underline{H}_3$), 2.03 (s, 3H, COC$\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.2 (C$\underline{O}$CH$_3$), 153.1 (carbamate, C=O), 146.3 (C-1), 136.5 (C-4), 122.0 (2C, C-2/6 or C-3/5), 119.7 (2C, C-2/6 or C-3/5), 54.3, 54.1 (C$\underline{H}_2$NCH$_3$), 45.7 (C$\underline{H}_3$-N), 44.0, 43.5 (CONC$\underline{H}_2$), 23.9 (CO$\underline{C}$H$_3$). IR ν_{max} (cm^{-1}) 3245, 1710, 1647, 1194, 752. APCI-HRMS m/z: calculated for $C_{14}H_{20}N_3O_3$, 278.1499, *[M+H]*$^+$ found 278.1487. Purity (HPLC): 86%.

4-(acetylamino)phenyldiphenylcarbamate (**10e**)



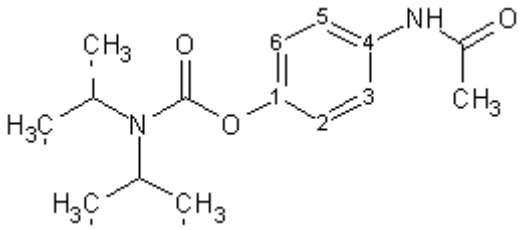
The title compound was prepared from 4-acetamidophenol and diphenylcarbamoyl chloride in a yield of 37%: mp. 211.6-213.4 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.99 (s, 1H, NH), 7.58 (d, *J* = 8.9 Hz, 2H, H2/6 or H-3/5), 7.46 – 7.38 (m, 8H, H-2',H-6', H-3', H-5'), 7.28 (t, *J* = 6.7 Hz, 2H, H-4'), 7.12 (d, *J* = 8.9 Hz,2H, H-2/6 or H-3/5), 2.03 (s, 3H, COC$\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.2 ($\underline{C}OCH_3$), 152.7 (C=O, carbamate), 146.0 (C-1), 142.2 (C-1'), 136.8 (C-4), 129.1 (4C, C-2'/6' or C-3'/5'), 127.2 (4C, C-2'/6' or C-3'/5'), 126.7 (2C, C-4'), 121.9 (2C, C-2/6 or C-3/5), 119.6 (2C, C-2/6 or C-3/5), 23.9 (CO$\underline{C}H_3$). IR ν_{max} (cm^{-1}) 3302, 1729, 1664, 1191, 749. APCI-HRMS m/z: calculated for $C_{21}H_{19}N_2O_3$, 347.1390, *[M+H]*$^{+}$ found 347.1387. Purity (HPLC): 97%.

4-(acetylamino)phenyl methyl(phenyl)carbamate (**10f**)



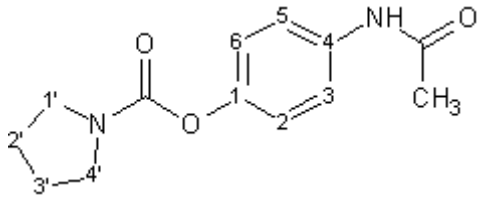
The title compound was prepared from 4-acetamidophenol and N-methyl-N-phenylcarbamoyl chloride in a yield of 41%: mp. 177.1-179.3 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.98 (s, 1H, NH), 7.56 (d, *J* = 8.7 Hz, 2H, H2/6 or H-3/5), 7.47 – 7.38 (m, 4H, H-2', H6', H-3′, 5′), 7.27 (t, *J* = 7.2 Hz, 1H, H-4'), 7.06 (d, *J* = 8.4 Hz, 2H, H-2/6 or H-3/5), 3.33 (s, 3H, N-C$\underline{H}_3$), 2.03 (s, 3H, COC$\underline{H}_3$).^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.2 ($COCH_3$), 153.3 (carbamate, C=O), 146.2 (C-1), 142.8 (C-1'), 136.6 (C-4), 128.9 (2C, C-2'/6' or C-3'/5'), 126.4 (C-4'), 125.9 (2C, C-2'/6' or C-3'/5'), 122.0 (2C, C-2/6 or C-3/5), 119.6 (2C, C-2/6 or C-3/5), 37.9 (N-CH_3), 23.9 (CO$\underline{C}H_3$). IR ν_{max} (cm^{-1}) 3307, 1689, 1676, 1197, 756. APCI-HRMS m/z: calculated for $C_{16}H_{17}N_2O_3$, 285.1234, *[M+H]*$^{+}$ found 285.1225. Purity (HPLC): 100%.

4-(acetylamino)phenyl dipropan-2-ylcarbamate (**10g**)



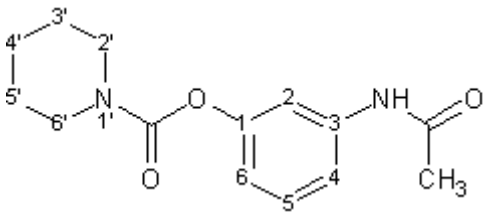
The title compound was prepared from 4-acetamidophenol and N,N-diisopropylcarbamoyl chloride in a yield of 6%: mp. 153.8-155.7 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.96 (s, 1H, NH), 7.55 (d, *J* = 8.9 Hz, 2H, H-3/5), 7.00 (d, *J* = 8.9 Hz, 2H, H-2/6), 3.97 (br s, 2H, isopropyl C<u>H</u>), 2.03 (s, 3H, COC<u>H$_3$</u>), 1.23 (d, *J* = 16.9 Hz, 12H, isopropyl CH$_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.1 (<u>C</u>OCH$_3$), 153.0 (carbamate, C=O), 146.3 (C-1), 136.2 (C-4), 121.9 (C-2/6), 119.7 (C-3/5), 45.6 (isopropyl CH), 23.9 (CO<u>C</u>H$_3$), 21.3, 20.2 (isopropyl CH$_3$'s). IR v_{max} (cm^{-1}) 3246, 1704, 1662, 1195, 756. APCI-HRMS m/z: calculated for $C_{15}H_{21}N_2O_3$, 279.1703, *[M+H]*$^+$ found 279.1713. Purity (HPLC): 96%.

4-(acetylamino)phenyl pyrrolidine-1-carboxylate (**10h**)



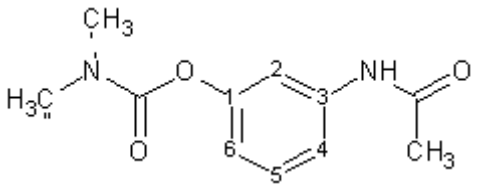
The title compound was prepared from 4-acetamidophenol and 1-pyrrolidinecarbonyl chloride in a yield of 24%: mp. 176.7-177.8 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.97 (s, 1H, NH), 7.55 (d, *J* = 8.9 Hz, 2H, H-3/5), 7.03 (d, *J* = 8.9 Hz, 2H, H2/6), 3.47 (t, *J* = 6.7 Hz, 2H, NC<u>H$_2$</u>), 3.31 (t, *J* = 6.7 Hz, 2H, NC<u>H$_2$</u>), 2.03 (s, 3H, COC<u>H$_3$</u>), 1.93 – 1.80 (m, 4H, remaining CH$_2$'s of pyrrolidine ring). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.1 (<u>C</u>OCH$_3$), 152.4 (C=O, carbamate), 146.4 (C-1), 136.3 (C-4), 122.0 (C-2/6), 119.6 (C-3/5), 46.2, 46.0 (N<u>C</u>H$_2$), 25.3, 24.5 (remaining CH$_2$'s of pyrrolidine ring), 23.9 (CO<u>C</u>H$_3$). IR v_{max} (cm^{-1}) 3206, 1702, 1684, 1190, 754. APCI-HRMS m/z: calculated for $C_{13}H_{17}N_2O_3$, 249.1234, *[M+H]*$^+$ found 249.1228. Purity (HPLC): 100%.

3-(acetylamino)phenyl piperidine-1-carboxylate (**11a**)



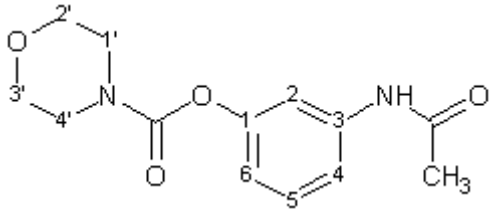
The title compound was prepared from 3-acetamidophenol and 1-piperidinecarbonyl chloride in a yield of 61%: mp. 153.4-154.7 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.04 (s, 1H, NH), 7.48 (t, *J* = 2.1 Hz, 1H, H-2), 7.32 – 7.24 (m, 2H, H-4and H-5), 6.76 (ddd, *J* = 7.7, 2.4, 1.3 Hz, 1H, H-6), 3.58 – 3.36 (m, 4H, NCH_2), 2.04 (s, 3H, $COC\underline{H}_3$), 1.62 – 1.49 (m, 6H, remaining CH_2's of piperidine ring). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.4 ($\underline{C}OCH_3$), 152.7 (C=O, carbamate), 151.4 (C-1), 140.1 (C-3), 129.1 (C-5), 116.3 (C-6), 115.4 (C-4), 112.4 (C-2), 45.0, 44.5 ($N\underline{C}H_2$), 25.5, 25.1 (CH_2's of piperidine ring), 24.0 (CH_3), 23.7 (CH_2 of piperidine ring). IR ν_{max} (cm^{-1}) 3318, 1704, 1684, 1162, 750. APCI-HRMS m/z: calculated for $C_{14}H_{19}N_2O_3$, 263.1390, $[M+H]^+$ found 263.1384. Purity (HPLC): 97%.

3-(acetylamino)phenyldimethylcarbamate (**11b**)



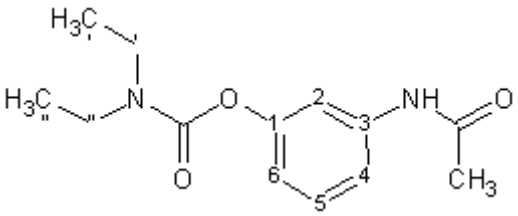
The title compound was prepared from 3-acetamidophenol and dimethylcarbamoyl chloride in a yield of 17%: mp.109.9-113.0 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.05 (s, 1H, NH), 7.49 (t, *J* = 2.1 Hz, 1H, H-2), 7.32 – 7.24 (m, 2H, H-4and H-5), 6.77 (ddd, *J* = 7.5, 2.3, 1.5 Hz, 1H, H-6), 3.02 (s, 3H, $NC\underline{H}_3$), 2.90 (s, 3H, $NC\underline{H}_3$), 2.04 (s, 3H, $COC\underline{H}_3$).^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.5 ($COCH_3$), 154.0 (C=O,carbamate), 151.5 (C-1), 140.1 (C-3), 129.2 (C-5), 116.4 (C-6), 115.5 (C-4), 112.5 (C-2), 36.3 (NCH_3), 36.1 (NCH_3), 24.1 ($CO\underline{C}H_3$). IR ν_{max} (cm^{-1}) 3253, 1718, 1682, 1158, 750. APCI-HRMS m/z: calculated for $C_{11}H_{15}N_2O_3$, 223.1077, $[M+H]^+$ found 223.1066. Purity (HPLC): 84%.

3-(acetylamino)phenyl morpholine-4-carboxylate (**11c**)



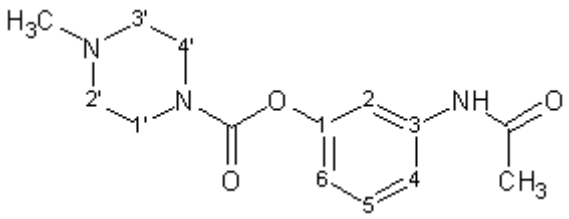
The title compound was prepared from 3-acetamidophenol and 4-morpholinecarbonyl chloride in a yield of 43%: mp. 172.2-174.2 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.05 (s, 1H, NH), 7.55 – 7.51 (m, 1H, H-2), 7.32 – 7.25 (m, 2H, H-4and H-5), 6.79 (dt, *J* = 6.1, 2.5 Hz, 1H, H-4), 3.64 (dd, *J* = 5.7, 4.0 Hz, 4H, OC$\underline{H}_2$), 3.57 (br s, 2H,NC$\underline{H}_2$), 3.41 (br s, 2H, NC$\underline{H}_2$), 2.04 (s, 3H, COC$\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.5 ($\underline{C}OCH_3$), 152.9 (C=O, carbamate), 151.2 (C-1), 140.2 (C-3), 129.2 (C-5), 116.3 (C-6), 115.6 (C-4), 112.4 (C-2), 65.8 (2 x O$\underline{C}H_2$), 44.5, 43.7 (N$\underline{C}H_2$), 24.0 (CO$\underline{C}H_3$). IR ν_{max} (cm^{-1}) 3301, 1676, 1607, 1165, 751. APCI-HRMS m/z: calculated for $C_{13}H_{17}N_2O_4$, 265.1183, *[M+H]*$^{+}$ found 265.1185. Purity (HPLC): 99%.

3-(acetylamino)phenyldiethylcarbamate (**11d**)



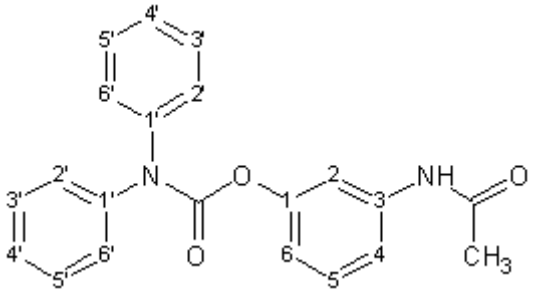
The title compound was prepared from 3-acetamidophenol and diethylcarbamoyl chloride in a yield of 15%: mp. 108.9-110.8 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.03 (s, 1H, NH), 7.46 (t, *J* = 2.1 Hz, 1H, H-2), 7.35 – 7.24 (m, 2H, H-4and H-5), 6.77 (ddd, *J* = 7.9, 2.4, 1.1 Hz, 1H, H-6), 3.38 (q, *J* = 7.1 Hz, 2H, NC$\underline{H}_2$), 3.29 (q, *J* = 7.1 Hz, 2H, NC$\underline{H}_2$'), 2.04 (s, 3H, COC$\underline{H}_3$), 1.18 (t, *J* = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 1.11 (t, *J* = 7.1 Hz, 3H, $CH_2C\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.4 ($\underline{C}OCH_3$), 153.2 (C=O, carbamate), 151.4 (C-1), 140.1 (C-3), 129.2 (C-5), 116.3 (C-6), 115.4 (C-4), 112.4 (C-2), 41.7 (N$\underline{C}H_2$), 41.5 (NCH_2), 24.0 ($COCH_3$), 14.2 (CH_2CH_3), 13.3 (CH_2CH_3). IR ν_{max} (cm^{-1}) 3308, 1698, 1678, 1171, 755. APCI-HRMS m/z: calculated for $C_{13}H_{19}N_2O_3$, 251.1390, *[M+H]*$^{+}$ found 251.1401. Purity (HPLC): 97%.

3-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**11e**)



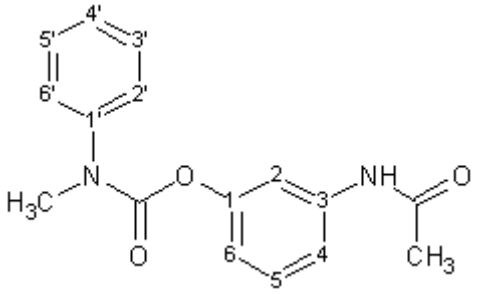
The title compound was prepared from 3-acetamidophenol and 4-methyl-1-piperazinecarbonyl chloride hydrochloride in a yield of 13%: mp. 136.5 - 138.5 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.05 (s, 1H, NH), 7.49 (t, *J* = 2.1 Hz, 1H, H-2), 7.32 – 7.24 (m, 2H, H-4 and H-5), 6.77 (dt, *J* = 7.5, 1.9 Hz, 1H, H-6), 3.56 (br s, 2H, CONC$\underline{H}_2$), 3.41 (br s, 2H, CONC$\underline{H}_2$), 2.34 (t, *J* = 5.7 Hz, 4H, C$\underline{H}_2$NCH$_3$), 2.21 (s, 3H, CH$_3$N), 2.04 (s, 3H, COC$\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.5 ($\underline{C}$OCH$_3$), 152.8 (C=O, carbamate), 151.3 (C-1), 140.1 (C-3), 129.2 (C-5), 116.3 (C-6), 115.5 (C-4), 112.4 (C-2), 54.3, 54.1 ($\underline{C}$H$_2$NCH$_3$), 45.7 ($\underline{C}$H$_3$N), 44.1, 43.5 (CON$\underline{C}$H$_2$), 24.0 (CO$\underline{C}$H$_3$). IR ν_{max} (cm^{-1}) 3308, 1678, 1600, 1168, 754. APCI-HRMS m/z: calculated for $C_{14}H_{20}N_3O_3$, 278.1499, *[M+H]*$^+$ found 278.1499. Purity (HPLC): 85%.

3-(acetylamino)phenyldiphenylcarbamate (**11f**)



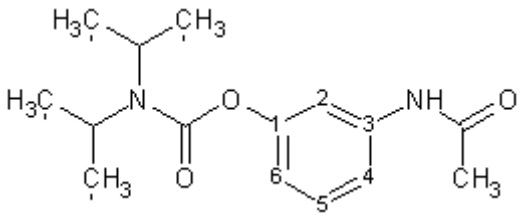
The title compound was prepared from 3-acetamidophenol and diphenylcarbamoyl chloride in a yield of 51%: mp.118.4-124.9 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.08 (s, 1H, NH), 7.54 (t, *J* = 2.1 Hz, 1H, H-2), 7.47 – 7.39 (m, 8H, H-2', H-3', H-5', H-6'), 7.35 – 7.26 (m, 4H, H-4, H-5 and H-4'), 6.89 (ddd, *J* = 7.9, 2.4, 1.2 Hz, 1H, H-6), 2.04 (s, 3H, COC$\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.5 ($\underline{C}$OCH$_3$), 152.4 (C=O, carbamate), 150.9 (C-1), 142.2 (2C, C-1'), 140.2 (C-3), 129.3(C-5), 129.2 (4C, C-2'/6' or C-3'/5), 127.2 (2C, C-4'), 126.8 (4C, C-2'/6' or C-3'/5'), 116.3 (C-6), 116.0 (C-4), 112.3 (C-2), 24.0 (CO$\underline{C}$H$_3$). IR ν_{max} (cm^{-1}) 3314, 1701, 1670, 1168, 749. APCI-HRMS m/z: calculated for $C_{21}H_{19}N_2O_3$, 347.1390, *[M+H]*$^+$ found 347.1382. Purity (HPLC): 98%.

3-(acetylamino)phenyl methyl(phenyl)carbamate (**11g**)



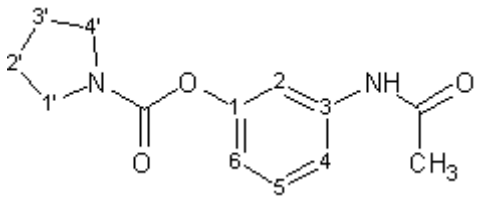
The title compound was prepared from 3-acetamidophenol and N-methyl-N-phenylcarbamoyl chloride in a yield of 47%: mp. 129.1 - 130.9 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.05 (s, 1H, NH), 7.49 (br s, 1H, H-2), 7.47 – 7.39 (m, 4H, H-2', H-3', H-5', H-6'), 7.33 – 7.24 (m, 3H, H-4,H-5 andH-4'), 6.84 – 6.79 (m, 1H, H-6), 3.33 (s, 3H, CH_3N), 2.04 (s, 3H, $COC\underline{H}_3$).^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.5 ($COCH_3$), 153.0 (C=O, carbamate), 151.2 (C-1), 142.8 (C-1'), 140.2 (C-3), 129.2 (C-5), 129.0 (C-2'/6' or C-3'/5'), 126.5 (C-4'), 126.0 (C-2'/6' or C-3'/5'), 116.3 (C-6), 115.7 (C-4), 112.3 (C-2), 37.9 (CH_3N), 24.0 ($CO\underline{C}H_3$). IR ν_{max} (cm^{-1}) 3327, 1697, 1680, 1170, 764. APCI-HRMS m/z: calculated for $C_{16}H_{17}N_2O_3$, 285.1234, $[M+H]^+$ found 285.1224. Purity (HPLC): 99%.

3-(acetylamino)phenyl dipropan-2-ylcarbamate (**11h**)



The title compound was prepared from 3-acetamidophenol and N,N-diisopropylcarbamoyl chloride in a yield of 4%: mp. 137.1-139.4 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.03 (s, 1H, NH), 7.43 (t, *J* = 2.2 Hz, 1H, H-2), 7.34 (ddd, *J* = 8.2, 2.0, 1.0 Hz, 1H, H-4), 7.27 (t, *J* = 8.1 Hz, 1H, H-5), 6.75 (ddd, *J* = 8.0, 2.4, 1.1 Hz, 1H, H-6), 3.97 (br s, 2H, isopropyl CH), 2.03 (s, 3H, $COC\underline{H}_3$), 1.28 – 1.21 (m, 12H, isopropyl $C\underline{H}_3$). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.4 ($\underline{C}OCH_3$), 152.7 (C=O,carbamate), 151.3 (C-1), 140.1 (C-3), 129.2 (C-5), 116.3 (C-6), 115.3 (C-4), 112.4 (C-2), 45.6 (2C, isopropyl CH), 24.0 ($CO\underline{C}H_3$), 21.3 (2C,isopropylCH_3), 20.1 (isopropyl CH_3). IR ν_{max} (cm^{-1}) 3307, 1671, 1606, 1171, 759. APCI-HRMS m/z: calculated for $C_{15}H_{23}N_2O_3$, 279.1703, $[M+H]^+$ found 279.1706. Purity (HPLC): 99%.

3-(acetylamino)phenyl pyrrolidine-1-carboxylate (**11i**)



The title compound was prepared from 3-acetamidophenol and 1-pyrrolidinecarbonyl chloride in a yield of 63%: mp.194.6-196.0 °C (ethanol), white powder. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.04 (s, 1H, NH), 7.51 (t, *J* = 2.1 Hz, 1H, H-2), 7.32 – 7.24 (m, 2H, H-4and H-5), 6.78 (dt, *J* = 7.3, 2.0 Hz, 1H, H-6), 3.48 (t, *J* = 6.7 Hz, 2H, NC$\underline{H}_2$), 3.32 (t, *J* = 6.7 Hz, 2H,NC$\underline{H}_2$), 2.04 (s, 3H, COC$\underline{H}_3$), 1.93 – 1.80 (m, 4H, remaining CH_2's of pyrrolidine ring).^{13}C NMR (151 MHz, DMSO-*d6*) δ 168.5 ($COCH_3$), 152.1 (C=O,carbamate,), 151.3 (C-1), 140.1 (C-3), 129.1 (C-5), 116.4 (C-6), 115.4 (C-4), 112.5 (C-2), 46.2, 46.0 (N$\underline{C}H_2$), 25.3 , 24.5 (remaining CH_2's of pyrrolidine ring), 24.0 (CO$\underline{C}H_3$). IR ν_{max} (cm^{-1}) 3252, 1709, 1654, 1166, 750. APCI-HRMS m/z: calculated for $C_{13}H_{17}N_2O_3$, 249.1234, $[M+H]^+$ found 249.1229. Purity (HPLC): 99%.

3.4 SUMMARY

In this chapter the design, synthesis and characterisation of the dihydroquinolinone-carbamate and acetomidophenol-carbamate compounds were described. All compounds were synthesised in one step using literature procedures. Eleven dihydroquinolinone-carbamate derivatives were successfully synthesised using the commercially available 7-hydroxy-3,4-dihydro-2(1*H*)-quinolinone or 6-hydroxy-3,4-dihydro-2(1*H*)-quinolinone and the appropriate carbamoyl chloride. Seventeen acetomidophenol-carbamate derivatives were successfully synthesised from commercially available 4-acetoamidophenol and 3-acetoamidophenoland the appropriate carbamoyl chloride. Thus, a total of twenty-eight compounds were synthesised.

Compounds were characterised with NMR spectroscopy, IR spectroscopy and mass spectrometry. HPLC were used to determine the purity of the synthesised products and melting points were determined.

^{1}H NMR and ^{13}C NMR data indicated that the proposed structures were correct, and this was also confirmed by the experimentally determined masses which correlated with calculated masses. Purities were generally above 80%, and were deemed suitable for biological analyses.

CHAPTER 4

BIOLOGICAL EVALUATION

4.1 ENZYME KINETICS

Enzyme kinetics is the study of the chemical reactions that are catalysed by enzymes. In enzyme kinetics, the reaction rate is measured and the effects of changes in the conditions of the reaction are examined. Enzyme kinetics is also used for determination of the interaction potency between an inhibitor and a specific enzyme (Kennelly & Rodwell, 2009).

Enzymes are proteins that originate from living cells and are capable of generating certain chemical changes through a catalytic action in organic substances. Enzymes bind to a substrate and lower the activation energy that is needed to convert the substrate to a product. Several factors influence enzyme working rates including: temperature, pH, activation energies, presence of inhibitors and the concentration of a substrate [S] (Alberts *et al.*, 2002).

The rate limiting step during an enzyme catalysed transformation of a substrate [S] to a product [P] is the breakdown of the Enzyme-Substrate complex (ES) (Figure 4.1).

$$E+S \rightleftharpoons ES \rightleftharpoons E+P$$

Figure 4.1 Transformation of a substrate through an enzyme catalytic reaction (Kennelly & Rodwell, 2009).

Since the ES concentration cannot be easily measured experimentally, different equations are used as alternative expressions for the determination of enzymatic reaction rates.

4.1.1 The Michaelis-Menten equation

This equation is used as an alternative expression for the determination of enzymatic reaction rates and illustrates the relationship between the initial reaction velocity (V_i) and the substrate concentration [S].

Equation 4.1 The Michaelis-Menten equation

$$Vi = \frac{Vmax[S]}{Km + [S]}$$

Where K_m is the Michaelis-Menten constant which represents the substrate concentration where $V_i$is half of the maximum velocity at a specific concentration of the enzyme.

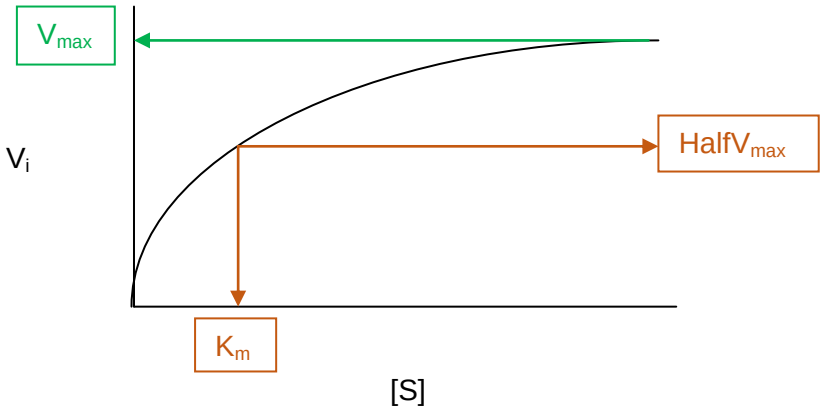


Figure 4.2 The Michaelis-Menten plot showing the effect of substrate concentration on V_i.

The enzyme concentration will almost always be directly proportional to V_i ([E] α V_i) because assays used to measure enzyme activity are usually done with an excess of the substrate over the enzyme. When V_i does not increase with an increase in [S], the enzyme is saturated (Murray *et al.*, 2003).

The Michaelis-Menten equation can be reduced to three different forms under the following circumstances:

When the value of K_m is much higher than [S]:

Equation 4.2

$$Vi = \frac{Vmax[S]}{Km}$$

When the value of K_m is much lower than [S]:

Equation 4.3

$$Vi = Vmax$$

When the value of K_m and [S] is equal:

Equation 4.4

$$Vi = \frac{Vmax}{2}$$

The determination of V_{max} and K_m values are not always possible with the Michaelis-Menten equation because of the impractically high concentrations of the substrate that is needed. Through inversion of the Michaelis-Menten equation, the Lineweaver-Burk equation is obtained which can be used to determine the V_{max} and K_m values very accurately at a low substrate concentration.

4.1.2 Lineweaver-Burk equation

Equation 4.5 Lineweaver-Burk equation

$$\frac{1}{Vi} = \left[\frac{Km}{Vmax}\right]\frac{1}{[S]} + \frac{1}{Vmax}$$

When the inverse of the initial velocity ($1/V_i$) and the inverse of the substrate concentration (1/[S]) are graphically plotted, a straight line, in the form y = ax + b, is obtained, which is termed the double reciprocal or Lineweaver-Burk plot (Figure 4.3).

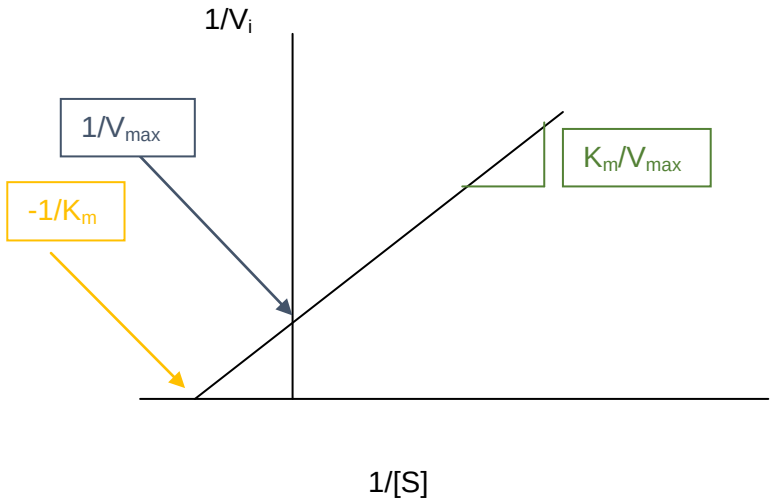


Figure 4.3 The Lineweaver-Burk plot.

This plot (Figure 4.3) shows that the y-axis intercept is equal to $1/V_{max}$, the x-axis intercept is equal to $-1/K_m$ and the slope is K_m/V_{max}. The K_m value will therefore be determined by solving x when setting y equal to zero (Kennelly & Rodwell, 2009; Rogers & Gibon, 2009).

The Lineweaver-Burk plot is used for the evaluation of inhibitory mechanisms to characterise whether a test compound is a competitive or non-competitive inhibitor. In competitive inhibition, the presence of the inhibitor decreases the ability of the enzyme to bind with a substrate. In non-competitive inhibition, the inhibitor binds to a distinct position on the enzyme, which is not the binding site of the substrate, and acts through the reduction of the turnover rate of the reaction (Rogers & Gibon, 2009).

4.1.2.1 Competitive inhibition

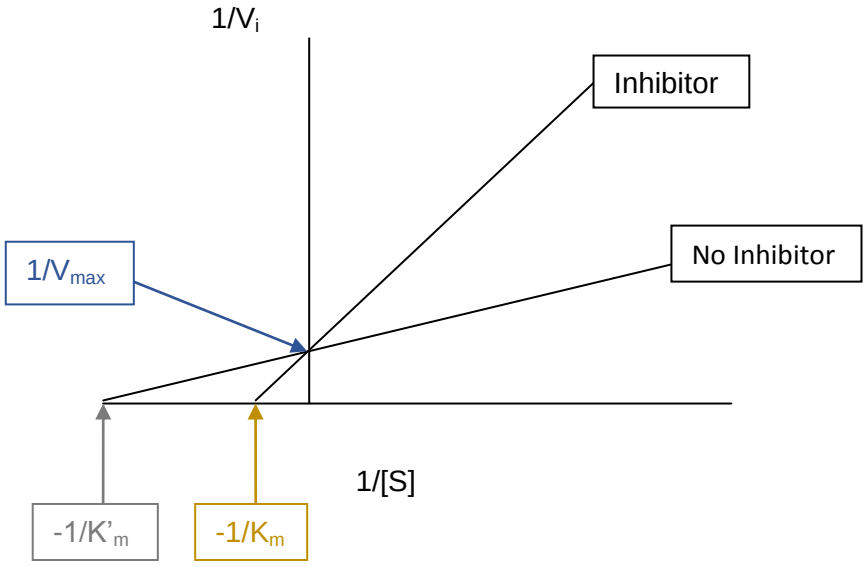


Figure 4.4 Lineweaver-Burk plot of competitive inhibition.

This plot (Figure 4.3) has two straight lines (one line corresponding to the presence of an inhibitor and the other representing the absence of an inhibitor). The x-intercept is $-1/K_m$ when no inhibitor is present, while, when an inhibitor is present, the x-intercept represents $-1/K'_m$. In both cases the y-intercept is $1/V_{max}$. During competitive inhibition, the V_{max} values will thus remain unchanged while the K_m values will increase (Murry*et al.*, 2003; Rodwell, 1993). It can also be deduced that the value of V_i is unaffected by the presence of the inhibitor as 1/[S] approaches 0, though the x-intercept is dependent on inhibitor concentration. The increase in concentration of competitive inhibitors will result in the increase in enzyme K_m value which has no effect on V_{max} (Rogers & Gibon, 2009).

The K_i value may be calculated from x-axis after K_m is determined in the absence of an inhibitor (equation 4.6). The K_i value is used in the determination of inhibitor affinity for an enzyme. Low K_i values are suggestive of good inhibitory potency while high K_i values are suggestive of weaker inhibitory potency (Murray *et al.*, 2003).

Equation 4.6 An equation for the determination of K_i

$$\frac{1}{Km} = \frac{1}{Km' + \frac{[I]}{Ki}}$$

4.1.2.2 Non-competitive inhibition

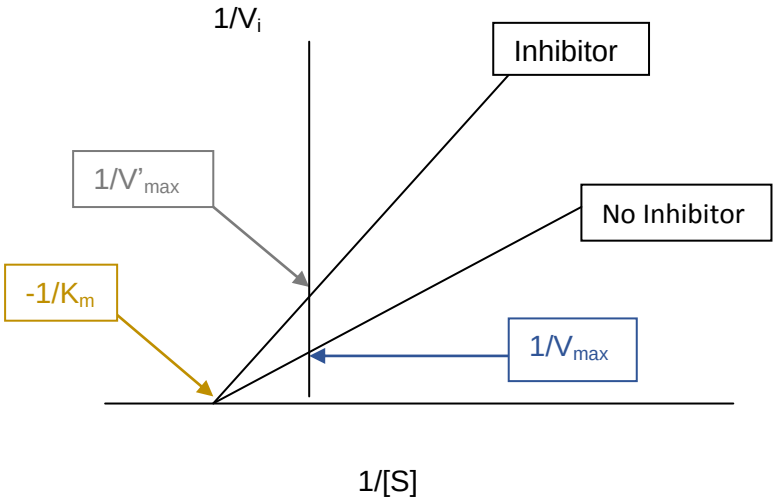


Figure 4.5 Lineweaver-Burk plot of non-competitive inhibition.

TheLineweaver-Burk plot for non-competitive inhibition (Figure 4.5) has two straight lines with the same x-intercept and different y-intercepts. The x-intercept is equal to $-1/K_m$ which remains constant, while the y-intercept varies, being $1/V_{max}$ in the presence of the inhibitor, and $1/V'_{max}$ in the absence of an inhibitor (Murray *et al.*, 2003). The V_{max} values can thus not be recovered by increasing the substrate concentration since the inhibitor does not bind to the active site (Rogers & Gibon, 2009).

4.1.3 IC_{50} value determination

Measuring IC_{50} values is another way of determining the effectiveness of inhibitors. In this study, IC_{50} values were used to measure the potencies of inhibitors forboth MAO and AChE inhibition. The IC_{50} value is defined as the concentration at which the inhibitor inhibits the activity of the enzyme by 50% (Vermeirssen *et al.*, 2002). The lower the IC_{50} value, the more potent the inhibitor. The following equation (equation 4.7) illustrates the relationship between the IC_{50} value and K_i.

Equation 4.7 Relationship between the IC_{50} value and K_i (Chen & Prusoff, 1973).

$$\mathrm{Ki} = \frac{IC50}{(1 + \frac{[s]}{Km})}$$

4.2 INTRODUCTION

In this section, the synthesised compounds were assessed for their ability to inhibit recombinant human MAO-A, MAO-B and AChE. There are several methods for measuring MAO-A, MAO-B and AChE inhibitory activities *in vitro*. In this study the IC_{50} values were

determined by fluorescence spectrophotometry for the inhibition of MAO-A and MAO-B (Strydom *et al.*, 2010; Legoabe *et al.*, 2014). On the other hand, a spectrophotometric assay, using 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB) as substrate, was used to determine AChE inhibitory activities with the inhibitory potencies also expressed as IC_{50} values.

4.3 BIOLOGICAL EVALUATION OF MAO INHIBITORS

4.3.1 General background

Most MAO activity measurements were developed in the 1960's. These studies are important for the improvement of clinical and pharmacological studies as well as for research. MAO activity measurements can be categorised as indirect or direct measurements with these studies being based on the measurement of the reaction products or by the measurement of the disappearance of reagent species (Nicotra & Parvez, 1999).

Polaropgraphic assay

Oxygen use by MAO-A and MAO-B can be measured polarographically. During this assay, oxygen-sensitive electrodes are used for the detection of oxygen consumption. The amount of oxygen consumed during the assay is an indicator of the degree to which oxidation takes place. The rapid processing of large quantities of a sample requires a well controlled assay environment, which is difficult to achieve (Averillbates *et al.*, 1993).

Fluorescence assay

Several MAO-B substrates, when oxidised, form fluorescent products. During this assay, the generation of these oxidised monoamine products is detected with a fluorescence spectrophotometer (Zhou *et al.*, 1996).

Hydrogen peroxide measurement

The detection of the co-products formed during catalysis, such as H_2O_2, forms the basis of this assay. The detection is possible through absorbance measurement of H_2O_2 at a wavelength of 230 nm. Biological compounds are capable of interferening during the assay because of their absorbance at 230 nm, which is disadvantageous (Stevanato *et al.*, 1995).

Horseradish peroxidase assay

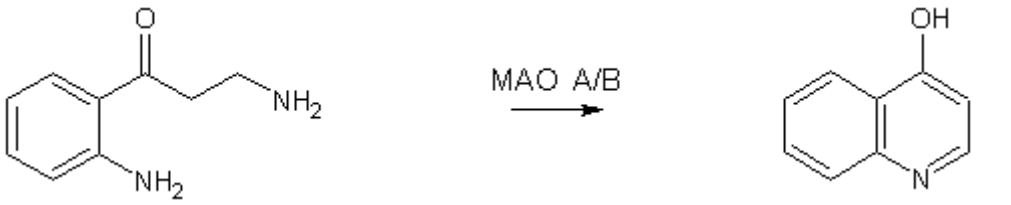
H_2O_2 is generated by the MAO enzyme. A horseradish peroxidase coupled reaction system detects the H_2O_2 generated by the MAO. The H_2O_2 is detected by using H_2O_2-sensitive probes (Morpurgo *et al.*, 1989). An extremely sensitive H_2O_2 probe, N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), is used to measure H_2O_2 in a horseradish peroxidase-

coupled reaction. Amplex Red is oxidised to a fluorescent dye, resorufin, by H_2O_2 with resorufin having an absorption and emission wavelength of a maximum of >560 nm (Zhou & Panchuk-Voloshina, 1997).

Kynuramine assay

This assay is based on the oxidative deamination of kynuramine by MAO to yield 4-hydroxyquinoline. The concentration of 4-hydroxyquinoline is determined by a spectrofluorometer with an excitation wavelength of 310 nm and an emission wavelength of 400 nm (Weissbach *et al.*, 1960). This assay will be used during this study for the measurement of MAO activity *in vitro*.

MAO-A and MAO-B activities were measured in this study by employing the MAO-A/B mixed substrate, kynuramine. The assay is based on the observation that certain MAO substrates can be oxidised by these enzymes to yield fluorescent products. Kynuramine is an example of such a substrate, and is oxidised by both MAO-A and MAO-B, to form 4-hydroxyquinoline, which fluoresces in an alkaline medium at a wavelength of λ_{ex} = 310 nm; λ_{em} = 400 nm (Figure 4.6) (Strydom *et al.*, 2010). In the first step of this reaction, MAO oxidatively deaminates kynuramine to yield an aldehyde. This aldehyde can either condense to yield 4-hydroxyquinoline, or undergo further oxidation to generate an acid or lactam (2,4-dihydroxyquinoline). *In vitro,* the non-enzymatic condensation of the aldehyde occurs faster than the further oxidation to the acid or lactam. Thus 4-hydroxyquinoline is generated by the action of the MAOs on kynuramine *in vitro*. Since 4-hydroxyquinoline fluoresces in alkaline media, it may be conveniently measured with a fluorescence spectrophotometer. This method has been developed into a reliable assay to determine MAO activity (Weissbach *et al.*, 1960). It was used in this study to determine whether the synthesised compounds exhibited MAO-A and MAO-B inhibitory activities.



Kynuramine 4-Hydroxyquinoline

Figure 4.6 The oxidation of kynuramine to 4-hydroxyquinoline.

4.3.2 Chemicals and instrumentation

MAO assay

A Varian Cary Eclipse fluorescence spectrophotometer was employed for the fluorometric measurements. Insect cell microsomes containing recombinant human MAO-A and MAO-B (5 mg/ml), kynuramine dihydrobromide, (*R*)-deprenyl and 4-hydroxyquinoline were obtained from Sigma-Aldrich. Milli-Q deionised water (Millipore) was used to prepare all the buffers and the following materials were obtained from Merck:

1. Polypropylene 96-well microtiter plates
2. Potassium phosphate (K_2HPO_4/KH_2PO_4)
3. KCl
4. Sucrose
5. NaOH
6. DMSO

4.3.3 Determination of the IC_{50}values

For the determination of the IC_{50} values of the synthesised compounds (Table 4.1), sigmoidal dose-response curves were constructed and IC_{50} values were calculated using the Prismsoftware package (version 5.0) (GraphPad Software, La Jolla, USA). The IC_{50} values were determined in triplicate and expressed as mean ± standard deviation (SD).

4.3.4 Method

Recombinant human MAO-A (5 mg/ml) and human MAO-B (5 mg/ml) were pre-aliquoted and stored at -70 °C. The enzymatic reactions were carried out in a white polypropylene 96-well microtiter plates.

- All reactions were carried out in white 96-well microtiter plates (Eppendorf). With the final volume of the enzymatic reactions consisting of 200 µl.
- Potassium phosphate buffer (100 mM, made isotonic with KCl) at pH 7.4 served as the reaction buffer.
- The enzymatic reactions consisted of the following:
    - The MAO-A/B mixed substrate, kynuramine (50 µM).
    - Different inhibitor concentrations spanning at least 3 orders of magnitude (0.0003–100 µM).
    - DMSO as co-solvent (4%). The inhibitors were prepared in DMSO and added to the reactions to yield a final DMSO concentration of 4%.

- The reactions were initiated with the addition of MAO-A or MAO-B (0.0075 mg protein/ml)

- The reactions were incubated in a convection oven for 20 min at 37 ºC.
- The reactions were subsequently terminated with the addition of 80 µl NaOH (2 N).
- The concentrations of 4-hydroxyquinoline formed in the reactions were measured by fluorescence spectrophotometry (λ_{ex} = 310; λ_{em} = 400 nm) (Novaroli *et al*., 2005).
- A linear calibration curve was constructed using authentic 4-hydroxyquinoline (0.047–1.56 µM). This curve was used to quantify the 4-hydroxyquinoline formed in the enzyme reactions. The enzyme catalytic rates were expressed as nmol 4-hydroxyquinoline formed/min.mg protein.
- The enzyme catalytic rates were fitted to the one site competition model incorporated into the Prism 5 software package (GraphPad). The IC_{50} values were determined in triplicate and are expressed as mean ± standard deviation (SD).

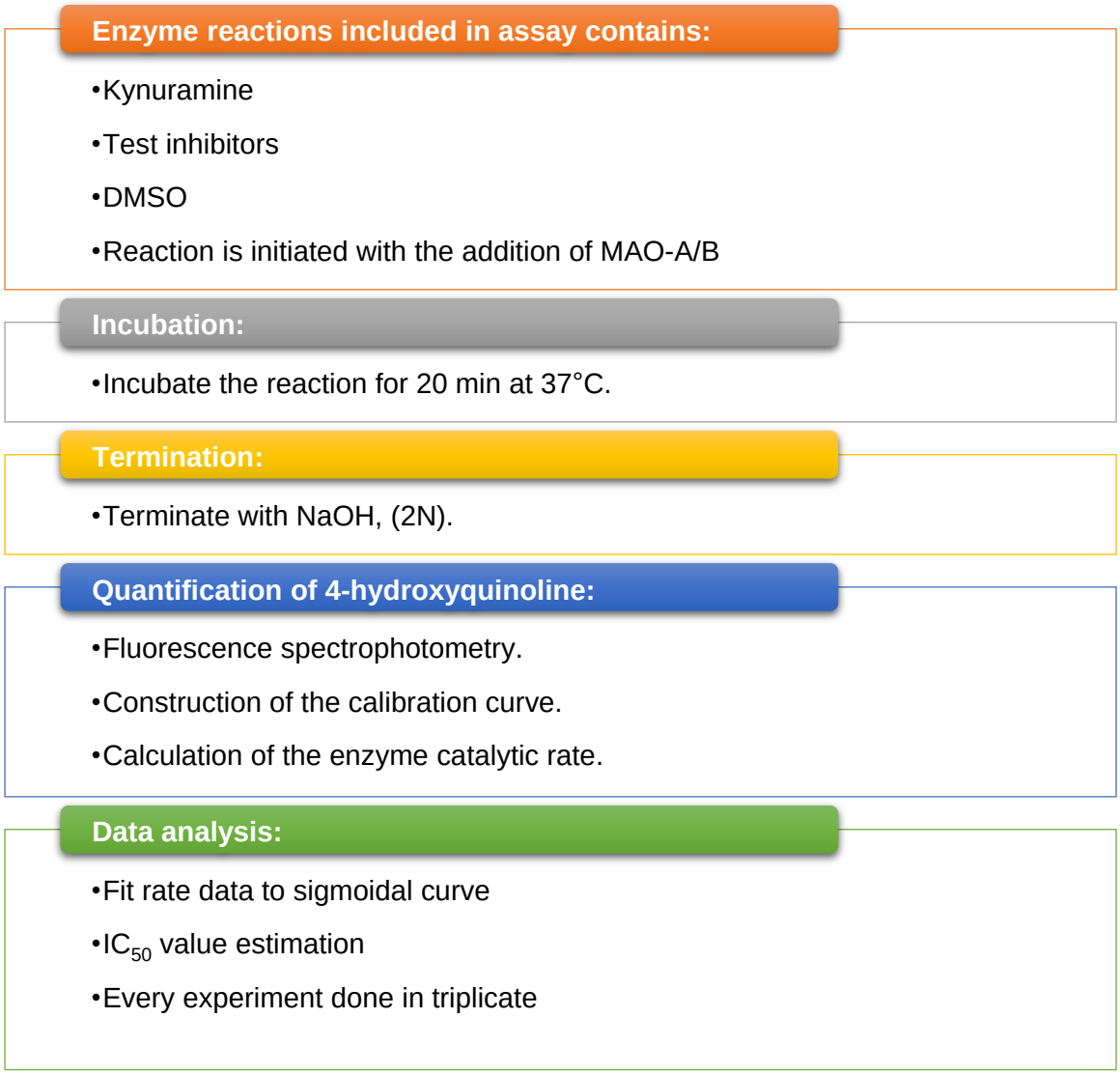


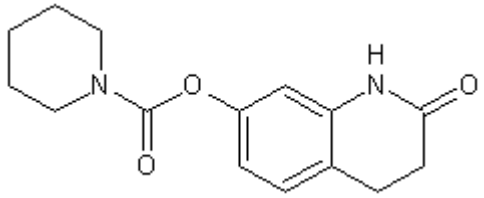
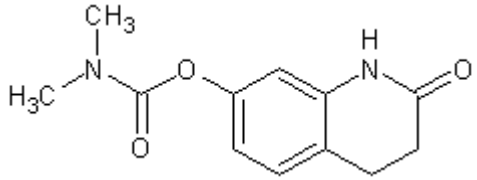
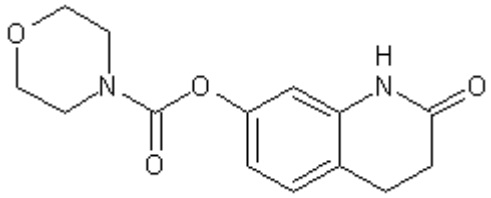
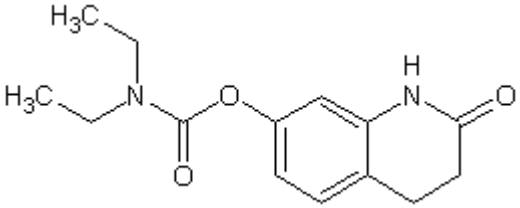
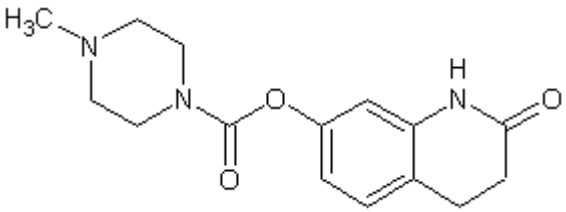
Figure 4.7 A summary for the method followed to determine IC_{50} values.

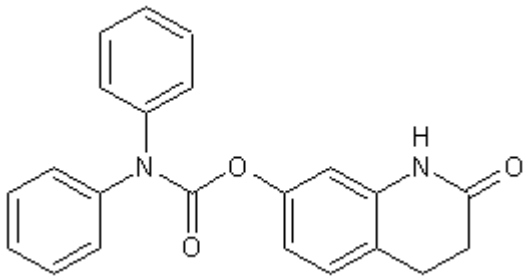
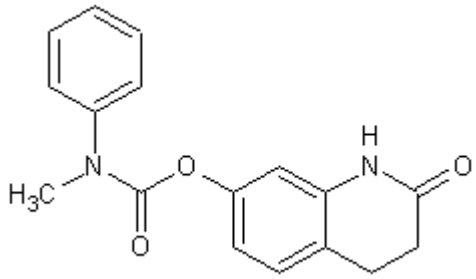
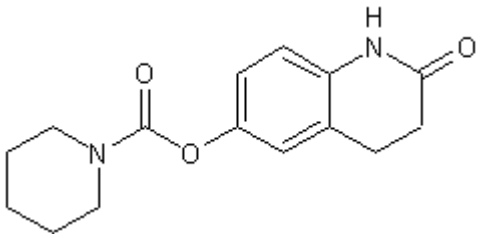
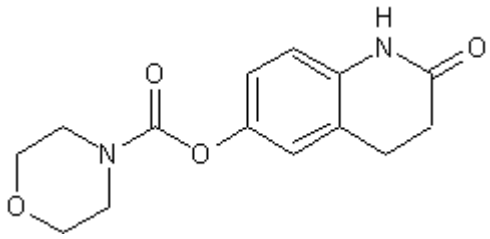
4.3.5 Results

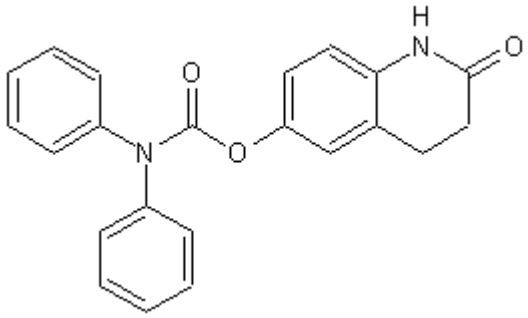
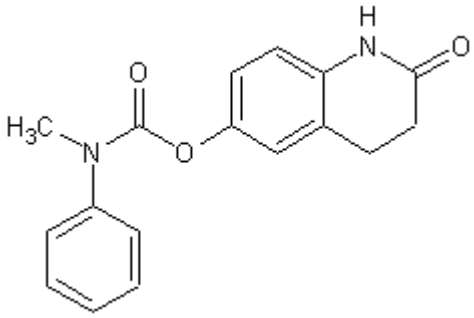
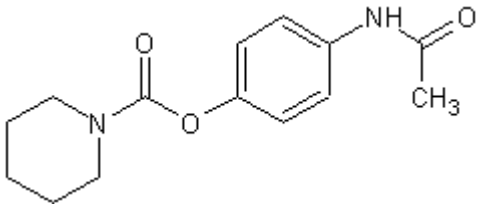
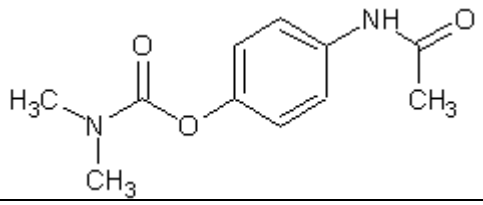
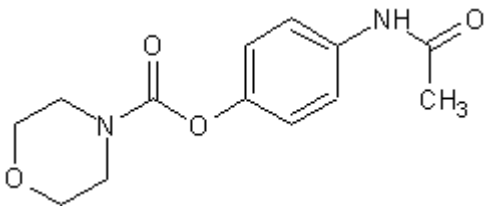
Both the dihydroquinolinone-carbamates (**8a**-**g**; **9a**-**d**) and acetamidophenol-carbamates (**10a**-**h**; **11a**-**i**) were screened for their ability to inhibit MAO-A and MAO-B. The IC_{50} values for MAO inhibition are presented in table 4.1. The lower the IC_{50} value, the higher the inhibition potency of the inhibitor.

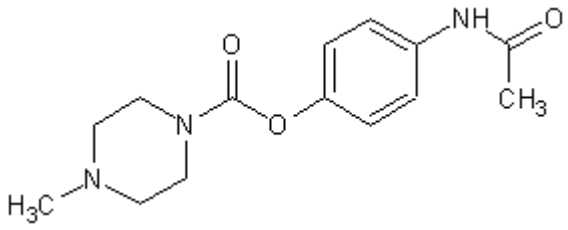
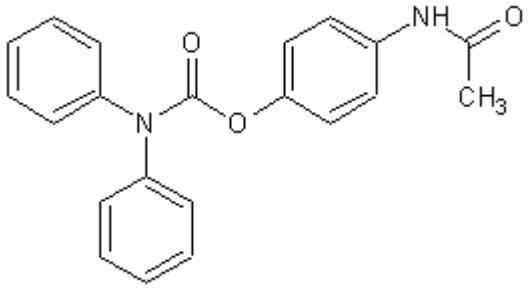
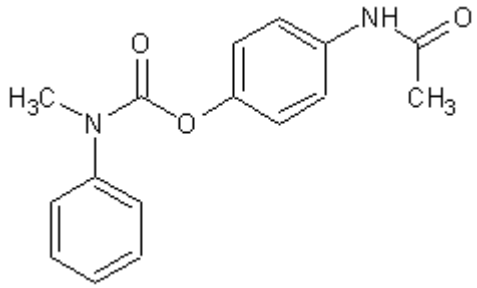
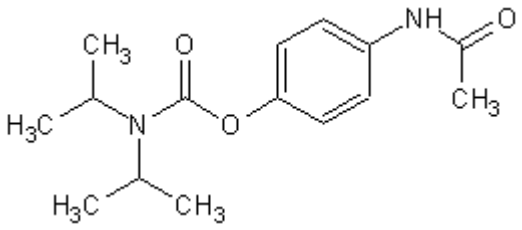
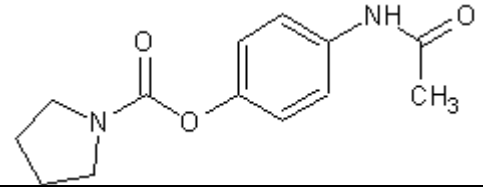
The selectivities of the inhibition of MAO-A versus MAO-B are represented by the selectivity index (SI) value in table 4.1. More selective inhibition for MAO-B is expressed by a higher SI value. The SI value is calculated by dividing the IC_{50} value of MAO-A inhibition by the IC_{50} value of MAO-B inhibition for each compound.

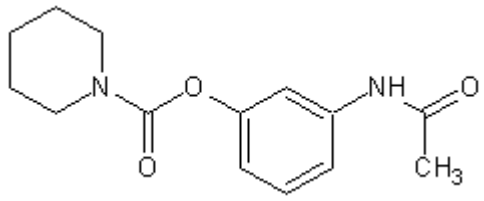
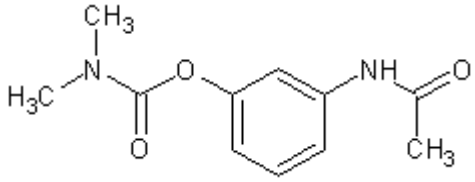
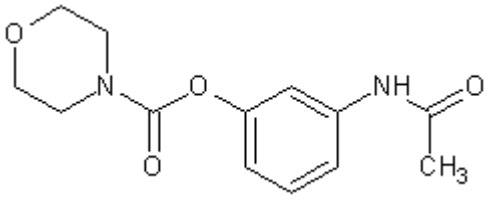
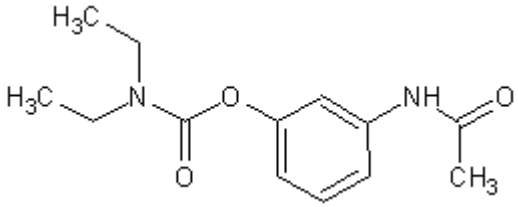
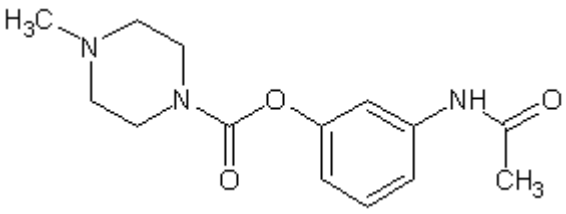
Table 4.1 IC_{50} values of the synthesised compounds for the inhibition of human MAO-A and MAO-B.

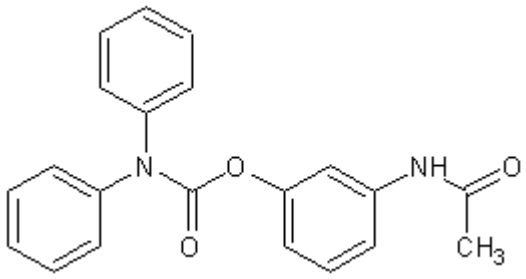
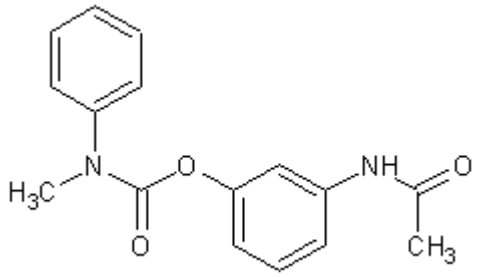
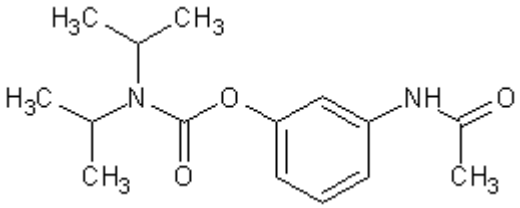
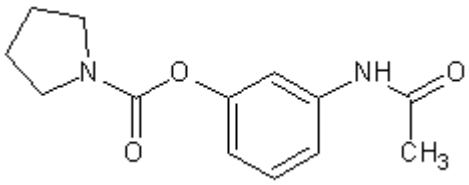
Compound		[a]MAO-A (μM)	[a]MAO-B (μM)	[b]SI
8a		[c]No inhibition	[c]No inhibition	-
8b		60.97±2.54	[c]No inhibition	-
8c		[c]No inhibition	10.65 ±2.70	-
8d		[c]No inhibition	7.88±1.40	-
8e		[c]No inhibition	[c]No inhibition	-

8f		[c]No inhibition	[c]No inhibition	-
8g		[c]No inhibition	3.73 ± 1.26	-
9a		89.10±3.80	7.30±1.20	12.20
9b		[c]No inhibition	[c]No inhibition	-

9c		[c]No inhibition	[c]No inhibition	-
9d		49.39 ± 6.32	5.11 ± 0.30	9.67
10a		[c]No inhibition	27.30 ± 7.00	-
10b		[c]No inhibition	[c]No inhibition	-
10c		[c]No inhibition	[c]No inhibition	-

10d		[c]No inhibition	[c]No inhibition	-
10e		33.99 ±8.99	5.36 ±1.57	6.34
10f		21.70 ± 6.04	[c]No inhibition	-
10g		[c]No inhibition	[c]No inhibition	-
10h		[c]No inhibition	[c]No inhibition	-

11a		[c]No inhibition	[c]No inhibition	-
11b		[c]No inhibition	[c]No inhibition	-
11c		[c]No inhibition	[c]No inhibition	-
11d		[c]No inhibition	[c]No inhibition	-
11e		[c]No inhibition	[c]No inhibition	-

11f		[c]No inhibition	[c]No inhibition	-
11g		[c]No inhibition	6.25± 1.75	-
11h		[c]No inhibition	[c]No inhibition	-
11i		[c]No inhibition	[c]No inhibition	-

[a]All the values are expressed as the mean ±SD of triplicate determinations.

[b]The SI gives an indication of the selectivity for the MAO-B isoform.

[c]No inhibition observed at maximum tested concentration of 100 µM.

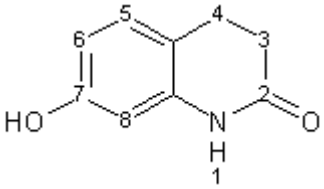
4.3.6 Discussion of Results

From the results in table 4.1 the following conclusions and general observations can be made:

- The synthesised dihydroquinolinone-carbamate derivatives exhibit poor to no inhibitory activities towards both MAO-A and MAO-B.
- Similarly, the synthesised acetamidophenol-carbamate derivatives exhibit poor to no inhibitory activities towards both MAO-A and MAO-B.
- The five most potent MAO-B inhibitors synthesised in this study, in decreasing order of activity are: **8g** (IC_{50} = 3.73 µM), **9d** (IC_{50} = 5.11 µM), **10e** (IC_{50} = 5.36 µM), **11g** (IC_{50} = 6.25 µM) and **9a** (IC_{50} = 7.30 µM).
- Compound **8g,** substituted with a methyl and phenyl group on the carbamate nitrogen**,** exhibited the best inhibition of MAO-B of all the compounds (IC_{50} = 3.73 µM). This inhibition is classified as weak since this compound is 37-fold less potent than the known selective reversible inhibitor, lazabemide, as determined in our laboratory under the same assay conditions (IC_{50} = 0.091 µM).
- Interestingly, two of the five most potent dihydroquinolinone-carbamate MAO-B inhibitors, namely **9d** (IC_{50} = 5.11µM) and **11g** (IC_{50} = 6.25 µM) are substituted with a methyl and phenyl group on the carbamate nitrogen, indicating that compounds bearing this substituent patern is accommodated by the enzyme active site, where most of the other compounds are not.
- Among all of the compounds evaluated, compound **10f** is the most potent human MAO-A inhibitor with an IC_{50} value of 21.70 µM.
- Consideration of the IC_{50} values and SI indices of compounds **9a**, **9d** and **10e** and the fact that it was not possible to calculate SI values for compounds **8c**, **8d**, **8g**, **10a** and **11g**, since no inhibition for MAO-A was observed, reveal that these compounds are weak, selective MAO-B inhibitors.
- On the other hand, for compounds **8b** and **10f**, no inhibition for MAO-B was observed, indicating that they are MAO-A selective, even though they exhibit weak MAO-A inhibition.
- Generally, the dihydroquinolinone-carbamate scaffold appears to be more useful for the design of MAO-B inhibitors than the acetamidophenol-carbamate derivatives. 3-Acetamidol derivatives (**11a – 11i**) in particular are notsuitable as MAO inhibitors.

Disappointingly, none of the synthesised compounds thus show IC_{50} values in the submicromolar range for the inhibition of MAO-B.

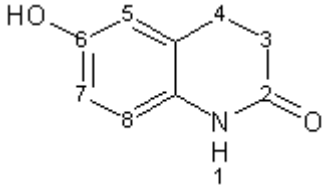
In a previous study done in our laboratories (Meiring *et al.*, 2013), results indicated that appropriate C7 and C6 substitution of 7-hydroxy-3,4-dihydro-2(1*H*)-quinolinone (**32a**) and 6-hydroxy-3,4-dihydro-2(1*H*)-quinolinone (**32b**) respectively, is a requirement for MAO inhibitory activity, since these compounds themselves are weak inhibitors of MAO.



32a

IC_{50} MAO-A 183 µM

No inhibition of MAO-B



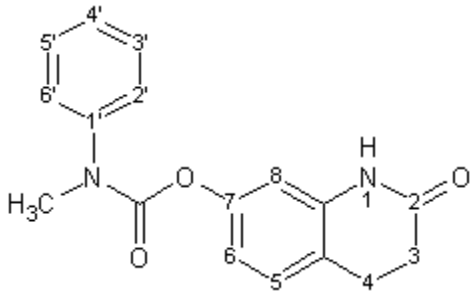
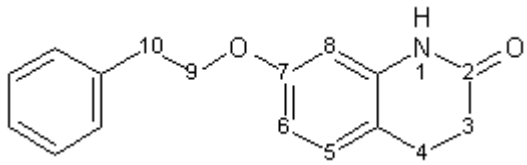
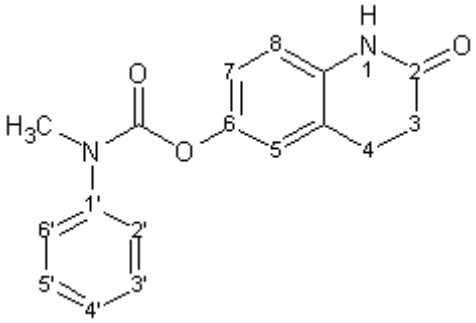
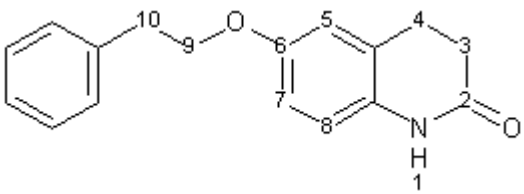
32b

IC_{50} MAO-A 161 µM

IC_{50} MAO-B 201 µM

Generally, substitution with a flexible phenylethoxyside chain on the C7 position of 3,4-dihydro-2(1*H*)-quinolinone yielded potentMAO inhibition activity. Although substitution with the phenylethoxy side chain on the C6 position also yielded good MAO-B inhibitors, C7 substitution, in general, led to better inhibition (Table 4.2).

Table 4.2 Comparison of IC_{50} values of the compounds synthesised in this study with those of the 3,4-dihydro-2(1*H*)-quinolinones reported in literature (Meiring *et al.*, 2013).

Compound	MAO-A (µM)	MAO-B (µM)	SI
	[c]No inhibition	3.73 ± 1.26	-
7-(2-Phenylethoxy)-3,4-dihydro-2(1*H*)-  quinolinone (Meiring *et al*., 2013)	53.7 ± 12.0	0.191 ± 0.041	281
	49.39 ± 6.32	5.11 ± 0.30	9.67
6-(2-Phenylethoxy)-3,4-dihydro-2(1*H*)-  quinolinone (Meiring *et al*., 2013)	22.5 ± 9.69	2.33 ± 1.33	10

Compounds **8g** and **1**are structurally similar with 3 atoms linking the dihydroquinolinone ring and the phenyl ring in the side chain. Compound **8g** does, however, also have an additional methyl group on the nitrogen atom in the side chain linker. The same is true for compounds **9d** and **2**. Interestingly, when the biological activities of these compounds are compared, it is clear that a significant loss of both MAO-A and MAO-B activity occurred for the carbamate derivatives. This loss of activity also appears to be more prononounced for the C7 derivatives (**8g** vs.**1**) than for the C6 derivatives (**9d** vs.**2**). Whether this decrease in activity is solely due to the loss of flexibility in the side chain or the combination of the loss of flexibility, introduction of additional heteroatoms, or the additional methyl substitent requires further investigation.

4.4 BIOLOGICAL EVALUATION OF AChE INHIBITORS

4.4.1 General background

Assay of AChEI activity is important for the diagnostic detection of nerve agents, pesticides and the *in vitro* characterisation of toxins and drugs such as probable AD treatments (Pohanka *et al.*, 2011). For example, AChEI activity serves as a diagnostic tool for potential contact with carbamate pesticides or organophosphorus and nerve agents (Lessenger & Reese, 1999; Bajgar, 2004). It is very effective for use in the verification of treatment efficiency (Sramek & Cutler, 2000). Antidotal therapy or novel drugs for AD, with AChE properties, are tested by *in vitro* methods (Hoskovcova *et al.*, 2008; Guo *et al.*, 2010). The method established by Ellman *et al.* (1961) is the most commonly used assay of AChE activity (Pohanka *et al.*, 2011).

The method used in this study to determine the AChE enzyme inhibitory activity is very sensitive and applicable to both low enzyme concentrations and small amounts of tissue, and detailed kinetic studies for AChE activity is thus possible. The protocol is based on determining the progress of a hydrolysis reaction, which is followed by the measurement of a product formed from are action catalysed by AChE (Ellman *et al.*, 1961).

The AChE enzyme is obtained from commercially available human erythrocytes. Acetylthiocholine is used as the substrate, and the rate of thiocholine production, generated by the hydrolysis of acetylthiocholine, is measured. This measurement is possible since the generated thiol reacts withthe 5,5-dithiobis-2-nitrobenzoate ion (DTNB) reagent, which results in the production of the yellow anion, 5-thio-2-nitro-benzoic acid. This yellow anion can be monitored with a spectrophotometer at 412 nm (Ellman *et al.*, 1961).

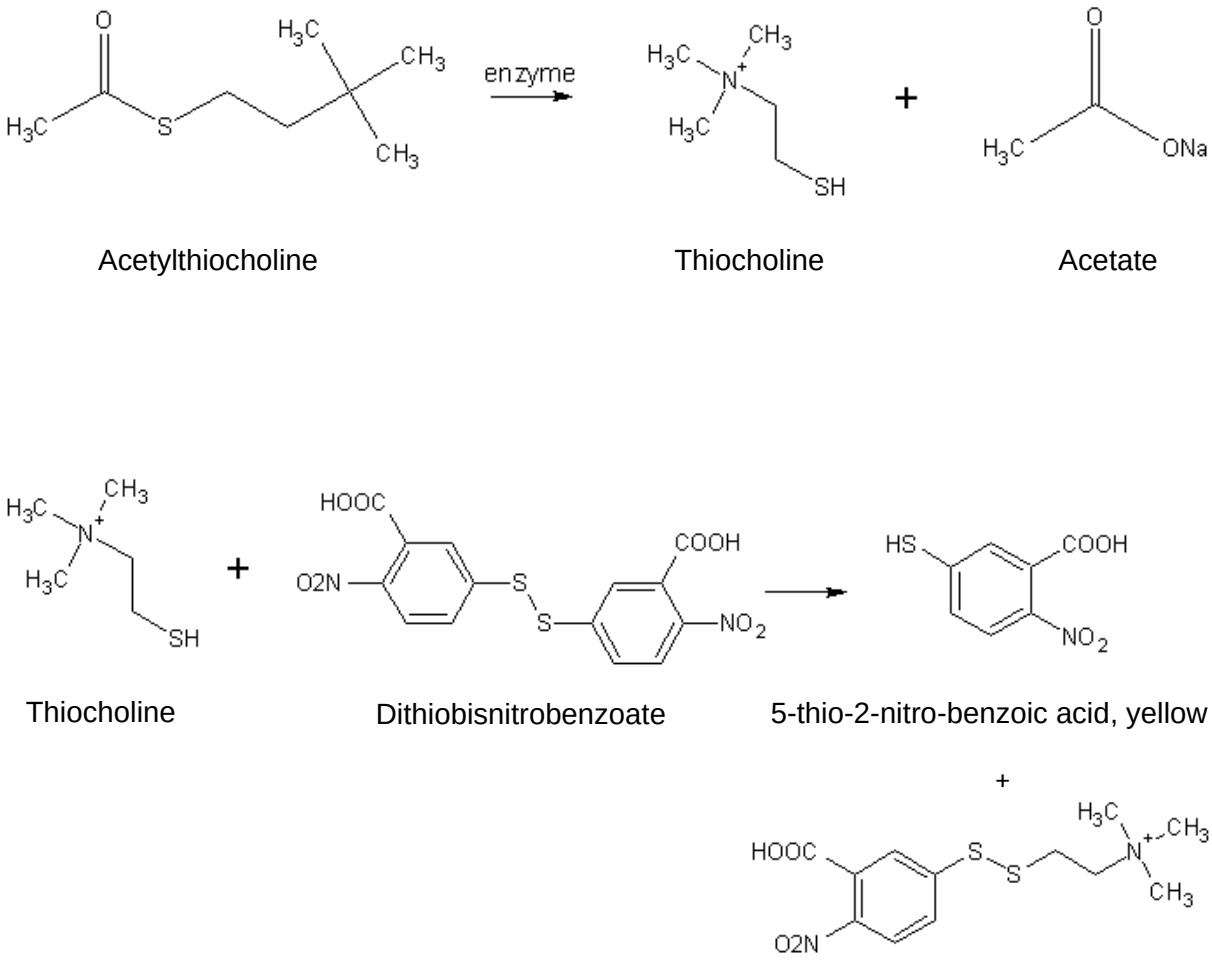


Figure 4.8 AChE activity determination through the reaction of thiocholine and DTNB.

4.4.2 Chemicals and instrumentation

AChE assay

AChE from human erythrocytes served as the enzyme source, and was obtained from Sigma-Aldrich. The following chemicals were used during the procedure and were also obtained from Sigma-Aldrich:

1. 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB)
2. Acetylthiocholine iodide (ATCh) (Substrate)
3. Methanol (Solvent for test inhibitors)
4. Potassium phosphate (K_2HPO_4/KH_2PO_4) (Buffer, pH 7.4)
5. Sodium bicarbonate ($NaHCO_3$) (Buffer, pH 7)

Spectrophotometric measurements were carried out with a Shimadzu MultiSpec-1501 photodiode array spectrophotometer.

4.4.3 Determination of the IC_{50} values

For the determination of IC_{50} values, sigmoidal dose-response curves were constructed and IC_{50} values were calculated using the Prismsoftware package (version 5.0) (GraphPad Software, La Jolla, USA). The IC_{50} values were determined in triplicate and expressed as mean ± SD.

4.4.4 Method

Recombinant human AChE was pre-aliquoted and stored at 5°C before determination of the IC_{50} values.

The enzyme reactions were prepared in potassium phosphate buffer (100 mM, pH 7.4) to a volume of 2000 µl. Stock solutions of DTNB (14 mM) were prepared freshly on the day of the experiment in potassium phosphate buffer (100 mM, pH 7.0).The reactions contained DTNB (50 µl; 350 µM final concentration), the test inhibitor (100 µl) and AChE (50 µl; 0.04 units/ml final concentration). Stock solutions of 0.003–100 µM of the test inhibitors were prepared in methanol and were added to the incubations to yield final concentrations of 5% methanol. Methanol (15 µl; 5%) was also added to reactions conducted in the absence of inhibitor. The reactions were preincubated for 15 min at 25 ºC and subsequently initiated with the addition of acetylthiocholine (50 µl; 500 µM). The formation of 5-thio-2-nitrobenzoic acid (ε = 13600 M^{-1}) were continuously monitored spectrophotometrically for a period of 8 min at 412 nm. The initial rates of 5-thio-2-nitrobenzoic acid formation were corrected by the rate of the nonenzymatic hydrolysis of acetylthiocholine recorded in the absence of enzyme. Sigmoidal dose–response curves were constructed using the one site competition model incorporated into the Prism version 5.0 software package (GraphPad Software, La Jolla, USA) and the IC_{50} values were determined in triplicate and are expressed as mean ± standard deviation (SD).

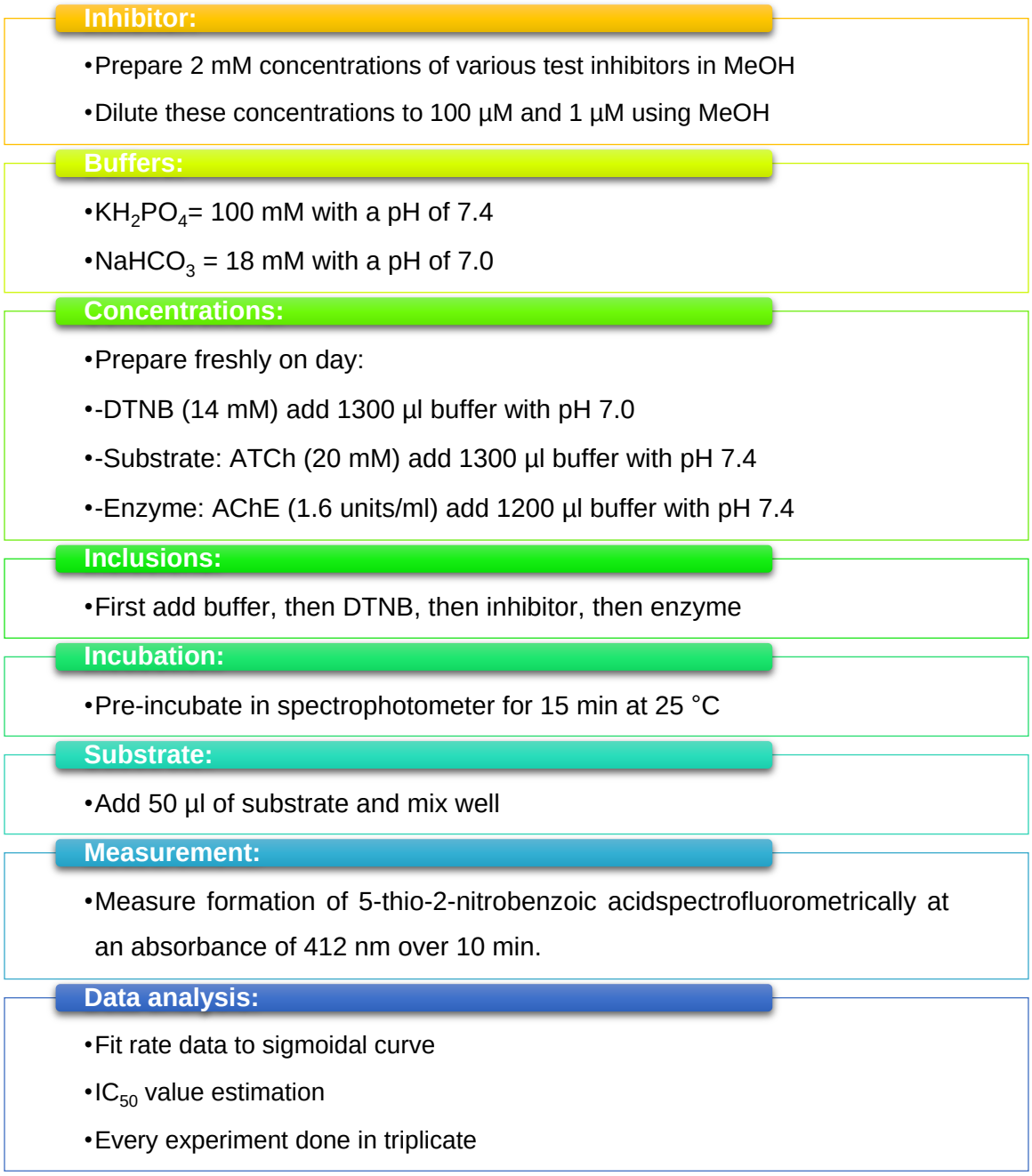


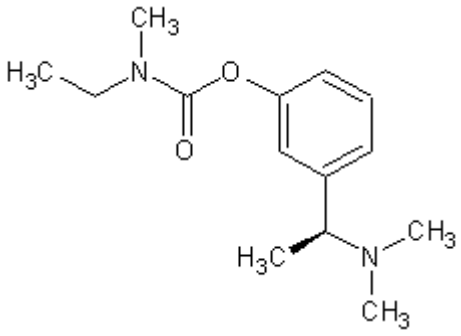
Figure 4.9 Flowdiagram representing the method for determination of IC_{50} values for the inhibition of AChE.

4.4.5 Results

Both the dihydroquinolinone-carbamates (**8a**-**g**; **9a**-**d**) and acetamidophenol-carbamates (**10a**-**h**; **11a**-**i**) were screened for their ability to inhibit AChE. Results from this assay was disappointing as none of the synthesised derivatives showed meaningful inhibitory activity with all compounds exhibiting IC_{50} values greater than 100 µM.

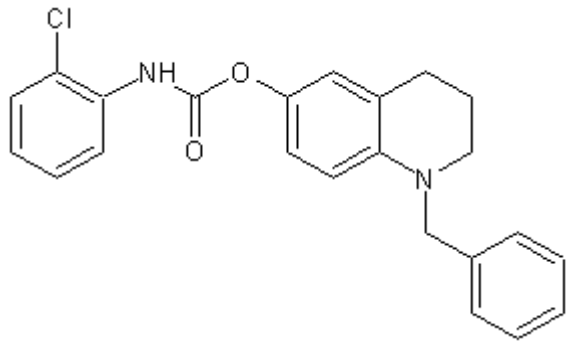
4.4.6 Discussion of results

As mentioned in chapter 1, the carbamate group often occurs in compounds with potent AChE inhibitory activity. Rivastigmine, which is used clinically, has a literature IC_{50} value of 0.011 µM (Li *et al.*, 2014).

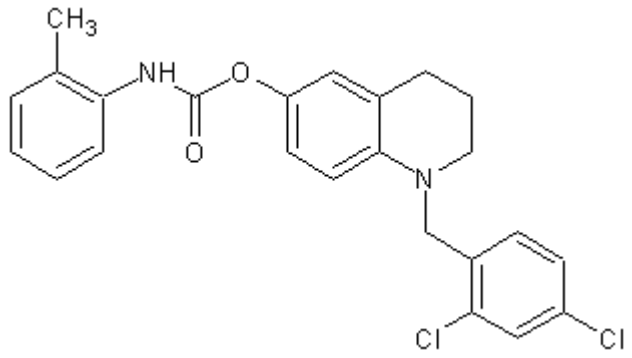


4 Rivastigmine (IC_{50} AChE: 0.011 µM)

Similarly, a series of carbamate substituted tetrahydroquinolines synthesised by Kuldeep and co-workers (Roy *et al.*, 2012) also showed promising AChE inhibitory activity. Although a direct comparison between the activities of the compounds synthesised in this study and those synthesised by Roy and co-workers (due to a difference in assay conditions), is not possible, it is clear that the structural features incorporated in their compounds are more suitable for inhibition of AChE. It is postulated that substitution on the quinolinenitrogen may be required for activity and that the additional carbonyl group as present in the dihydroquinolinone scaffold of the compounds of the current study, may affect activity adversely. Further investigation in this regard is required.



5 IC_{50} AChE: 3.31 µM



6 IC_{50}AChE: 2.57µM

4.5 SUMMARY

In this chapter, *in vitro* biological evaluation of the synthesised dihydroquinolinone-carbamate and acetamidophenol-carbamatederivatives were discussed. These derivatives were screened for their ability to inhibit MAO-A and MAO-B activity using a fluorescence assay with kynuramine as substrate. The compounds' ability to inhibit AChE activity using commercially available AChE from human erythrocytes with acetylthiocholine as substrate was also examined.

The results of the MAO inhibition study suggest that some of the synthesised compounds possess selective inhibitory activity for the MAO-B enzyme, but these potencies are generally rather poor. The results of the AChE inhibition study suggest that the synthesised compounds do not inhibit the AChE enzyme.

CHAPTER 5
CONCLUSION

Neurodegenerative diseases, such as AD and PD, severely impair the normal daily activities of sufferers and result in high social, family and financial costs. Current therapies are furthermore inadequate, highlighting the importance of research in this area (Trippier *et al.*, 2013).

AD is the most common neurological disorder in elderly patients and this irreversible disease affects more than 70% of neurodegenerative casese (Reitz & Mayeux, 2014). AD is clinically characterised by progressive cognitive impairment, the decline in learning ability and memory loss accompanied by a reduction in the ability to perform normal daily routines. Neuropsychiatric symptoms include apathy, irritability, depression, verbal and physical agitation, anxiety, hallucinations and delusions (Lyketsos *et al.*, 2011). Selective neuronal and synaptic loss, neurofibrillary tangles (NFT's) composed of hyperphosphorylated forms of the tau protein and extracellular neuritic plaques containing the Aβ peptide are characteristic neuropathological findings in AD (Klafki *et al.*, 2006).

The region of the brain which consists mainly of cholinergic neurons is most affected by the neuronal loss in AD, resulting in a decrease in ACh levels. ACh, a neurotransmitter that is important for cognitive function (Lane *et al.*, 2004), is metabolised by two types of cholinesterases namely, AChE and BuChE. AChE selectively hydrolyses ACh at the cholinergic synapses and inhibition of AChE will thus result in increased ACh concentrations in the brain. AChEIs thus form the mainstay of current AD treatment (Terry & Buccafusco, 2003). On the other hand, MAO-B inhibitors are used as additive symptomatic treatment in AD and are considered to be potentially neuroprotective (Guzior *et al.*, 2015). The multifactorial nature of the disease complicates therapy and currently there is no effective therapy that cures, stop or even slow the progression of AD (Bautista-Aguilera *et al.*, 2014).

The progressive neurodegenerative disorder, PD, is pathologically characterised by the death of dopaminergic neurons in the substantia nigra resulting in motor symptoms which include muscle rigidity, bradykinesia, an impairment of postural balance and resting tremor (Dauer & Przedborski, 2003). The MAO enzymes, MAO-A and MAO-B, are responsible for the oxidative degradation of amine neurotransmitters such as DA, epinephrine and 5-HT. Inhibition of these enzymes can thus modulate the levels of these neurotransmitters in the brain. The inhibition of the MAO-B enzyme in the brain, for example, will increase DA levels,

and MAO-B inhibitors are used in the symptomatic treatment of PD, while it is also considered as potentially neuroprotective (Youdim & Bakhle, 2006).

Non-motor symptoms associated with PD include sleep dysfunction, salivation, forgetfulness, urinary urgency, hyposmia, dysautonomia and constipation. During the latter stages of PD, cognitive impairment, better known as PDD, often develops, reducing the patient's quality of life and increasing the cost of care (Emre, 2003).

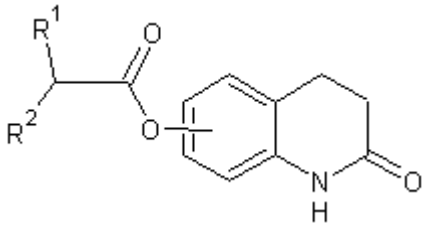
There is no cure for PDD and as for AD, treatment only gives symptomatic relief, there is thus an urgent need for novel drugs for the treatment and neuroprotection in AD, PD and PDD. The use of multitarget compounds has the additional advantage of acting at different targets without increasing the number of drugs that a patient has to take. Multitarget compounds also better address the multifactorial nature of these complicated neurodegenerative diseases.

Aim

The quinolinone scaffold has been established as privileged for the inhibition of MAO-B (Meiring *et al.*, 2013), while the carbamate moiety is often present in the scaffolds of AChE inhibitors. Thus, the aim of the current study was to design, synthesise and evaluate molecules that contained both carbamate and quinolinone moieties in order to obtain polyfunctional entities with inhibitory activities against both MAO and AChE. The ideal inhibitor in this case would be a selective, reversible MAO-B and reversible AChE inhibitor with potential use in the treatment of PD, PDD and AD.

Methods

Compounds were synthesised in one step using standard synthetic procedures obtained from literature. Commercially available 6-hydroxy-3,4-dihydro-2(1*H*)-quinolinone, 7-hydroxy-3,4-dihydro-2(1*H*)-quinolinone, 3- and 4-acetamidophenol were reacted with selected carbamoyl chlorides under basic conditions to yield the target compounds (**8-11**). Compounds were characterised using NMR and IR spectroscopy as well as mass spectrometry. Melting points were determined and purity determined with HPLC.



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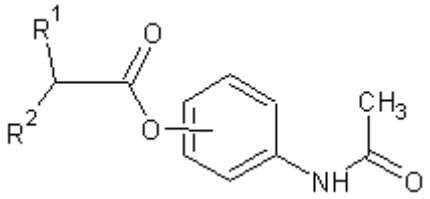


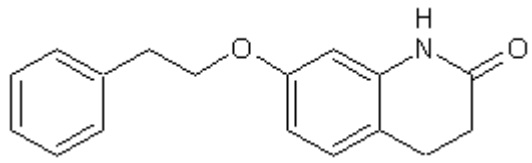
10,11

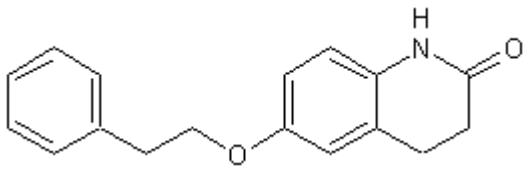
MAO inhibitory activities of the synthesised compounds were determined with a fluorometric assay by using recombinant human MAO-A and MAO-B. Kynuramine was used as substrate for both isoforms of MAO.

AChE inhibitory activities of the synthesised compounds were assessed with a spectrophotometric assay by using recombinant human AChE with acetylthiocholine as the substrate.

Results and Discussion

Twenty eight compounds comprising eleven dihydroquinolinone-carbamate derivatives and seventeen acetomidophenol-carbamate derivatives were successfully synthesised, generally in low yields. Disappointingly, the MAO assays revealed that most of the synthesised compounds were either devoid of MAO inhibitory activity or only showed weak activity. The most potent MAO-B inhibitor was compound **8g** with an IC_{50} value of 3.73 µM. This is in contrast to the results obtained by Meiring *et al.* (2013) where potent MAO-B inhibitory activity was illustrated for related dihydroquinolinones (**1**,**2**). It is postulated that the rigidity imposed by the carbamate side chain is at least, in part responsible for the loss in activity. The increase in the number of heteroatoms in the side chain could also be a contributing factor. The active site of MAO-B is quite hydrophobic due to the number of aromatic and alphatic amino acids that lines the substrate binding site (Walker & Edmondson, 1994), which can be undesirable for carbamate binding. These results clearly illustrate that substitution with carbamate-containing side chains is not a viable option when designing dihydroquinolinone MAO inhibitors. Furthermore, opening of the dihydroquinolinone ring also appears to be detrimental for MAO inhibition activity (for example compare **8g** vs.**11g**).





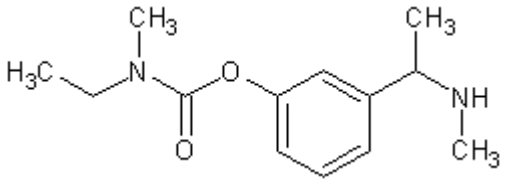
1 IC_{50} MAO-A: 53.7 µM

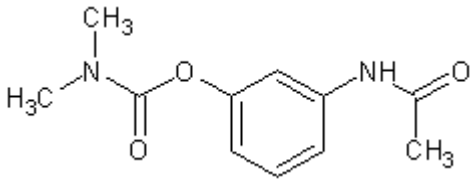
IC_{50} MAO-B: 0.191 µM

2 IC_{50} MAO-A: 22.5 µM

IC_{50} MAO-B: 2.33 µM

Similarly, the results of the AChE inhibition assays were also disappointing since none of the synthesised compounds displayed inhibitory activity. When the structures of the compounds from this study (e.g **11b**) are compared to those of rivastigmine (**4**) for example, the carbamate side chains are very similar. It may therefore be concluded that the replacement of the amine (as in rivastigmine) with amide-containing side chains, as present in the compounds of this study, results in loss of activity. It is hypothesised that, while protonation of the amine in rivastigmine is possible, the amides can no longer protonate at physiological pH, resulting in loss of binding and inhibition activity. The configuration of atoms in these compounds could also be a further contributing factor.





4 **11b**

Further structural modifications are thus required to optimise these compounds as potential dual-acting reversible inhibitors of MAO-B and AChE.

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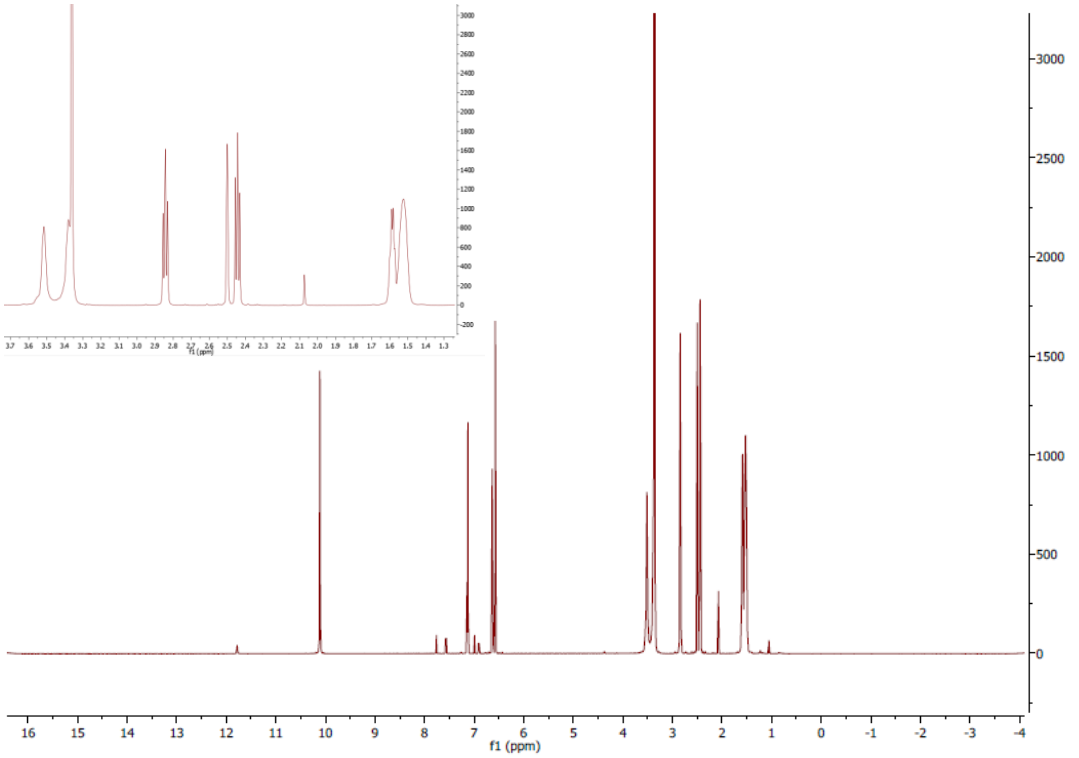
ADDENDUM

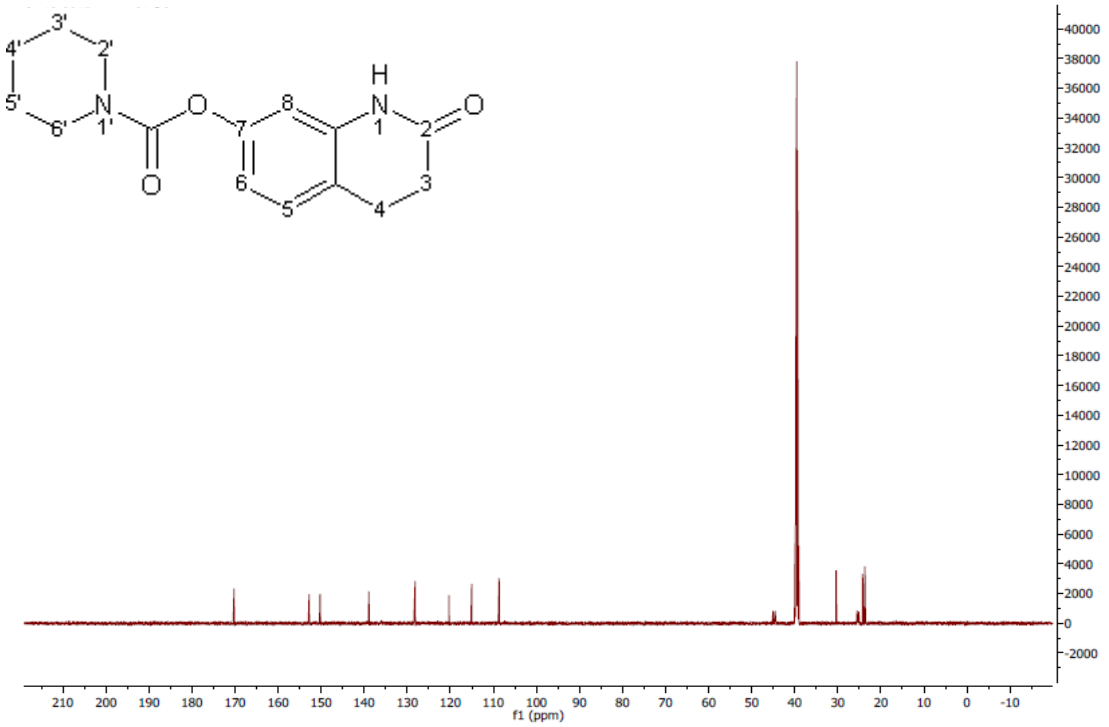
^{1}H NMR AND ^{13}C NMR SPECTRA

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl piperidine-1-carboxylate (**8a**)

^{1}H-NMR

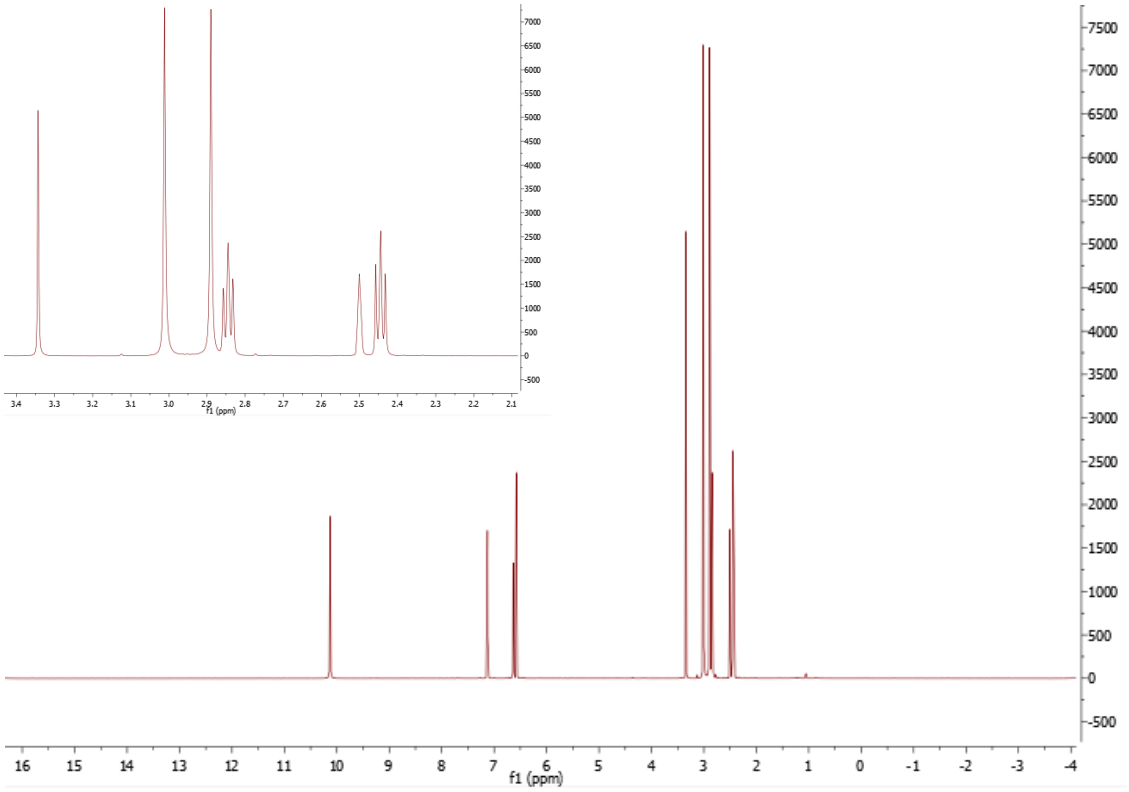


^{13}C-NMR



2-oxo-1,2,3,4-tetrahydroquinolin-7-yl dimethylcarbamate (**8b**)

^{1}H-NMR



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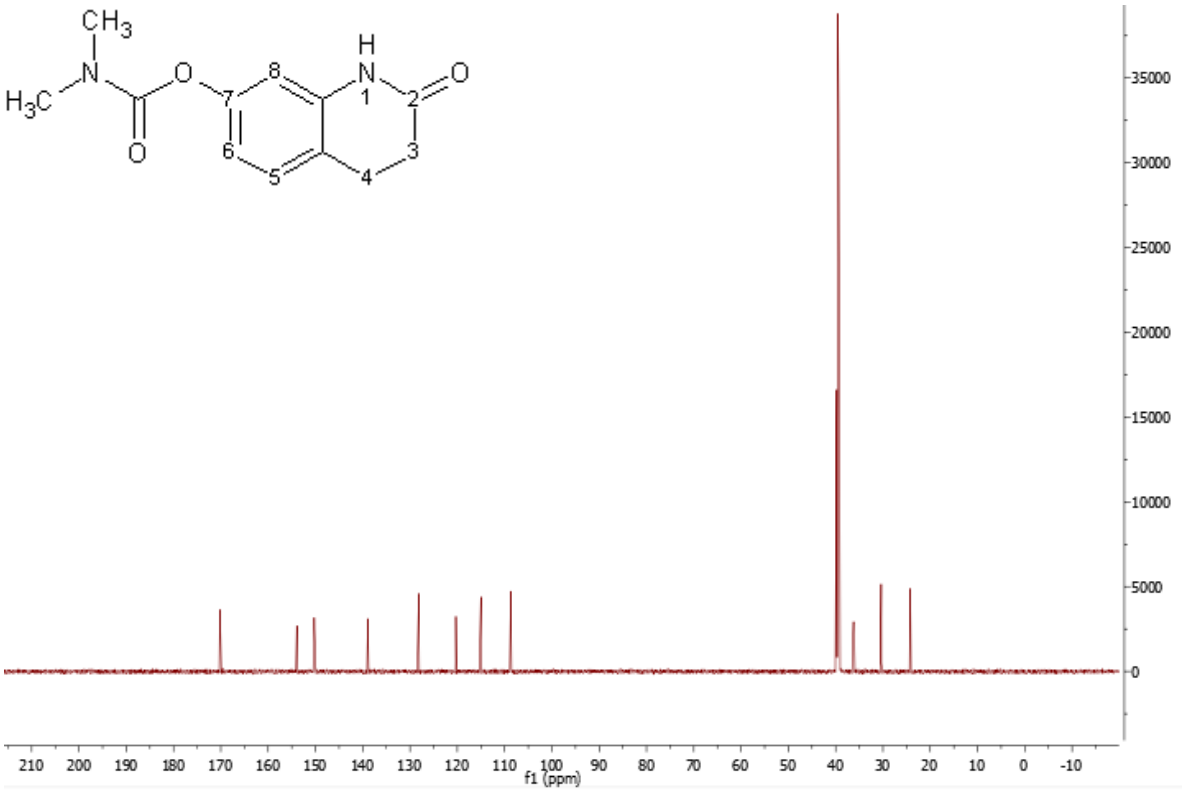
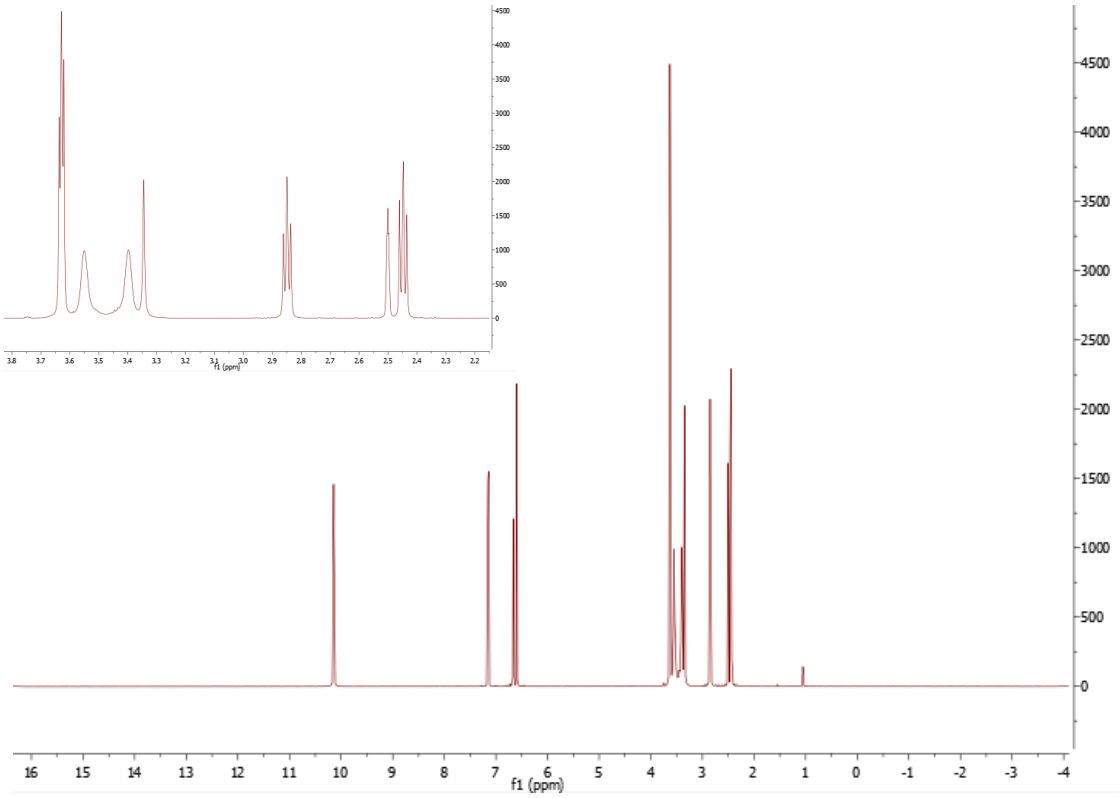


2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**8c**)

^{1}H-NMR



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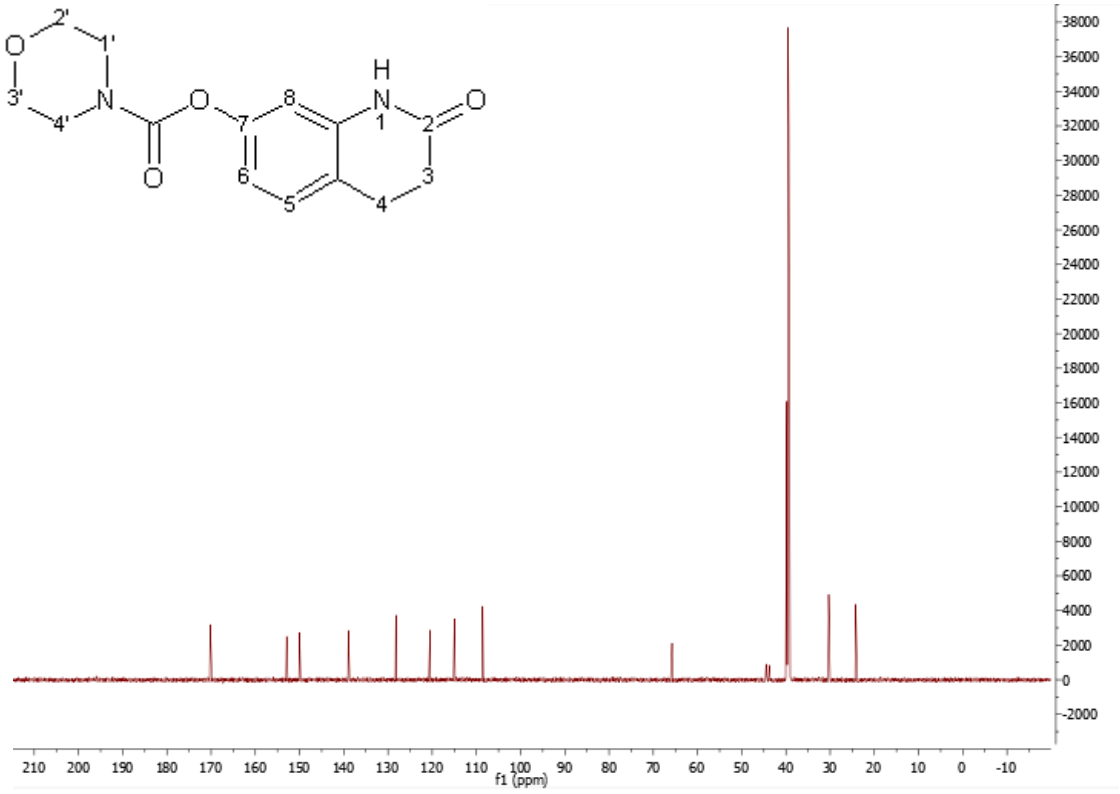
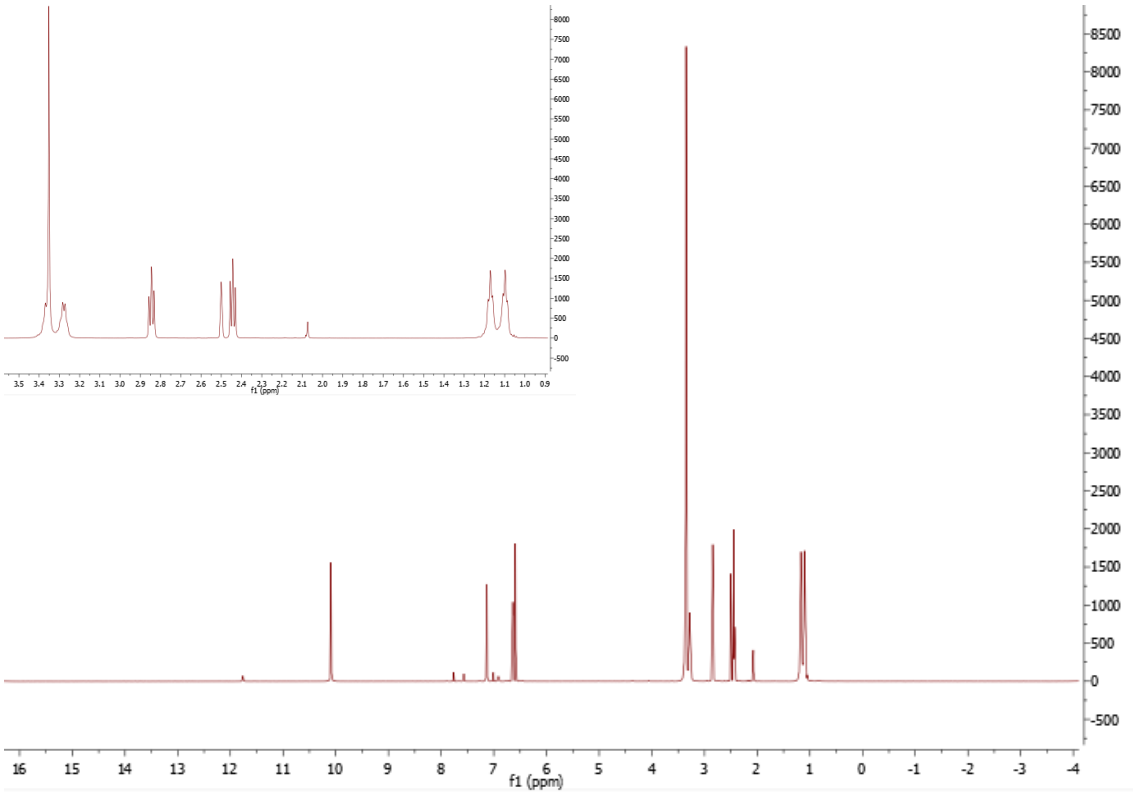


2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diethylcarbamate (**8d**)

^{1}H-NMR



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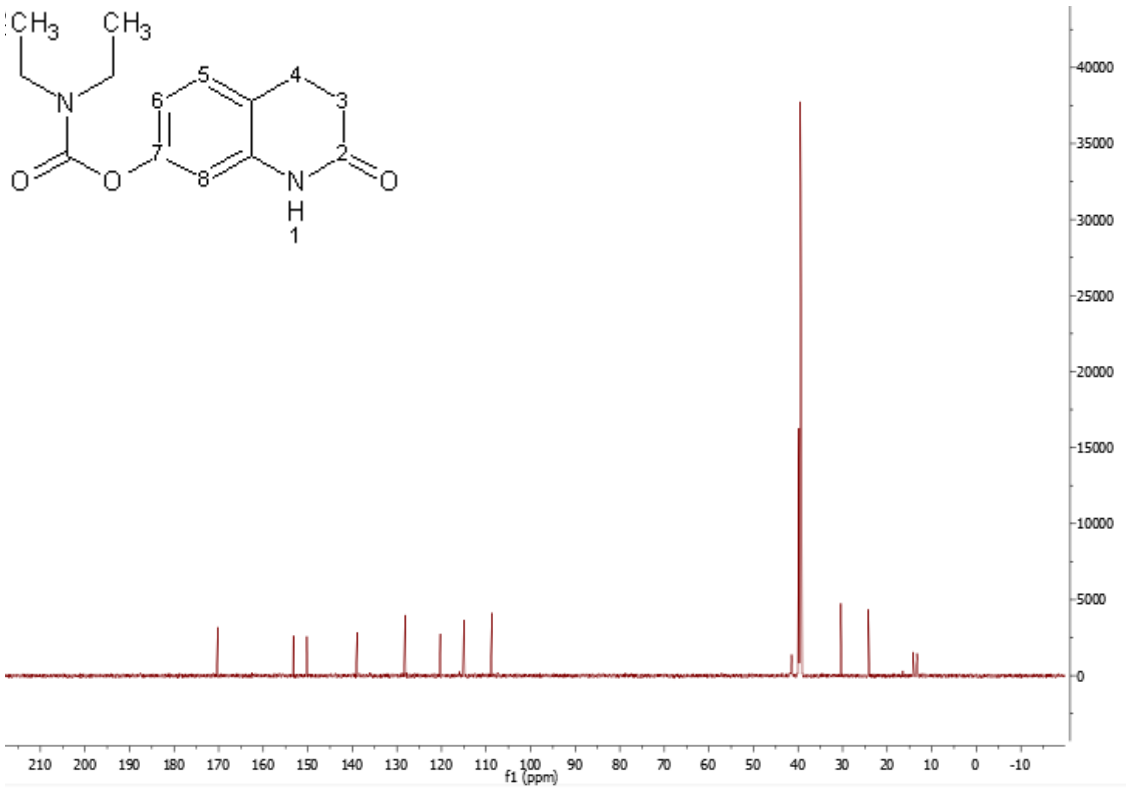
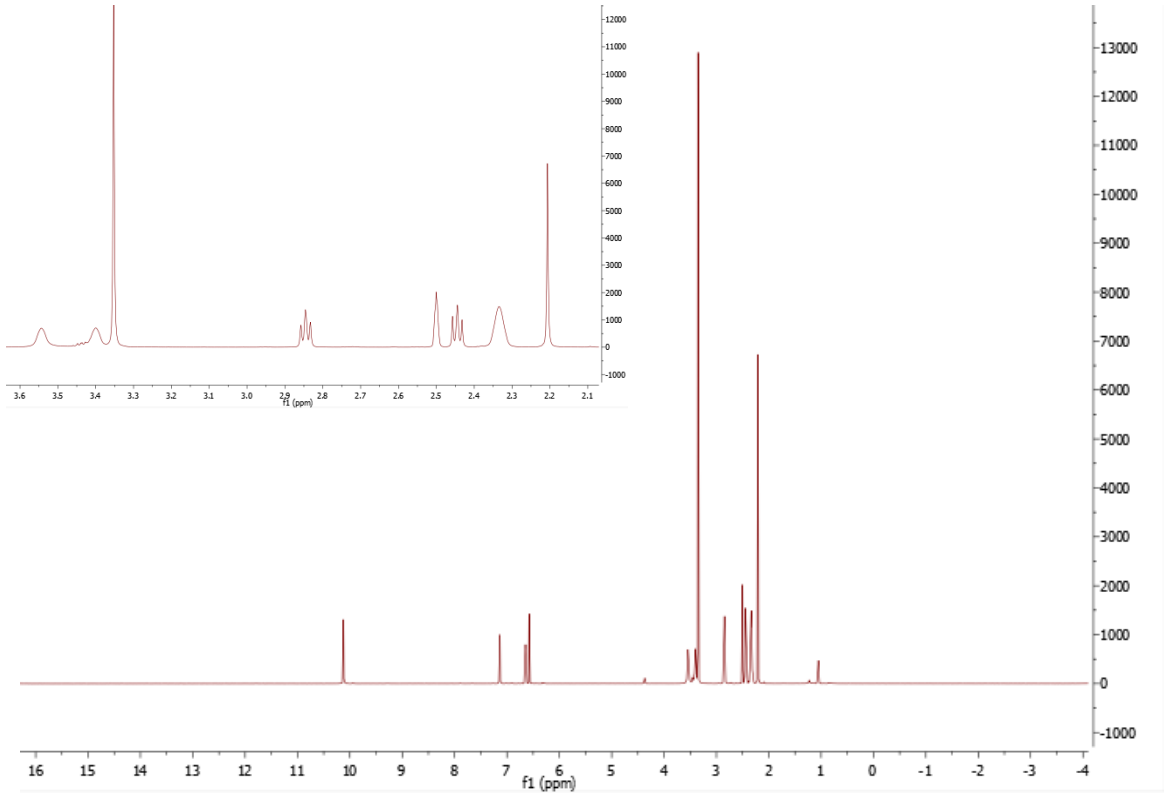


2-oxo-1,2,3,4-tetrahydroquinolin-7-yl 4-methylpiperazine-1-carboxylate (**8e**)

^{1}H-NMR



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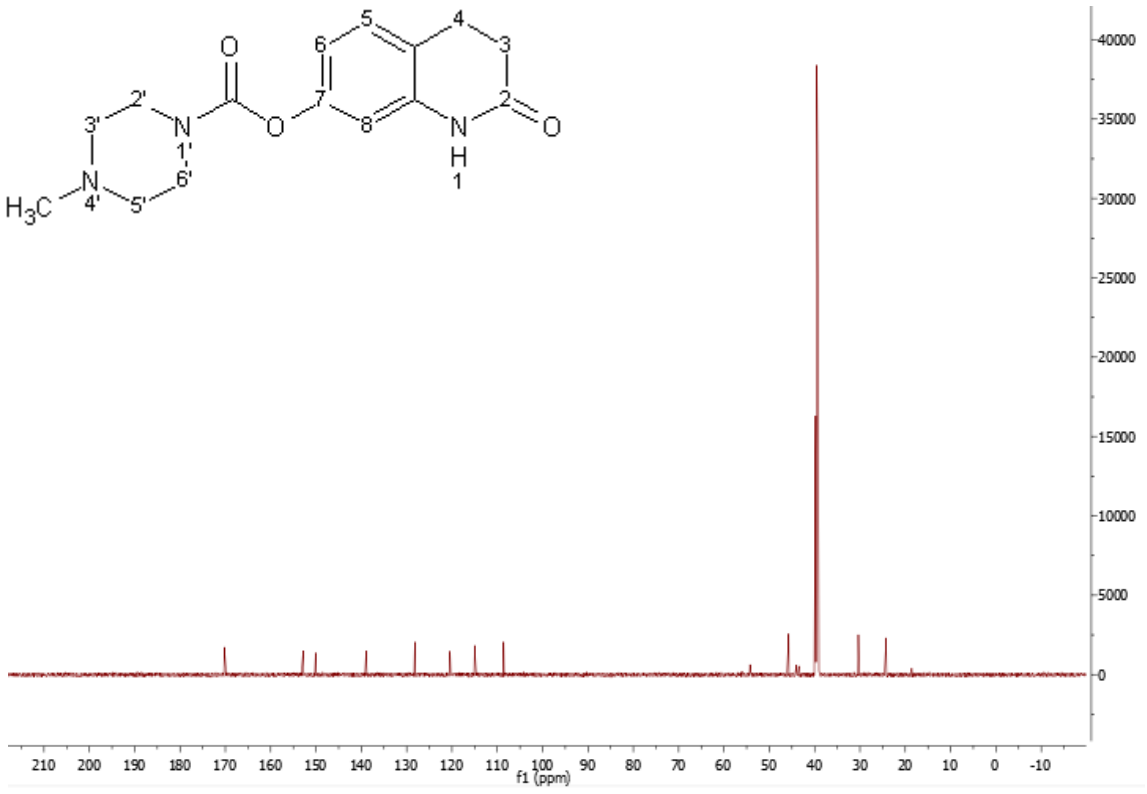

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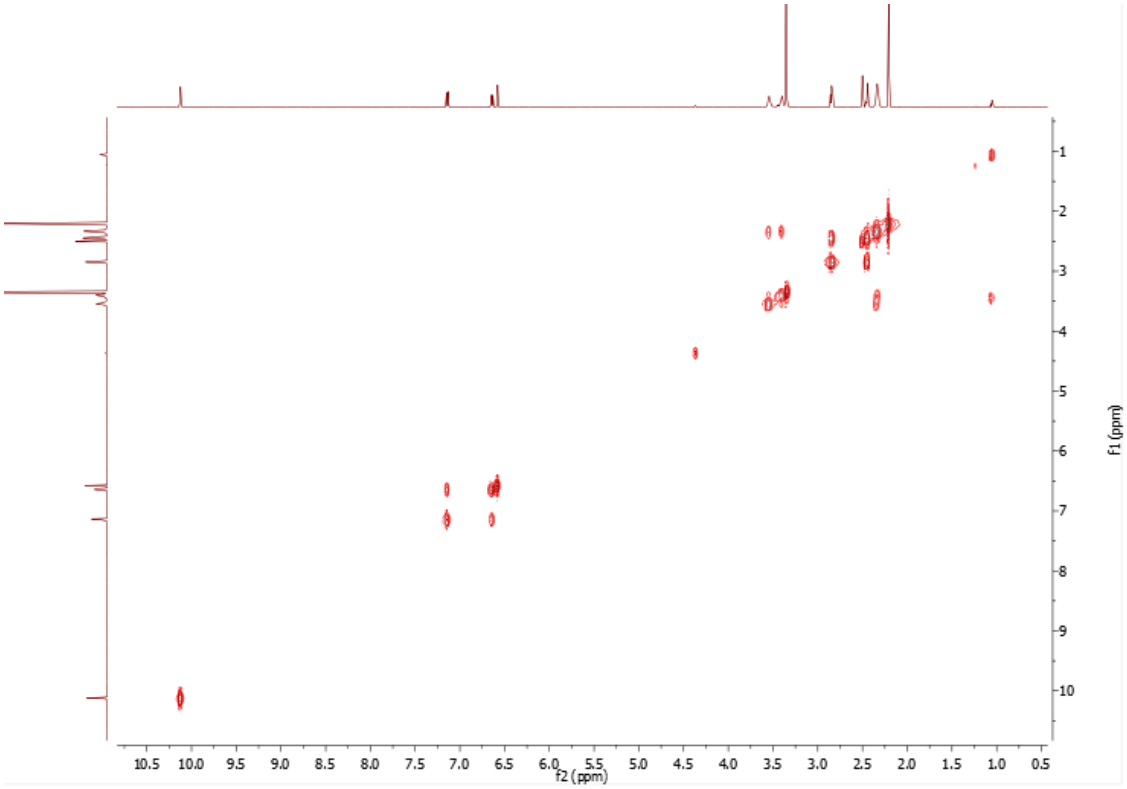

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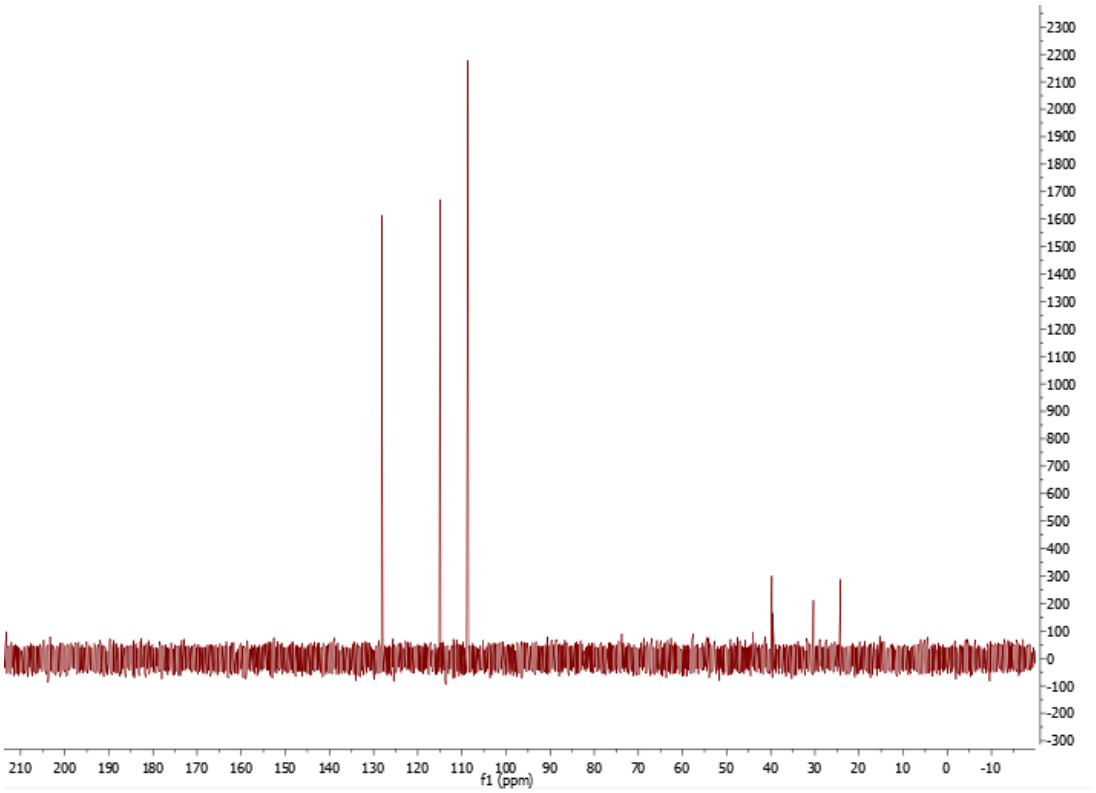

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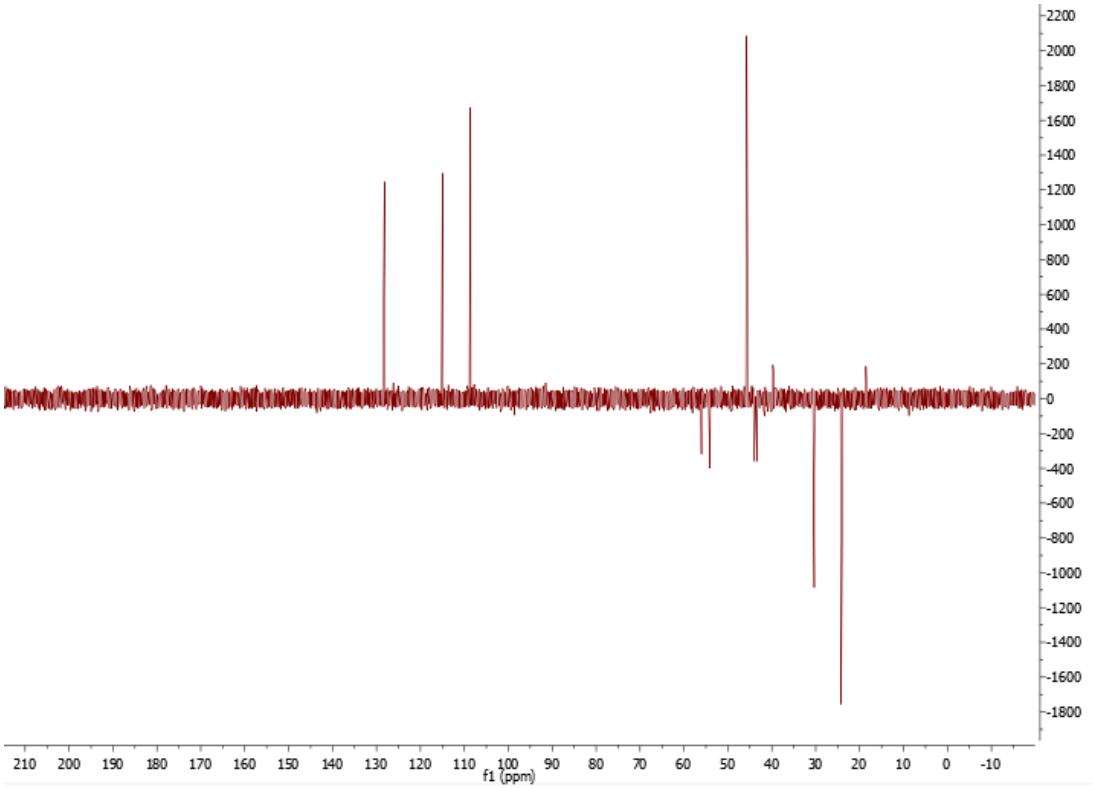

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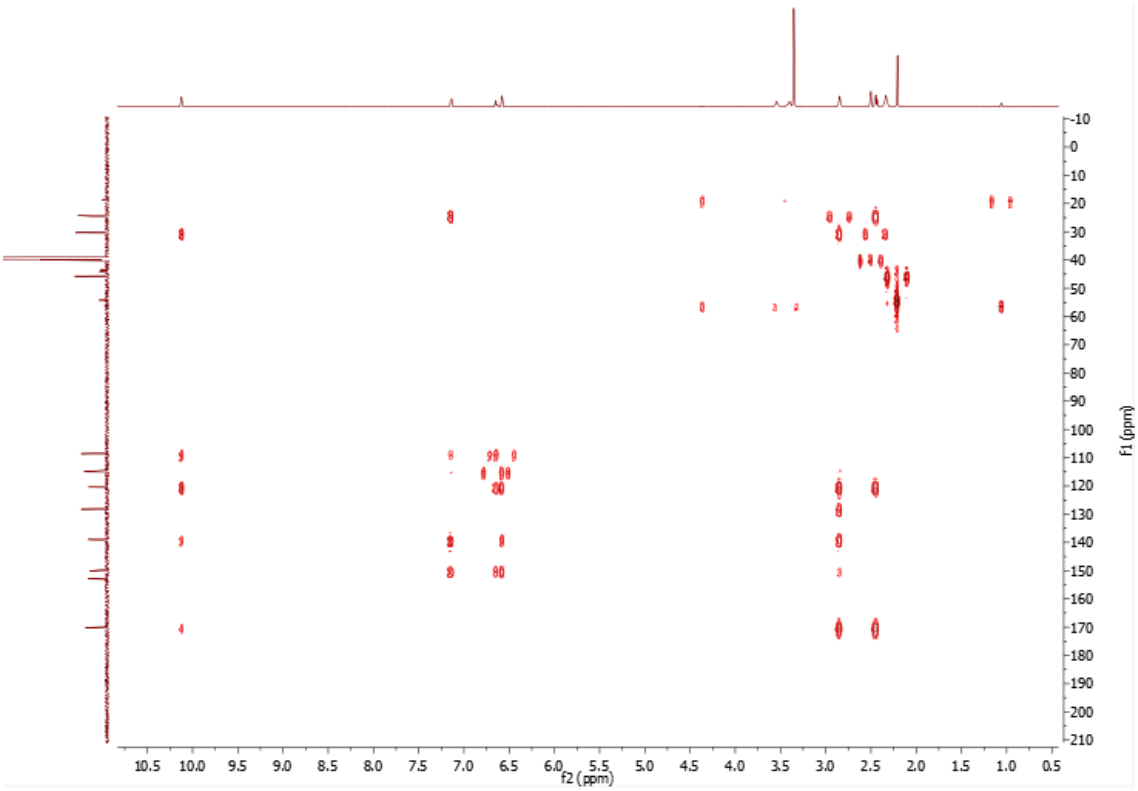

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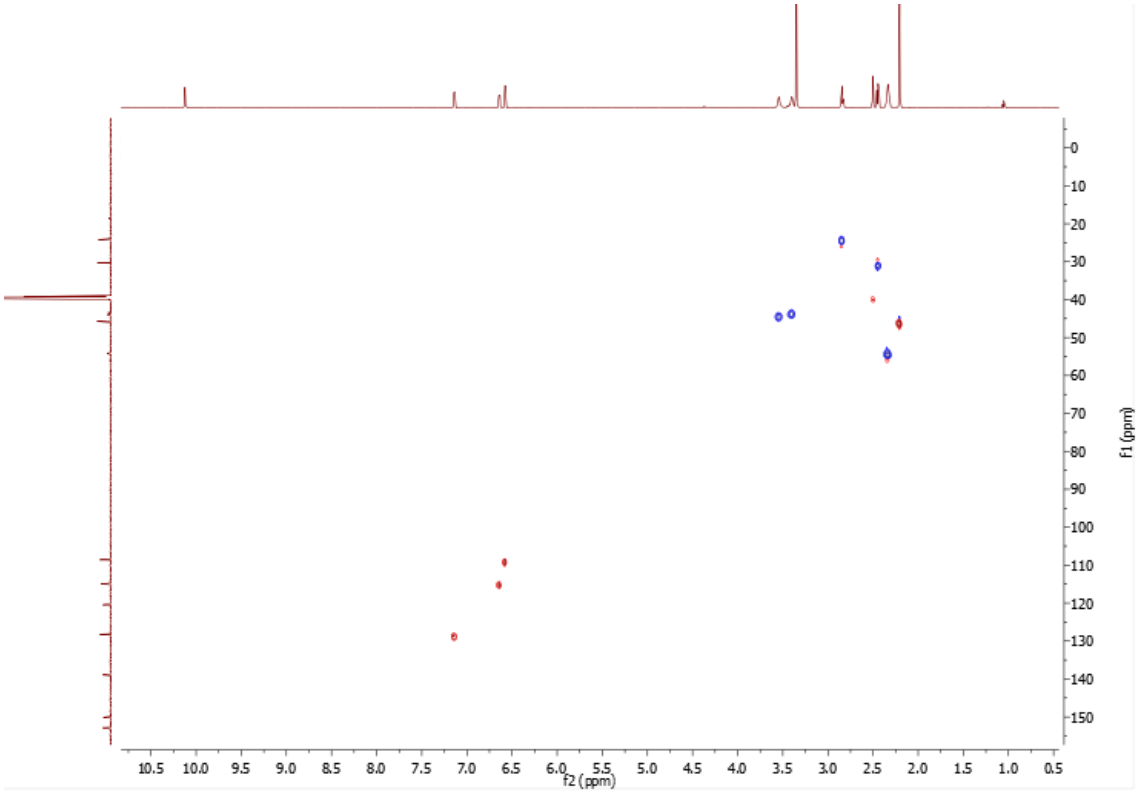
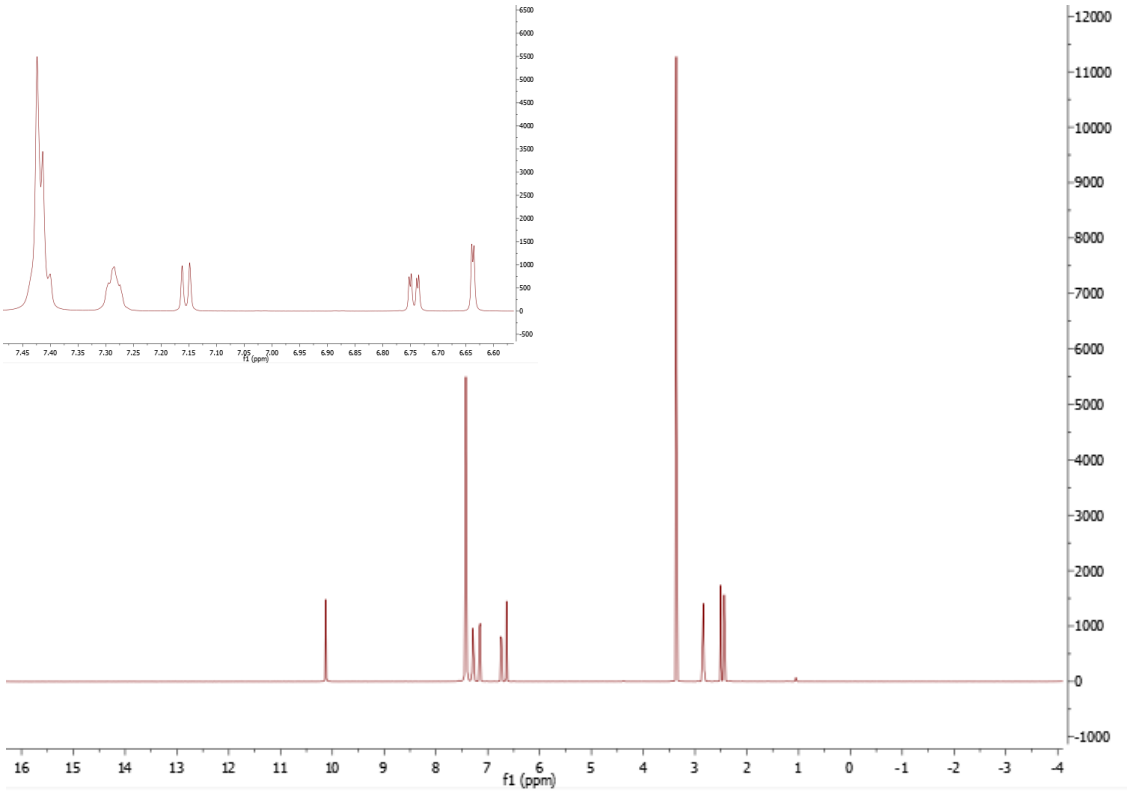


2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diphenylcarbamate (**8f**)

^{1}H-NMR



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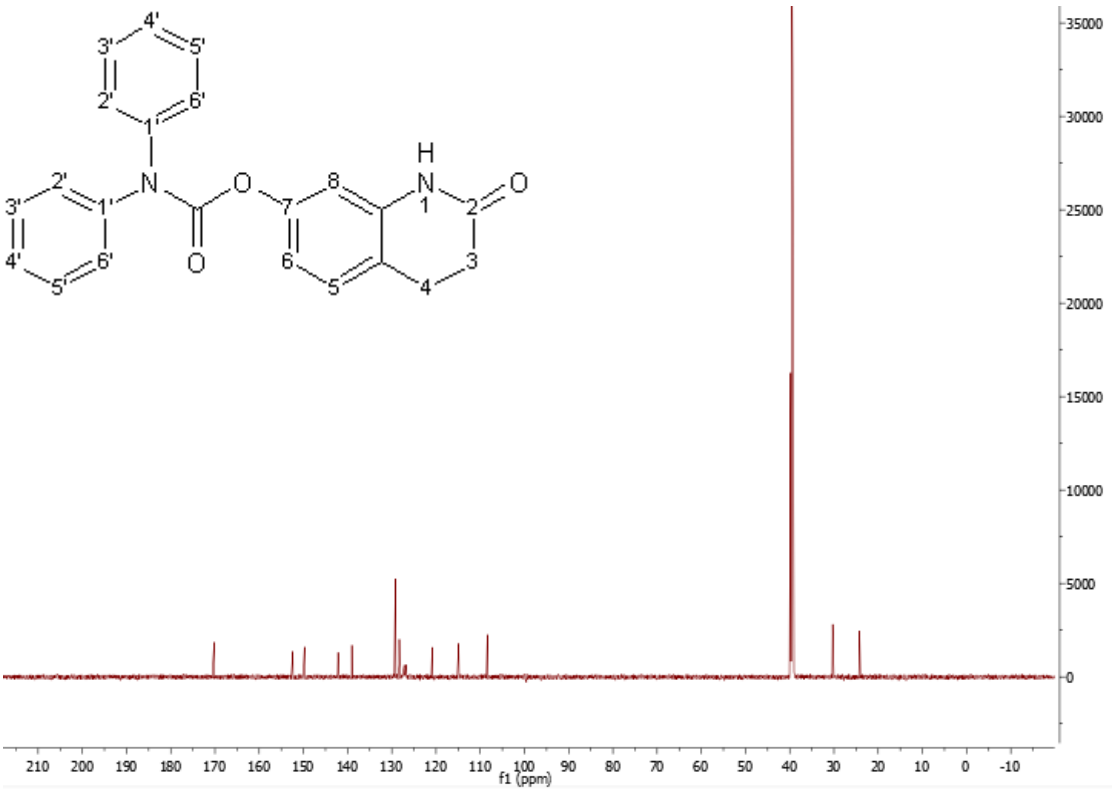
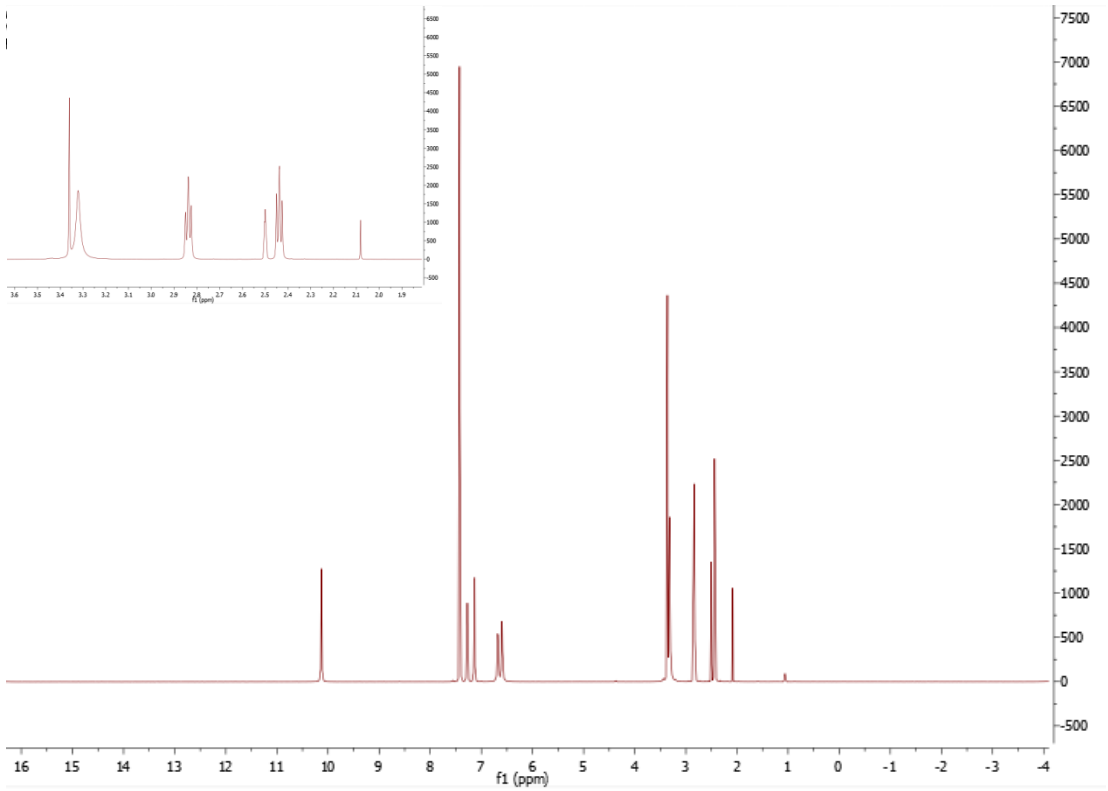


2-oxo-1,2,3,4-tetrahydroquinolin-7-yl methyl(phenyl)carbamate (**8g**)

^{1}H-NMR



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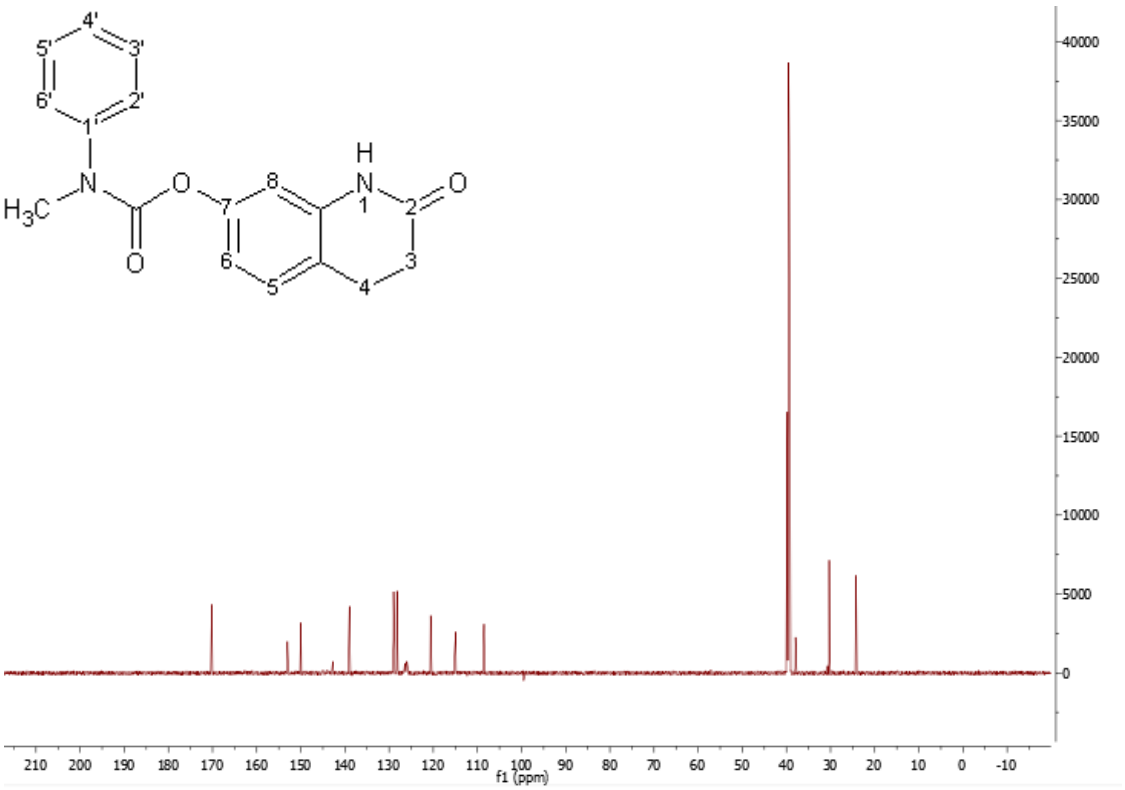

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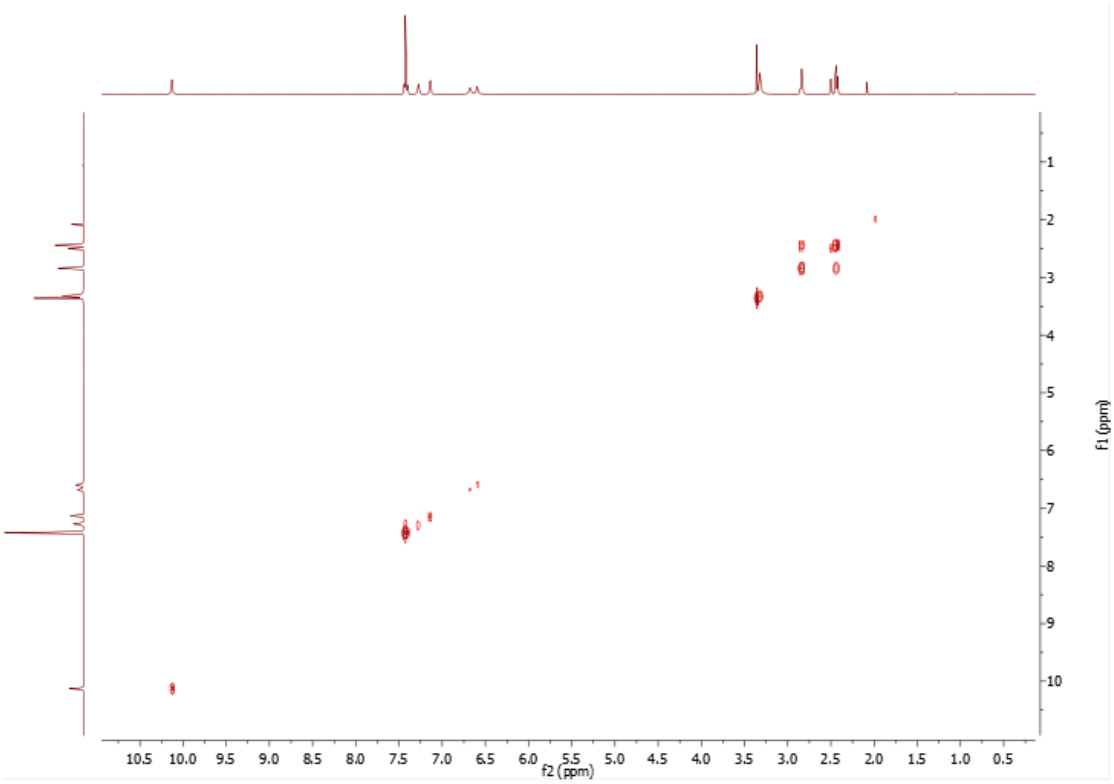

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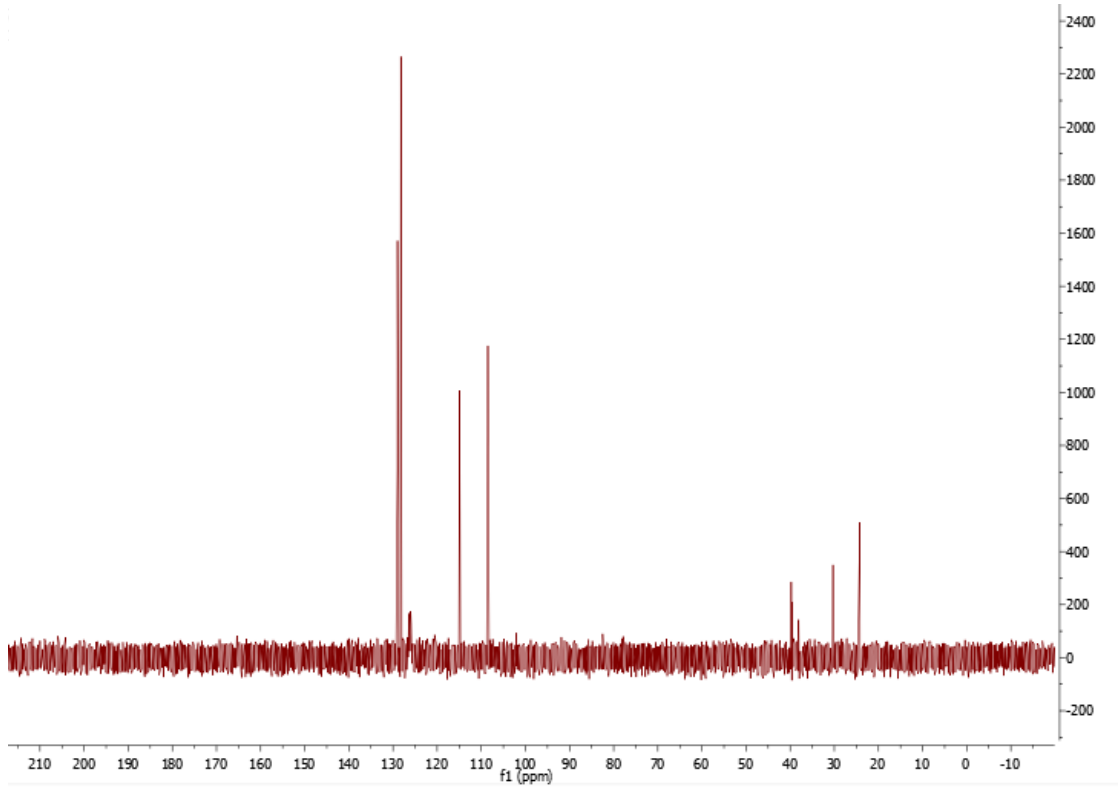

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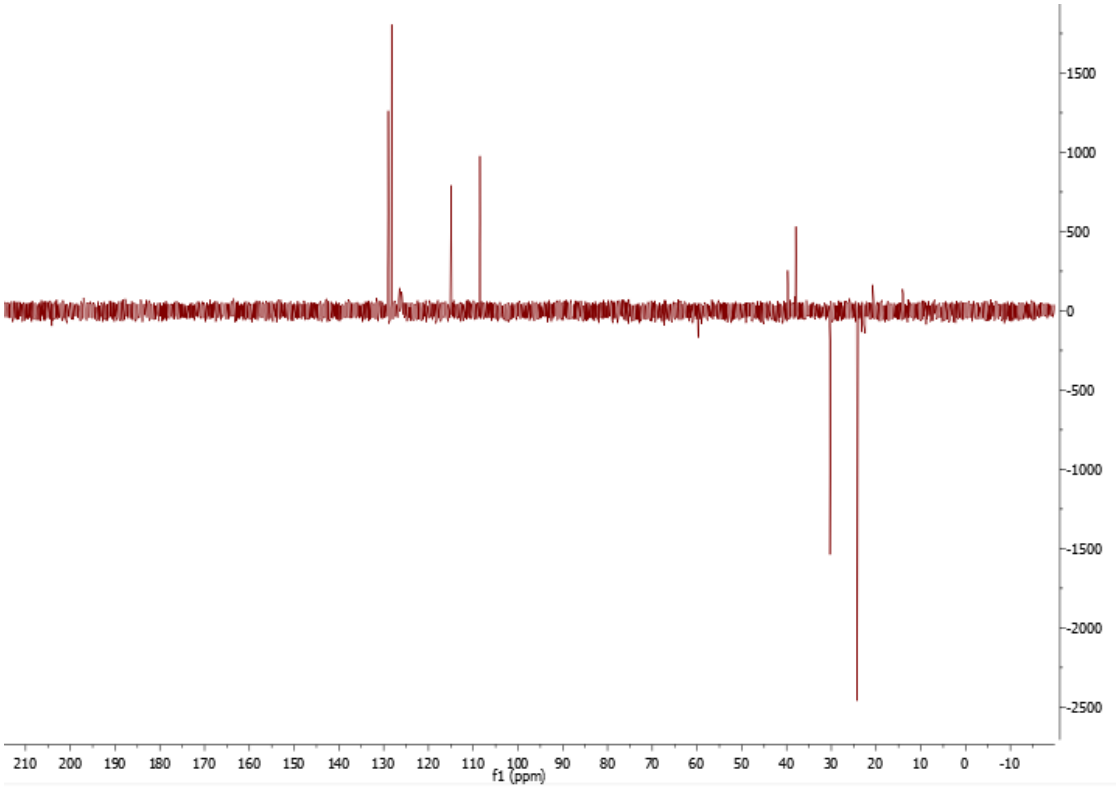

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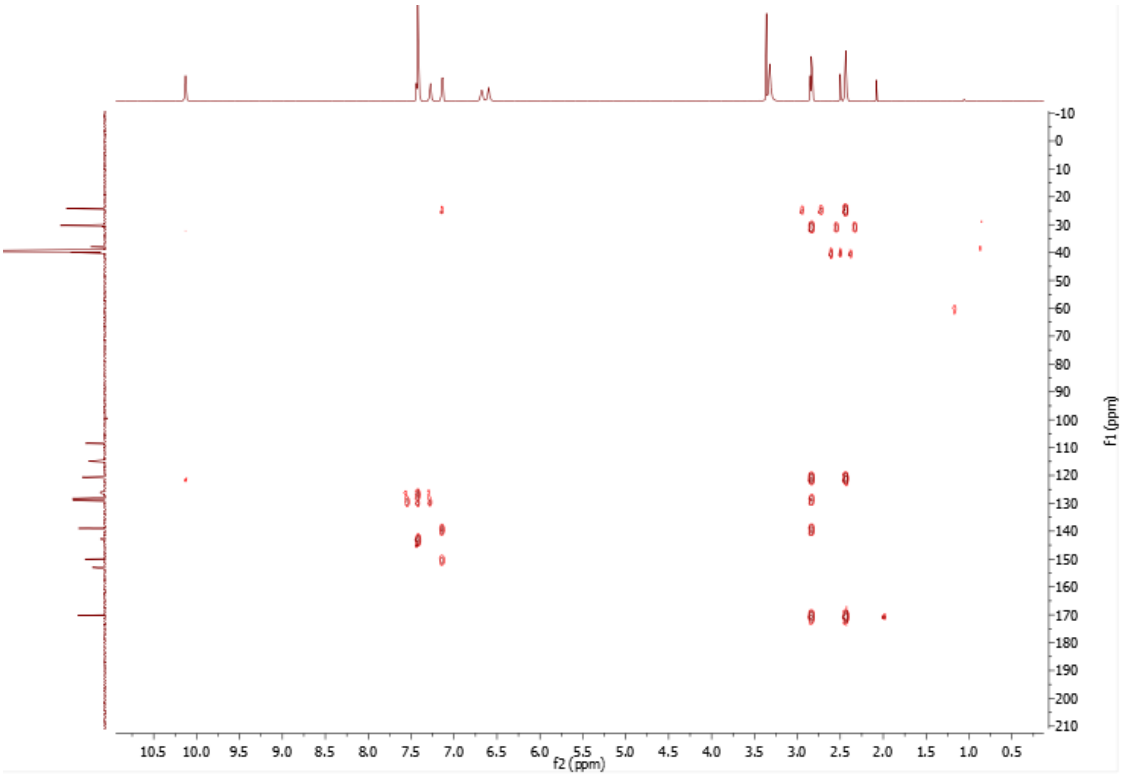

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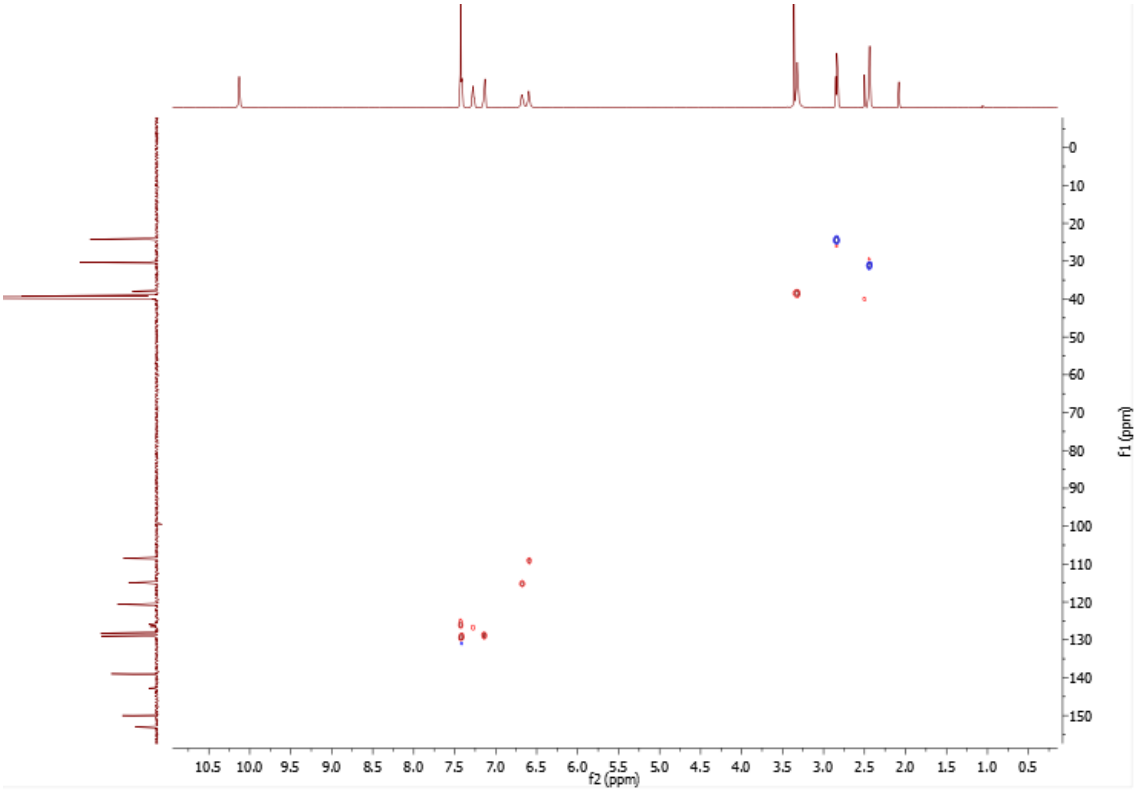
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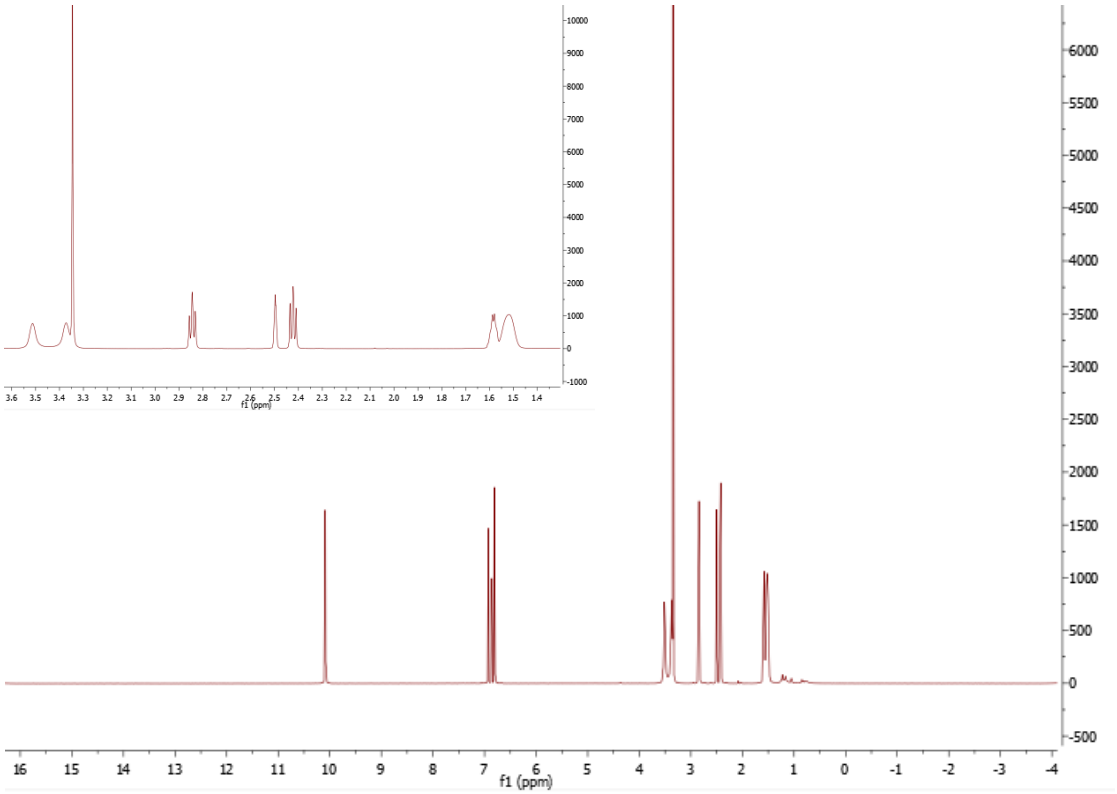
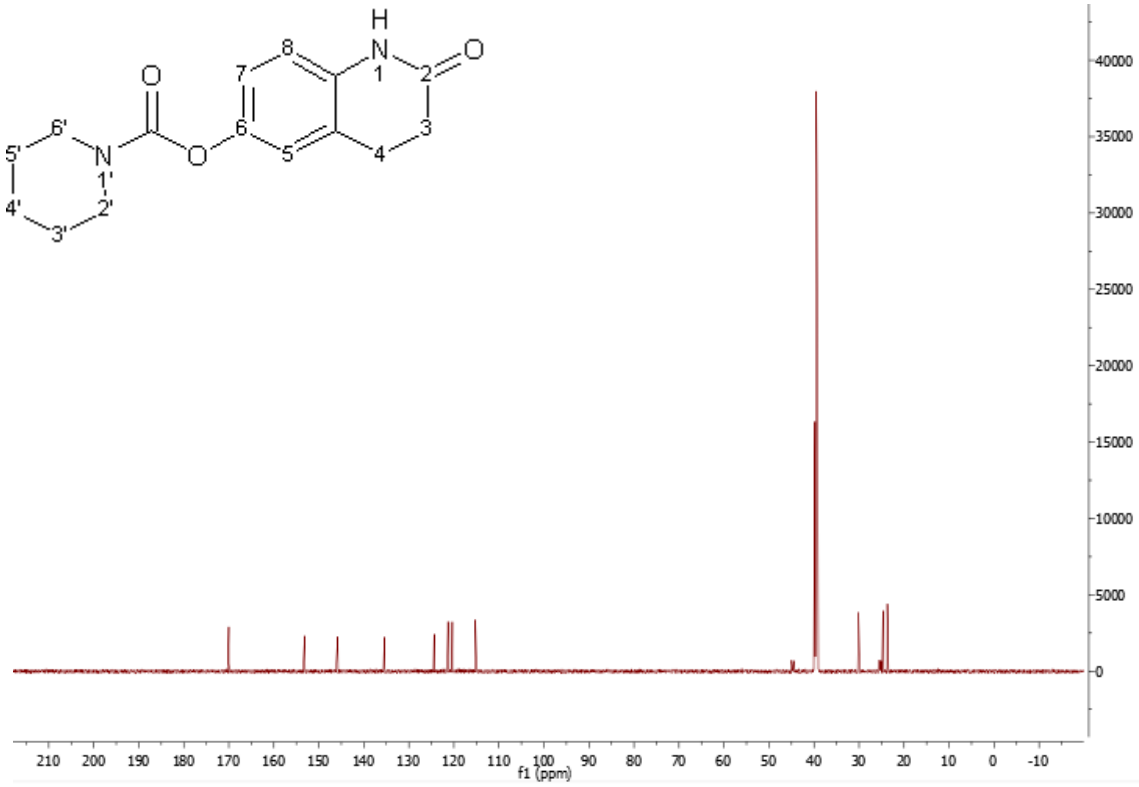
f1 (ppm)
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f2 (ppm)

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl piperidine-1-carboxylate (**9a**)

^{1}H-NMR

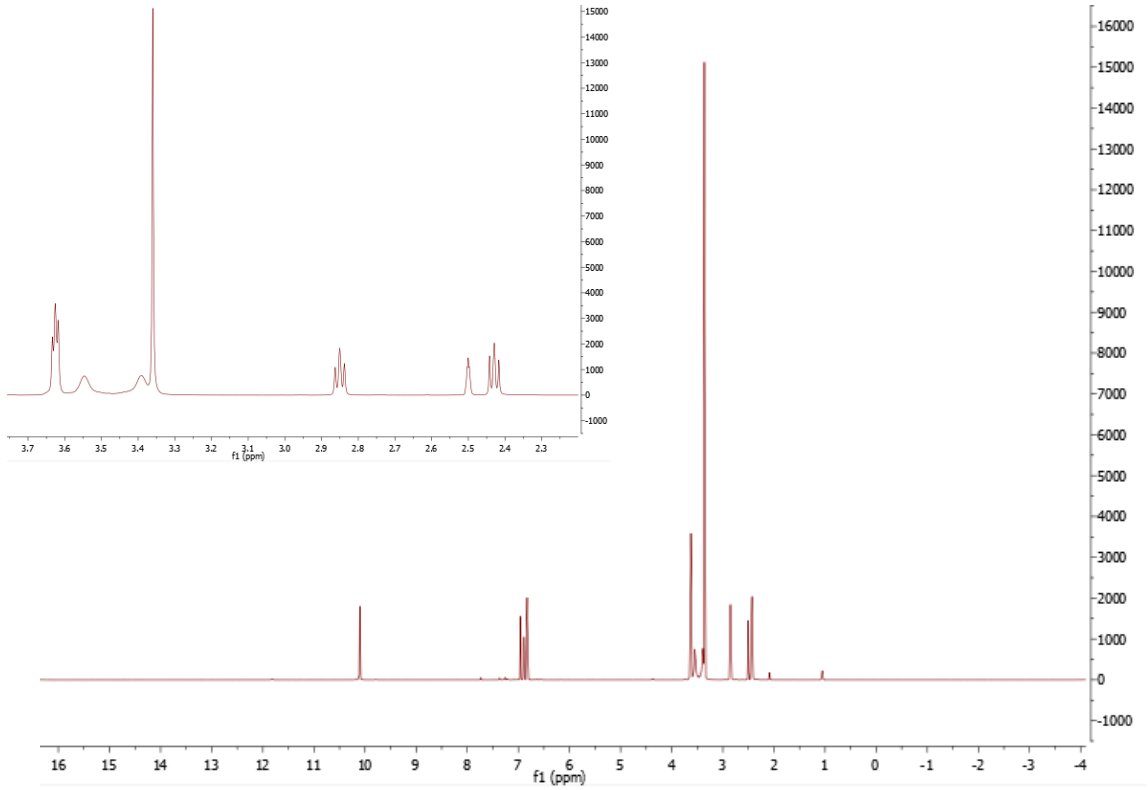


^{13}C-NMR

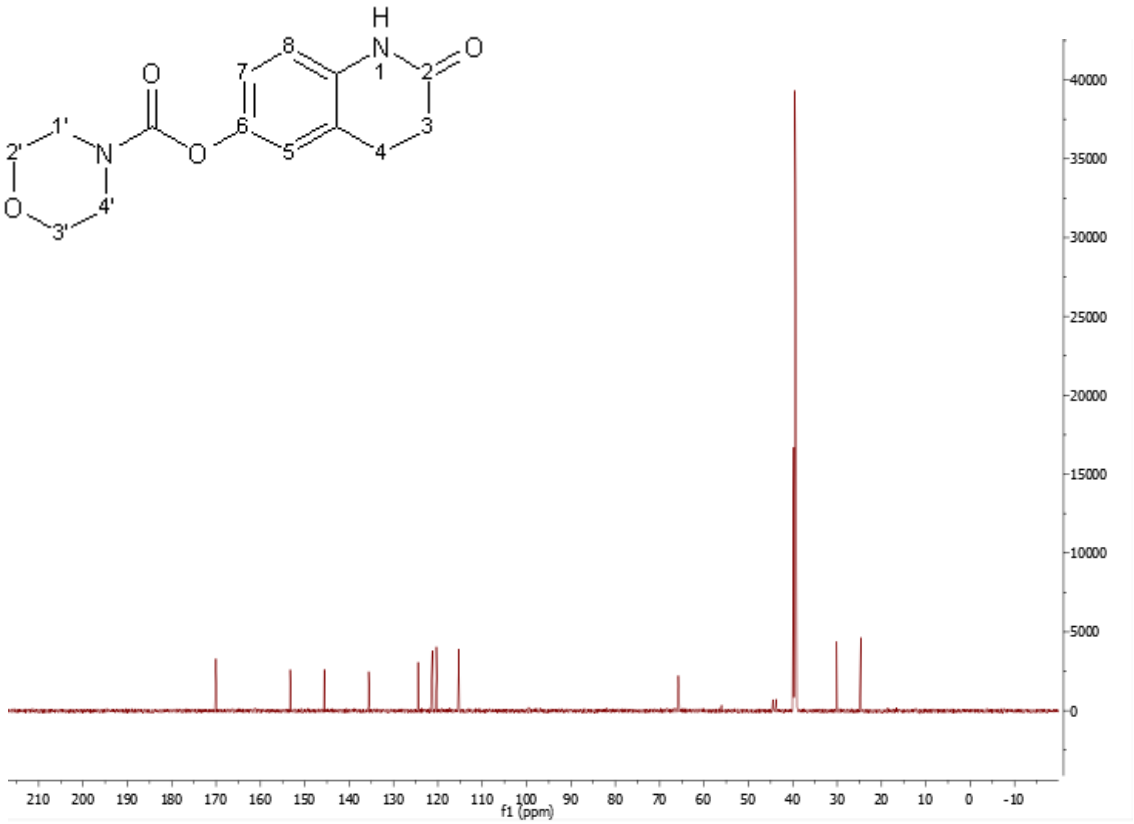


2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**9b**)

^{1}H-NMR

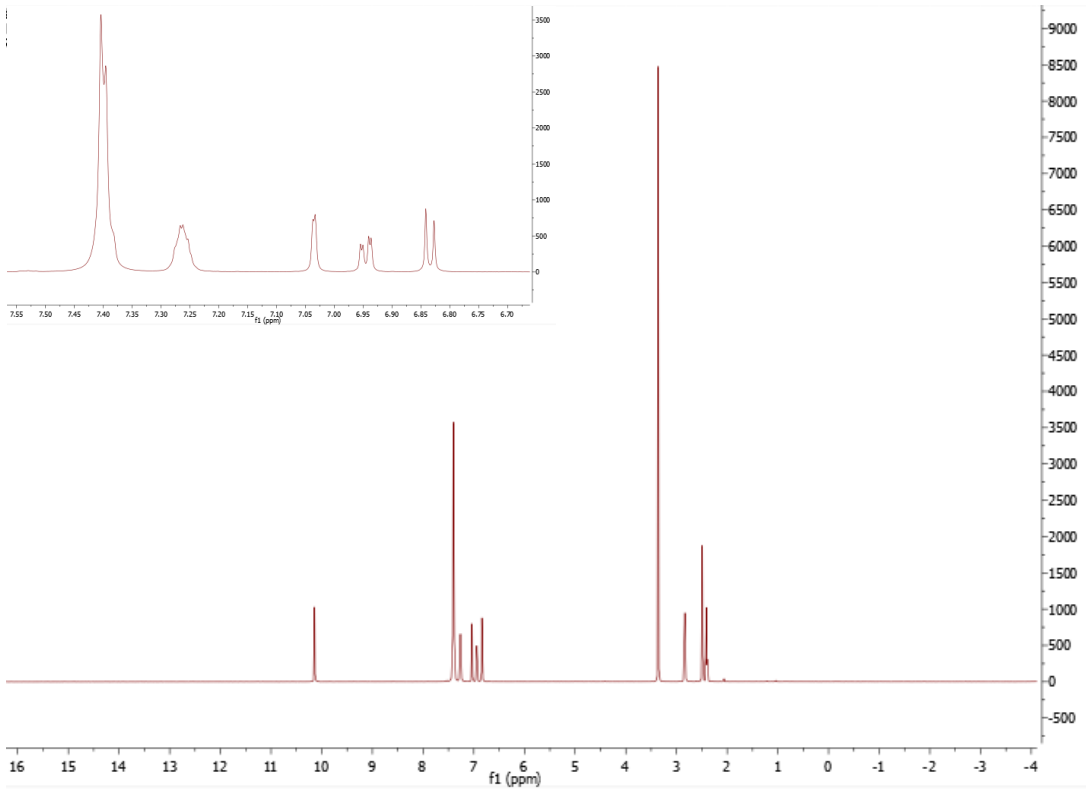


^{13}C-NMR

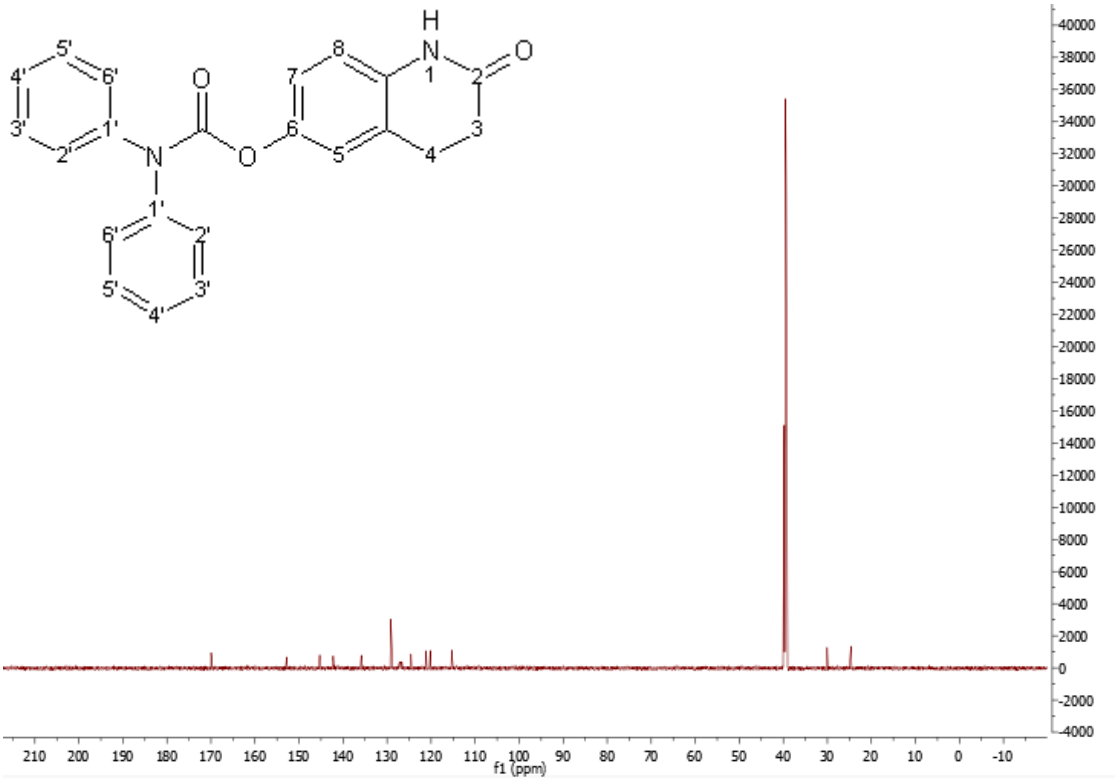


2-oxo-1,2,3,4-tetrahydroquinolin-6-yl diphenylcarbamate (**9c**)

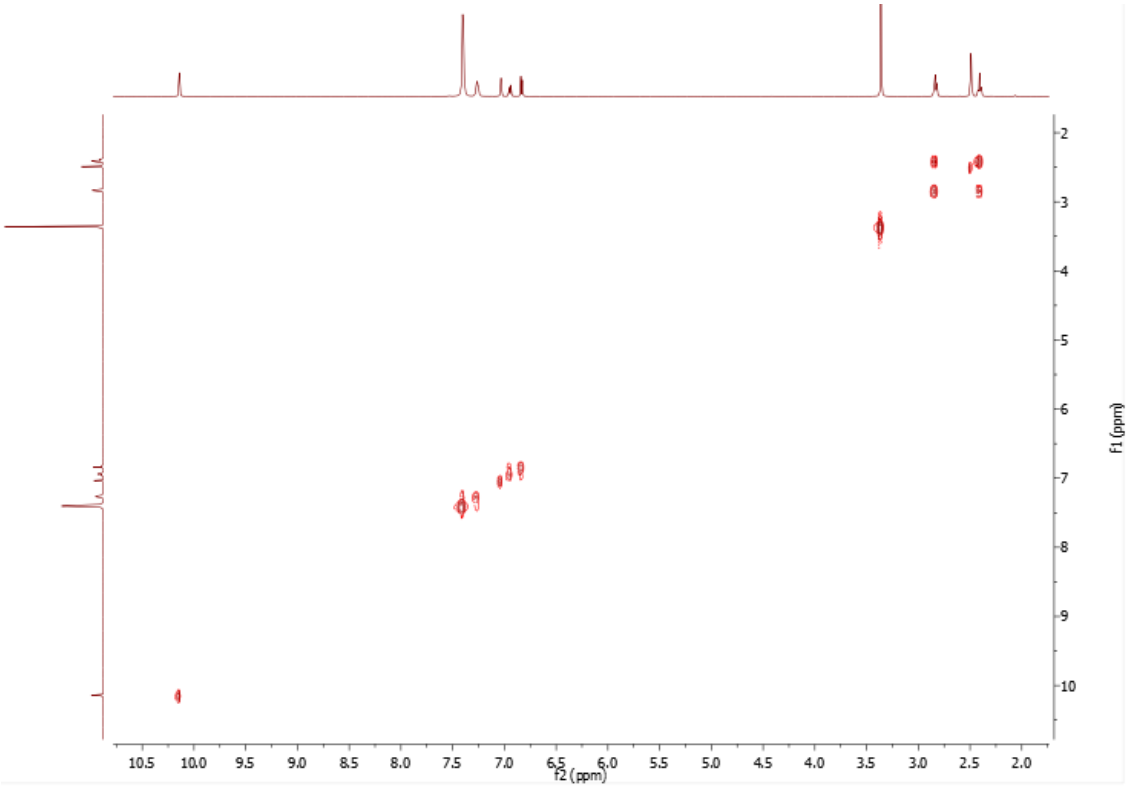
^{1}H-NMR



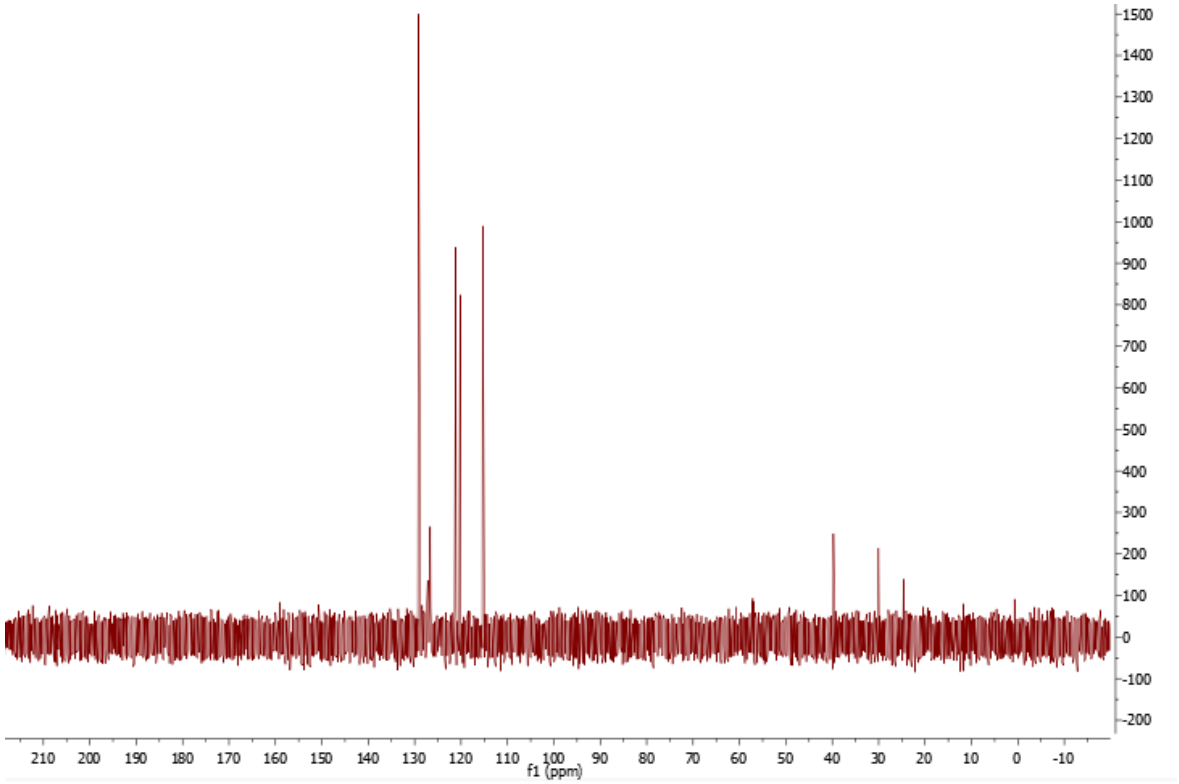
^{13}C-NMR



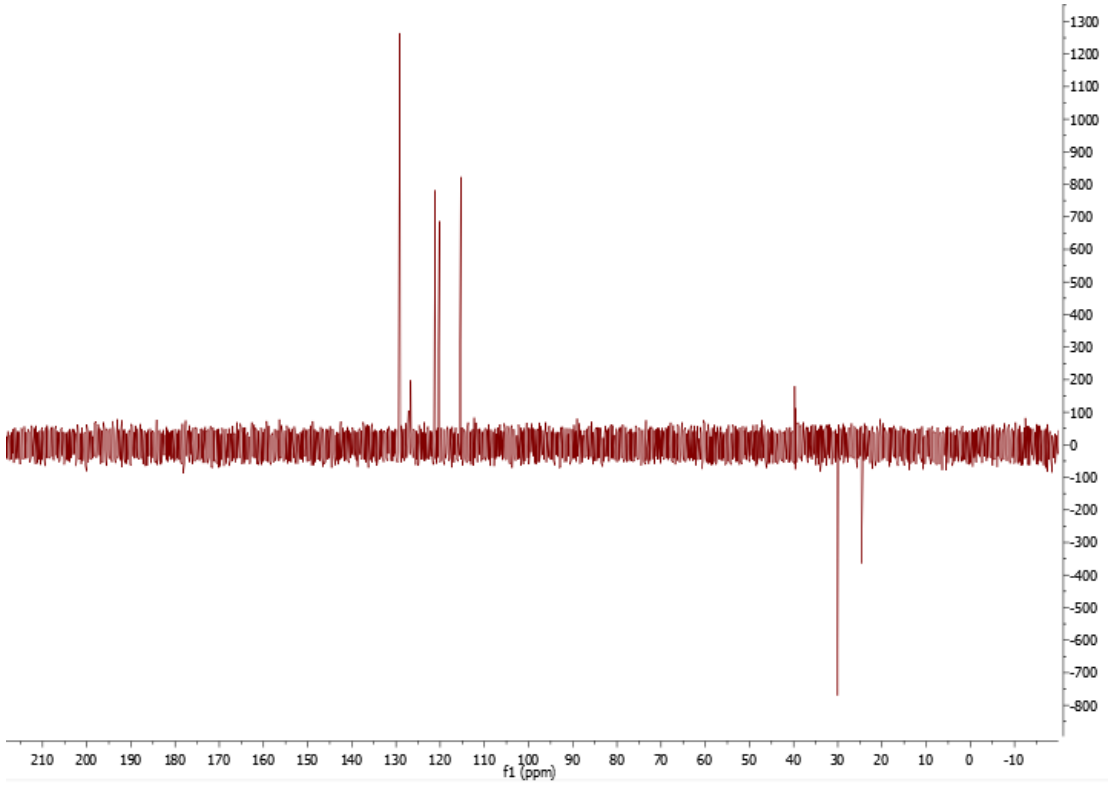
COSY



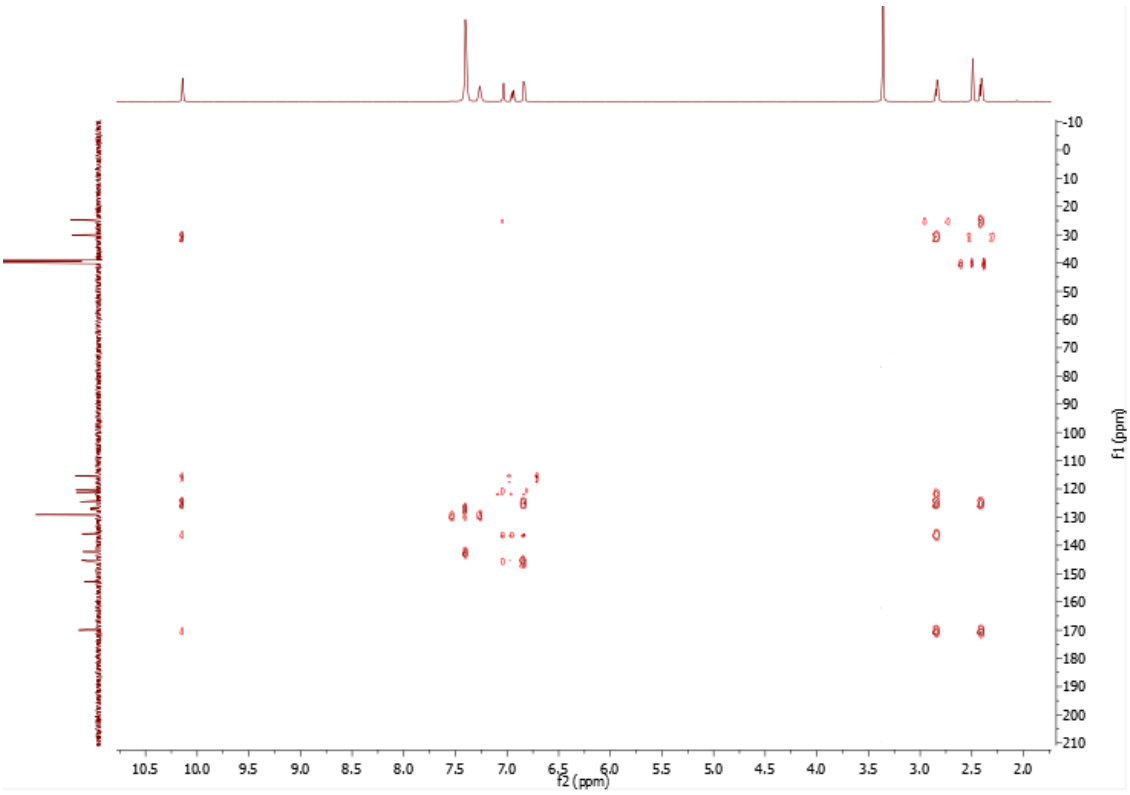
DEPT 90



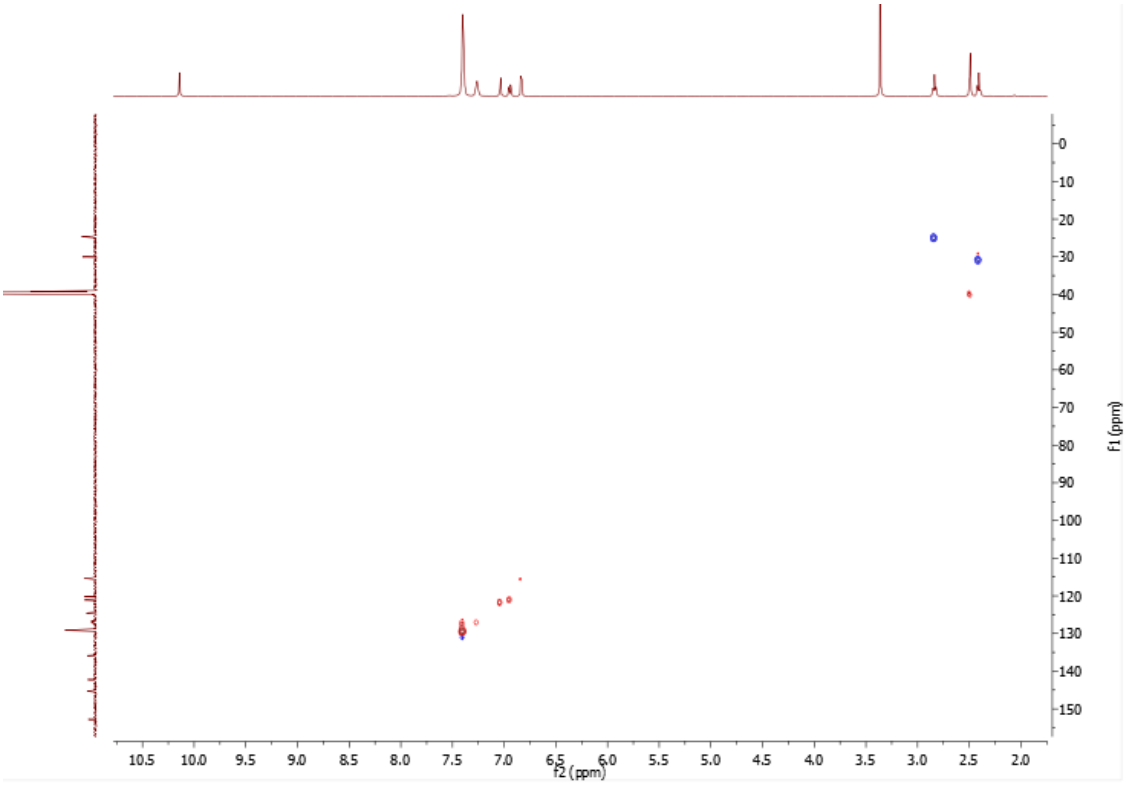
DEPT 135



HMBC



HSQC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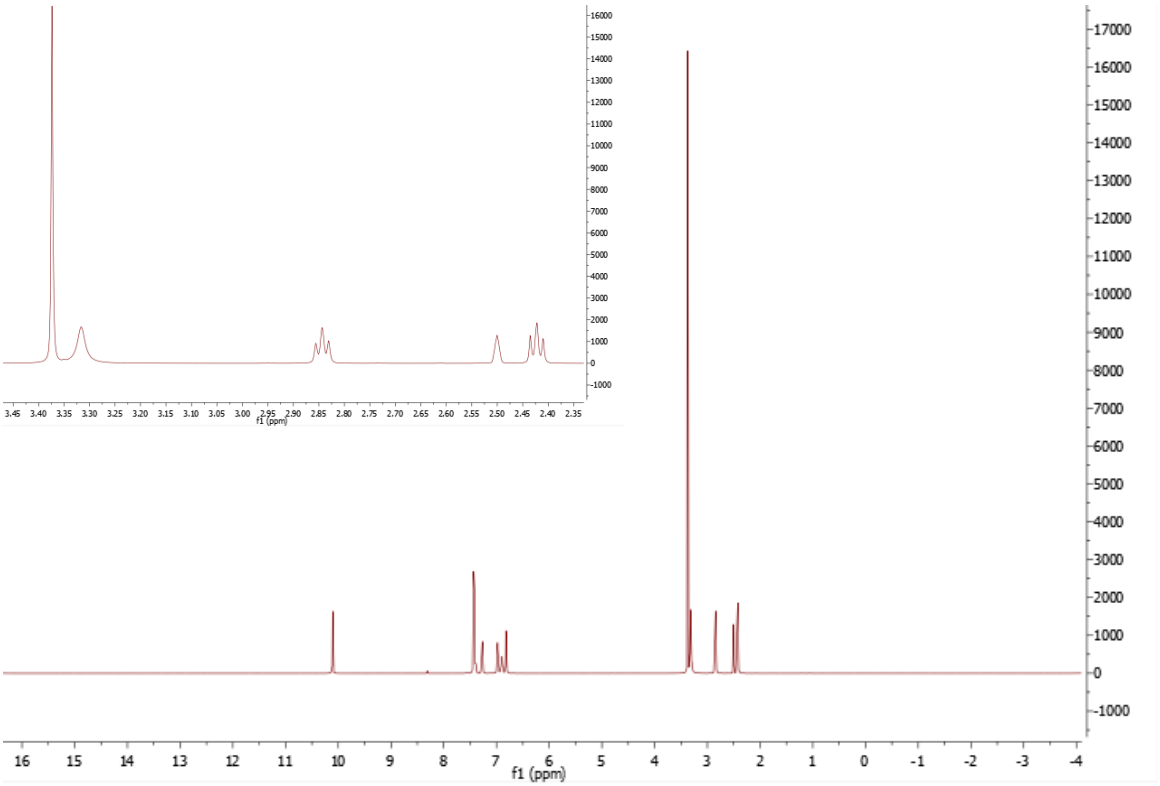
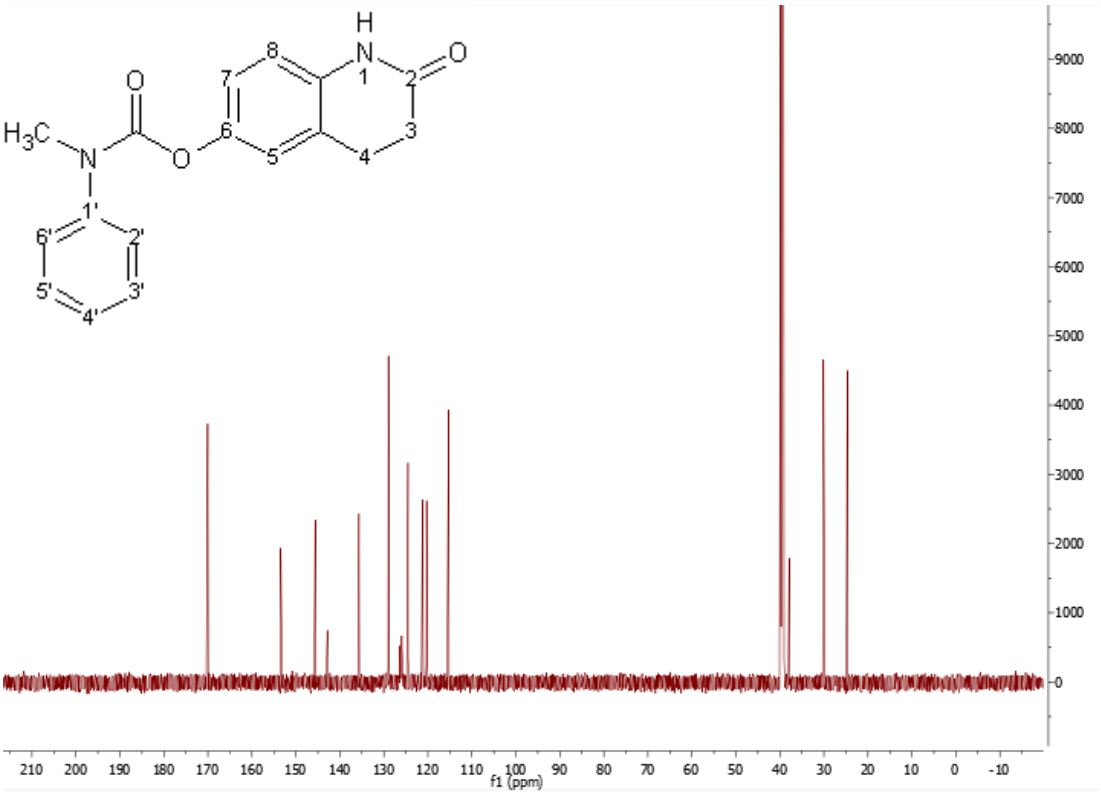
f2 (ppm)

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl methyl(phenyl)carbamate (**9d**)

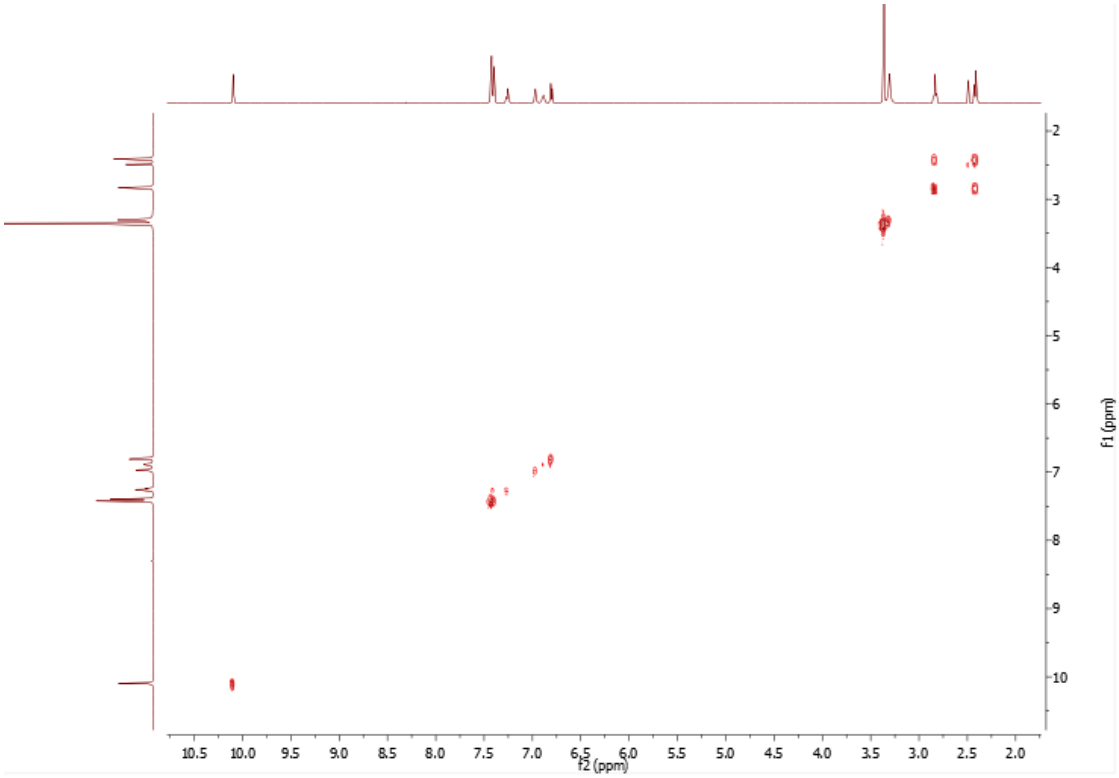
^{1}H-NMR



^{13}C-NMR



COSY



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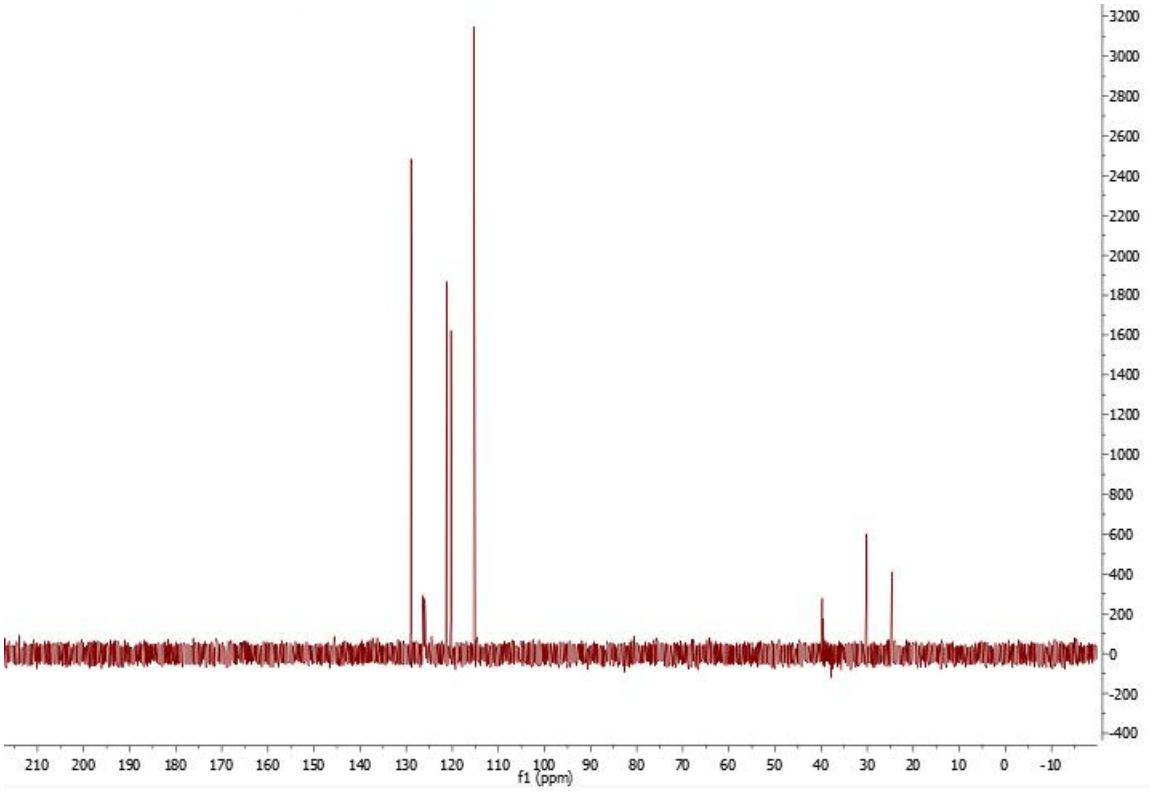
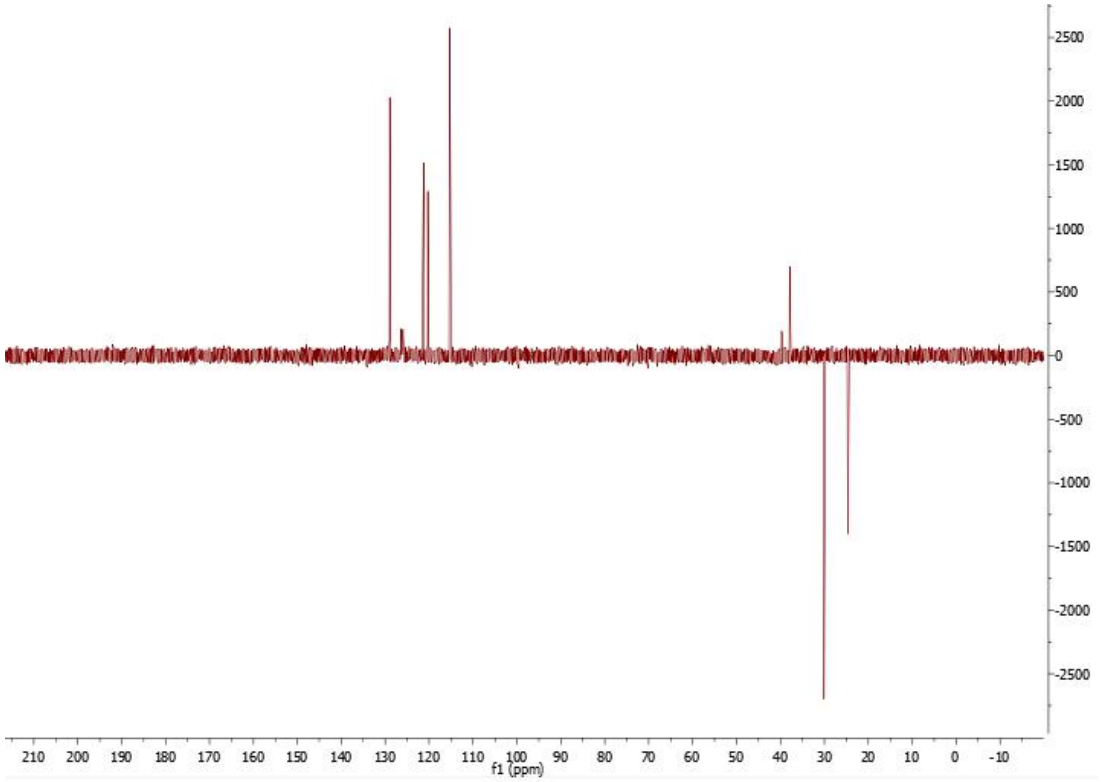
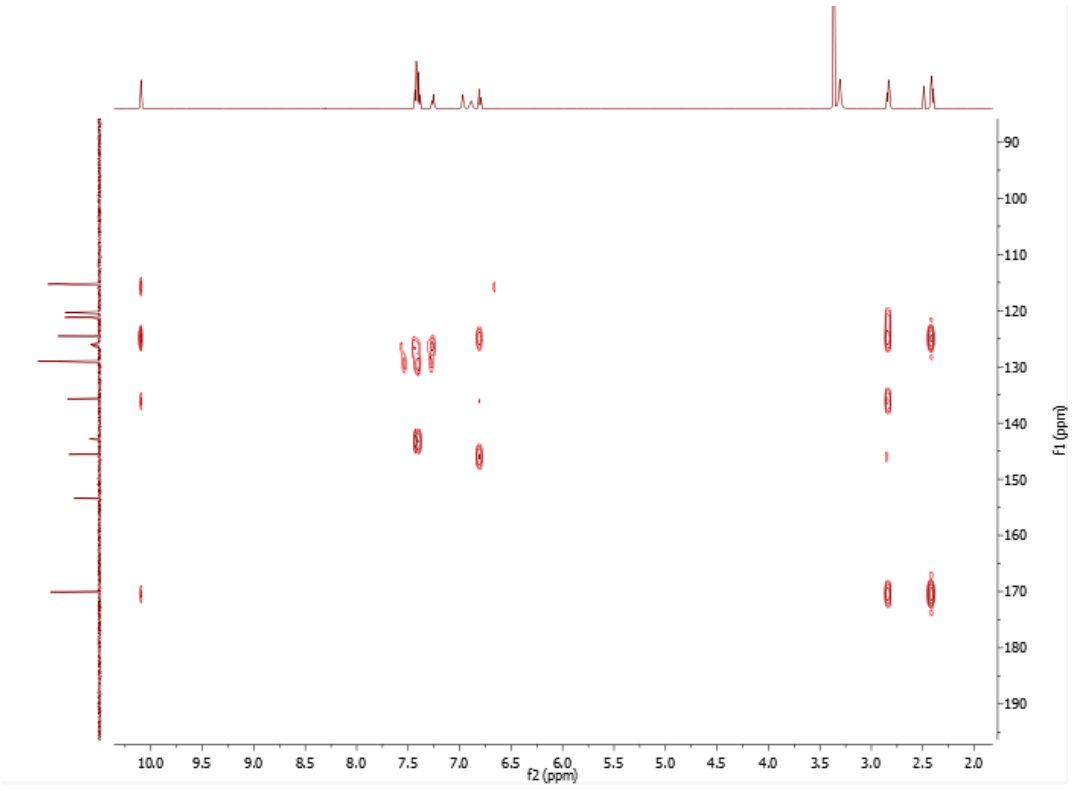


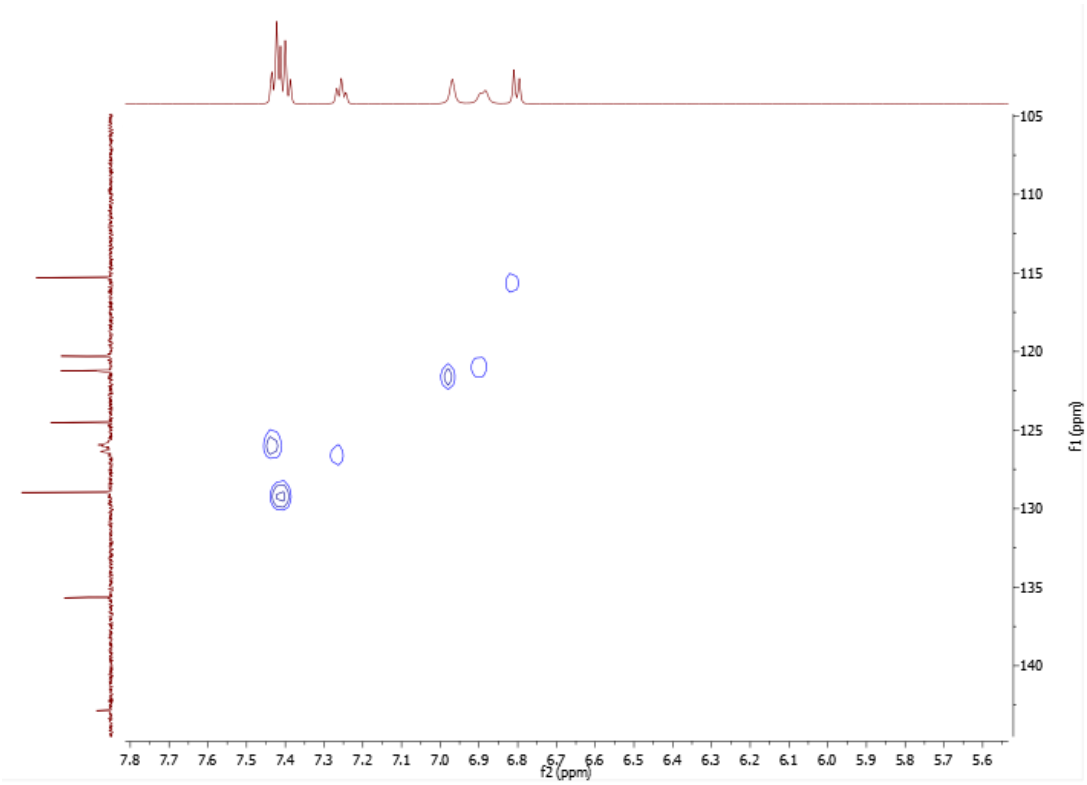
DEPT 135



HMBC



HSQC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6.5
6.4
6.3
6.2
6.1
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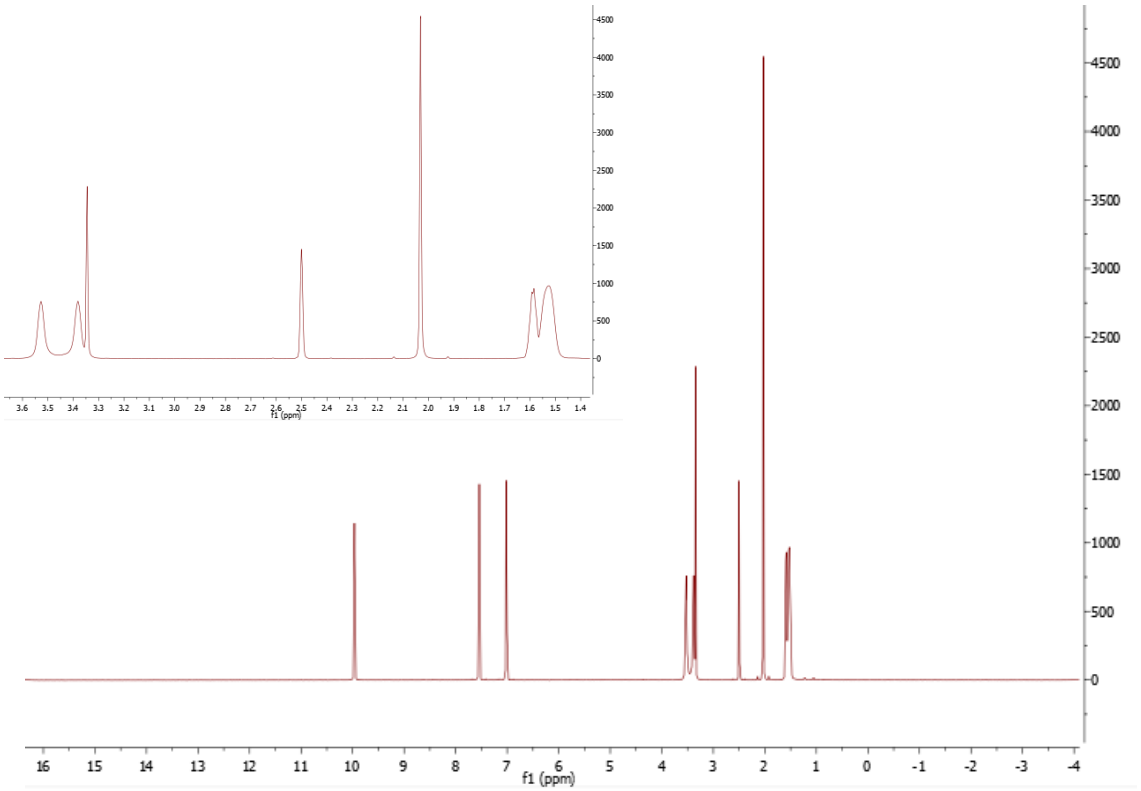
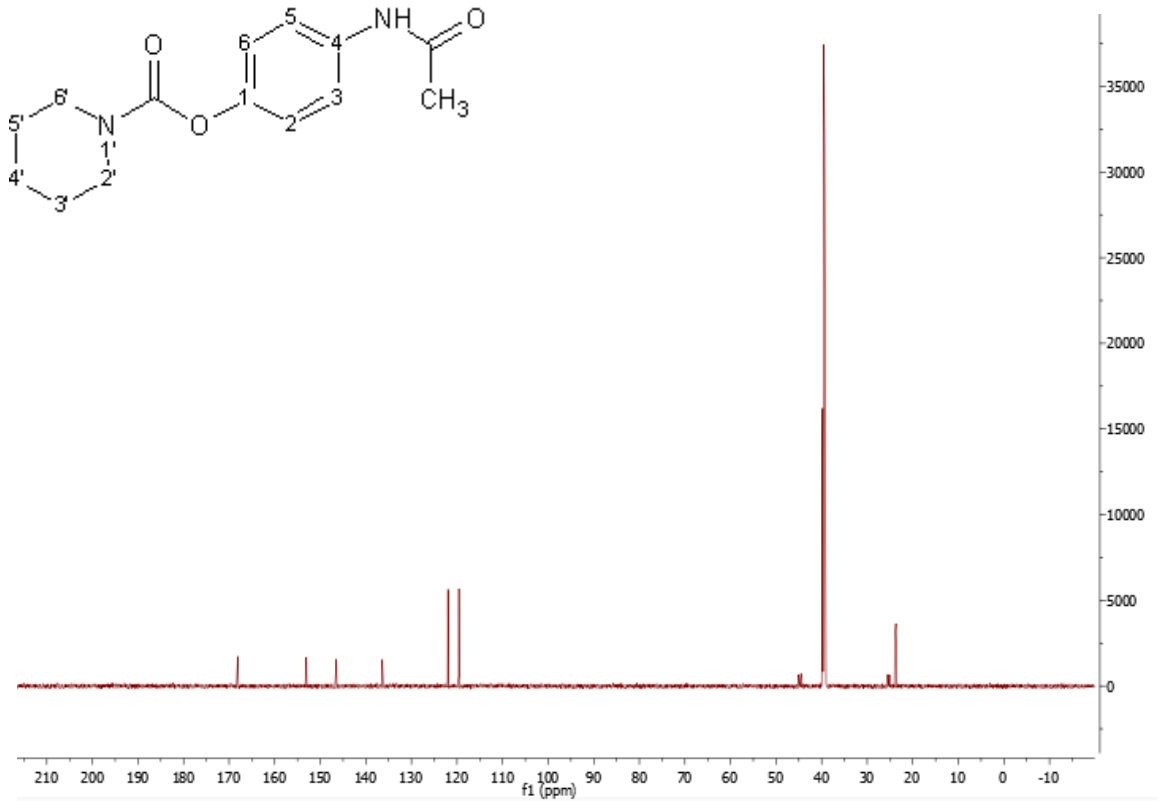
5.6
f2 (ppm)

4-(acetylamino)phenyl piperidine-1-carboxylate (**10a**)

^{1}H-NMR

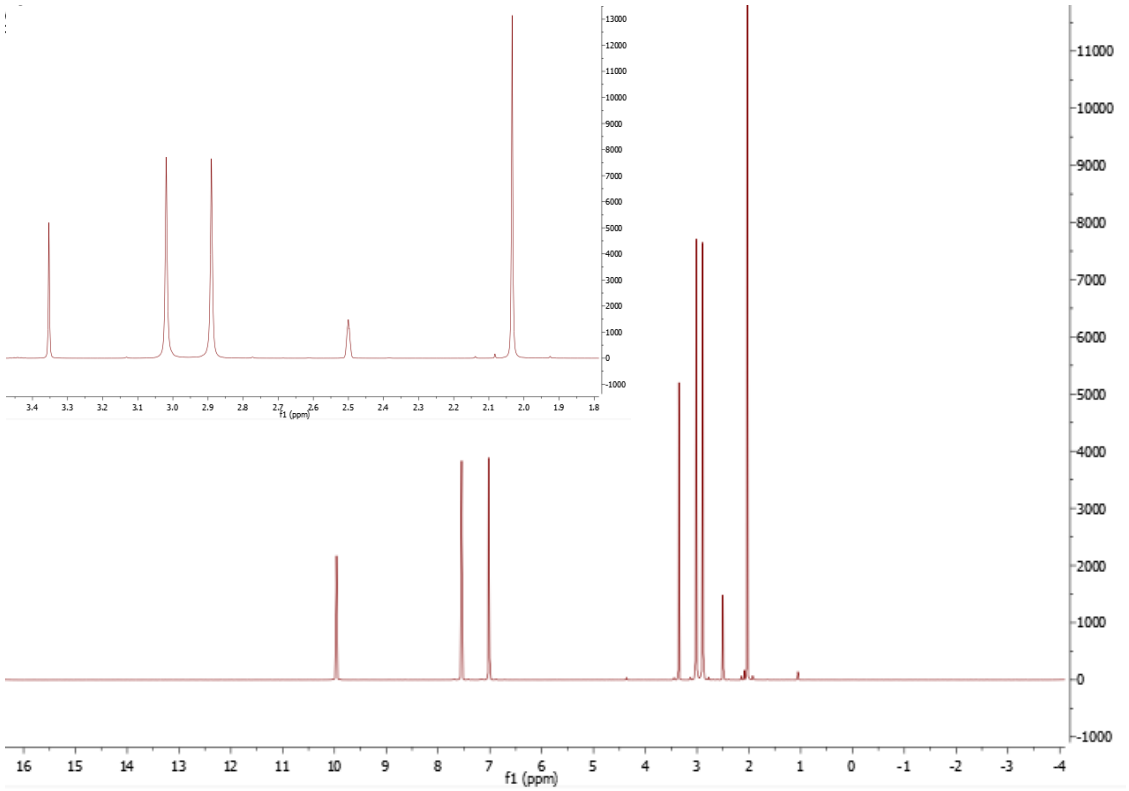


^{13}C-NMR

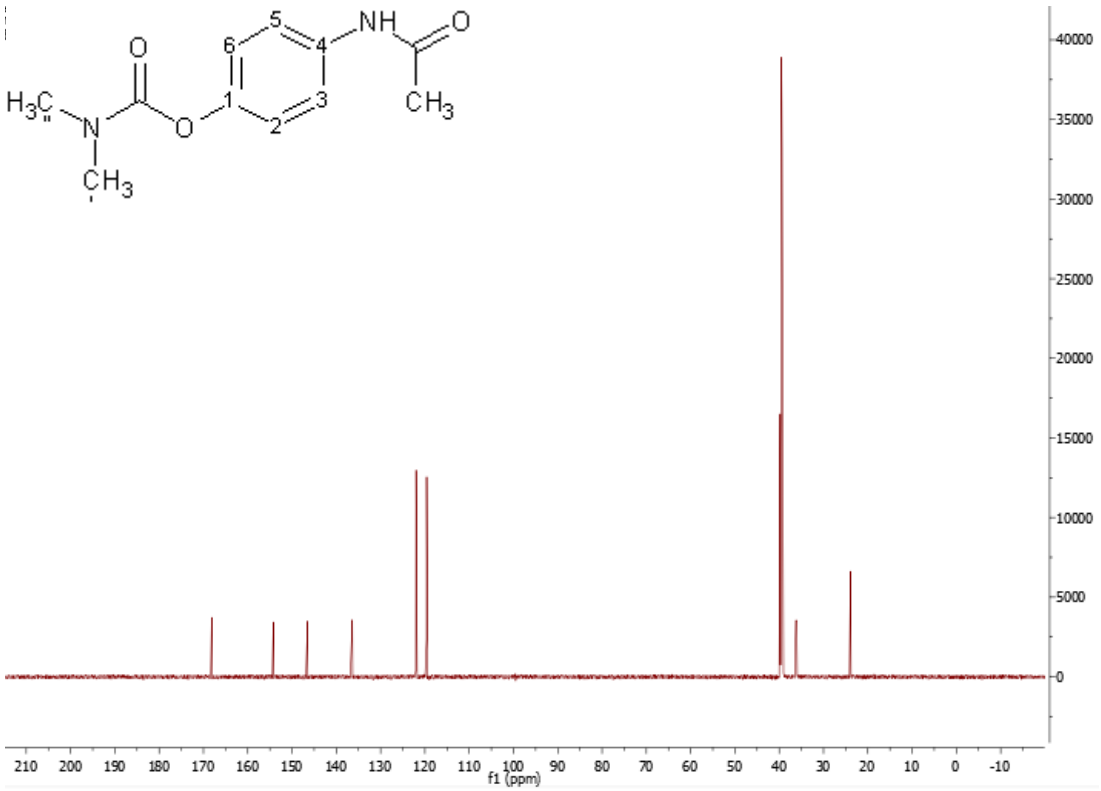


4-(acetylamino)phenyldimethylcarbamate (**10b**)

^{1}H-NMR

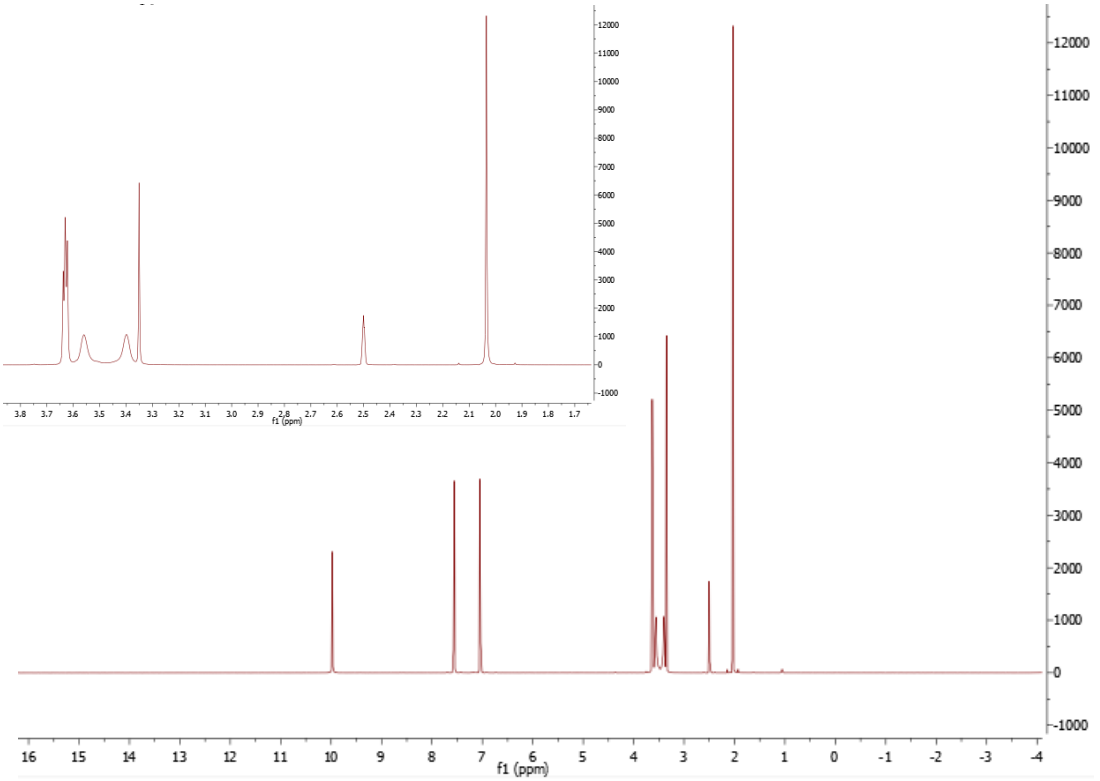


^{13}C-NMR

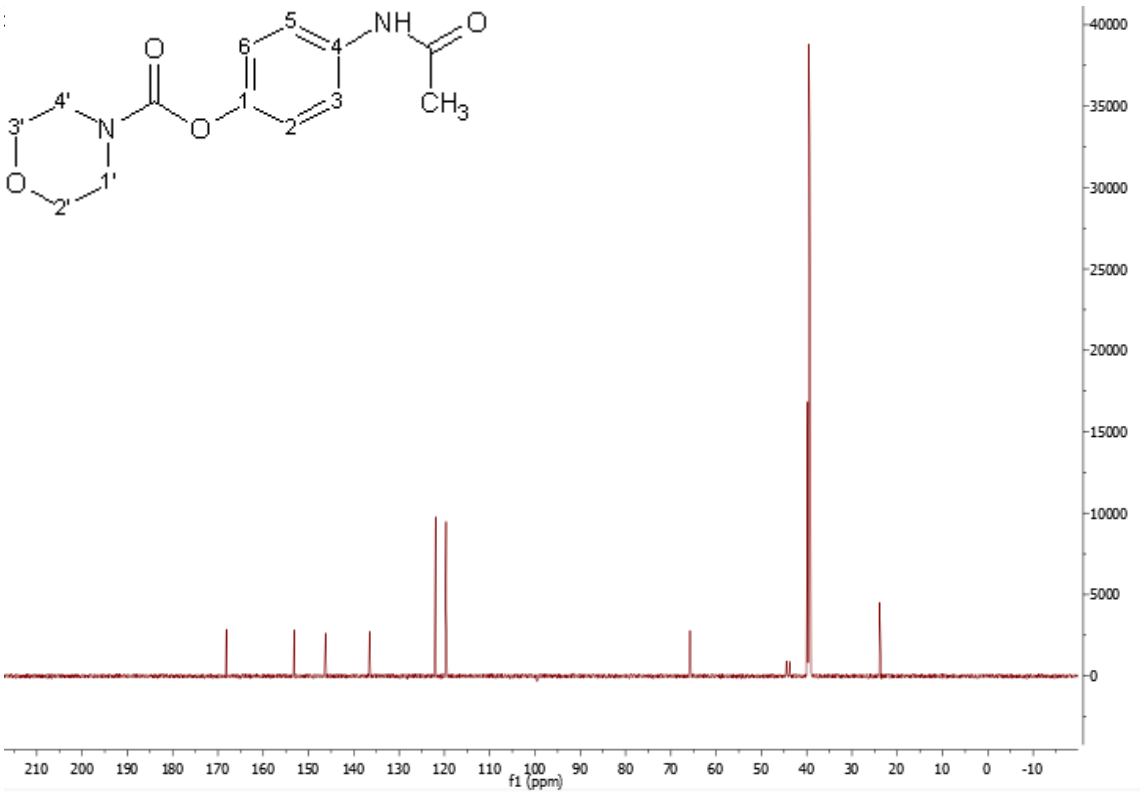


4-(acetylamino)phenyl morpholine-4-carboxylate (**10c**)

^{1}H-NMR

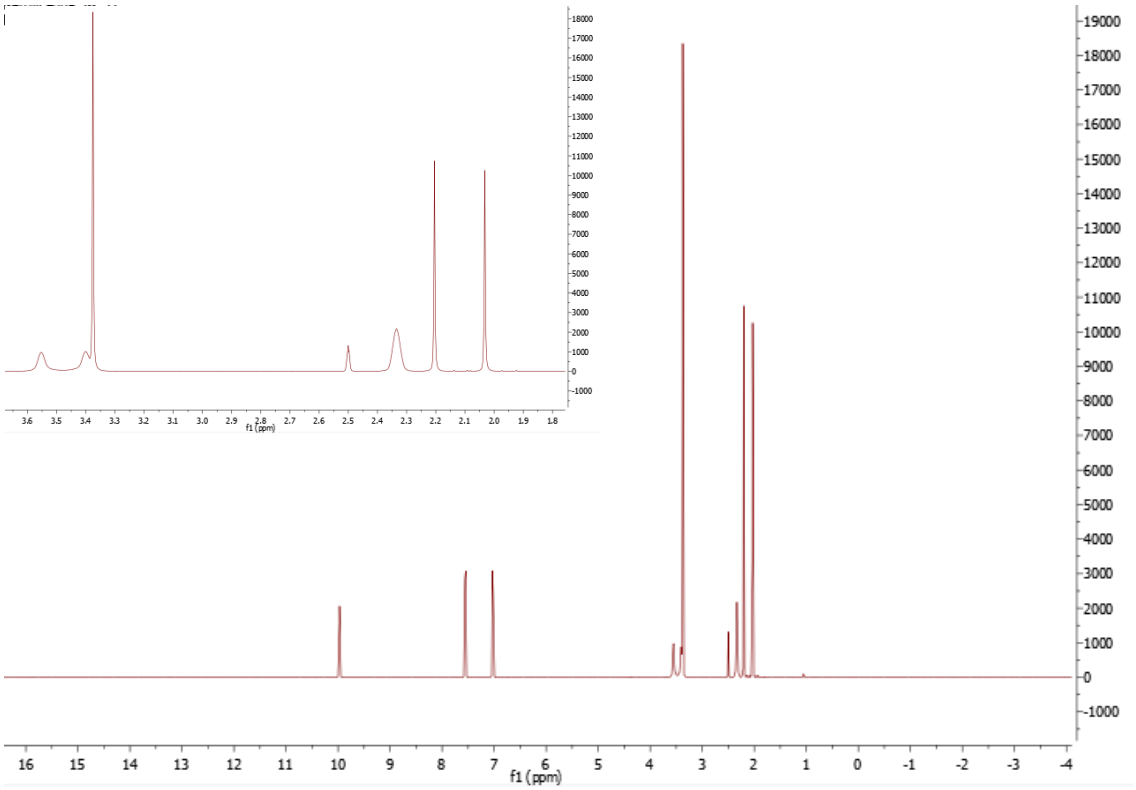


^{13}C-NMR



4-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**10d**)

^{1}H-NMR



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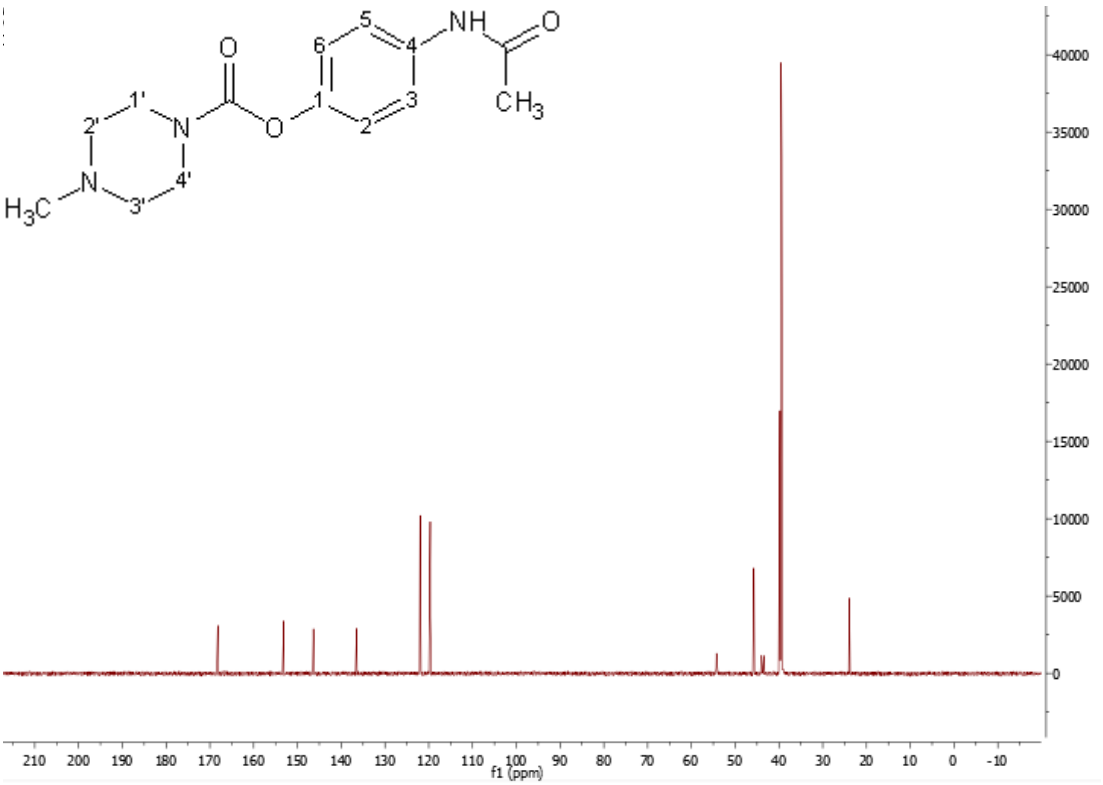
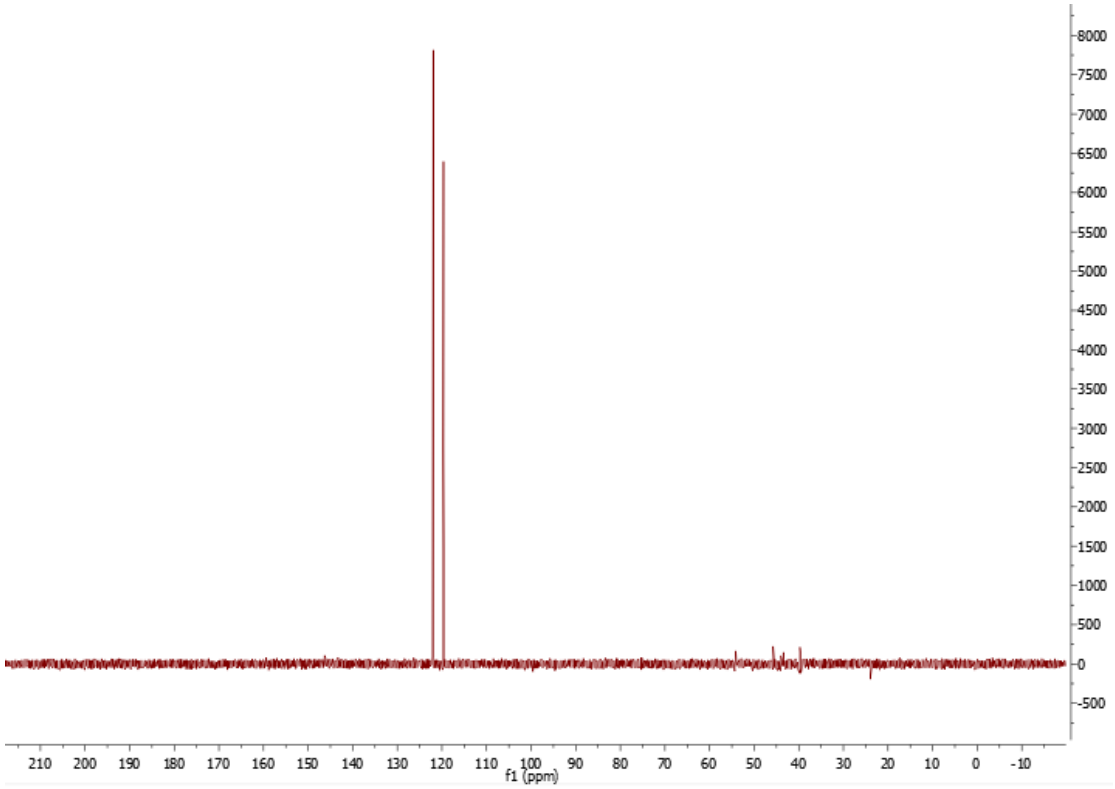
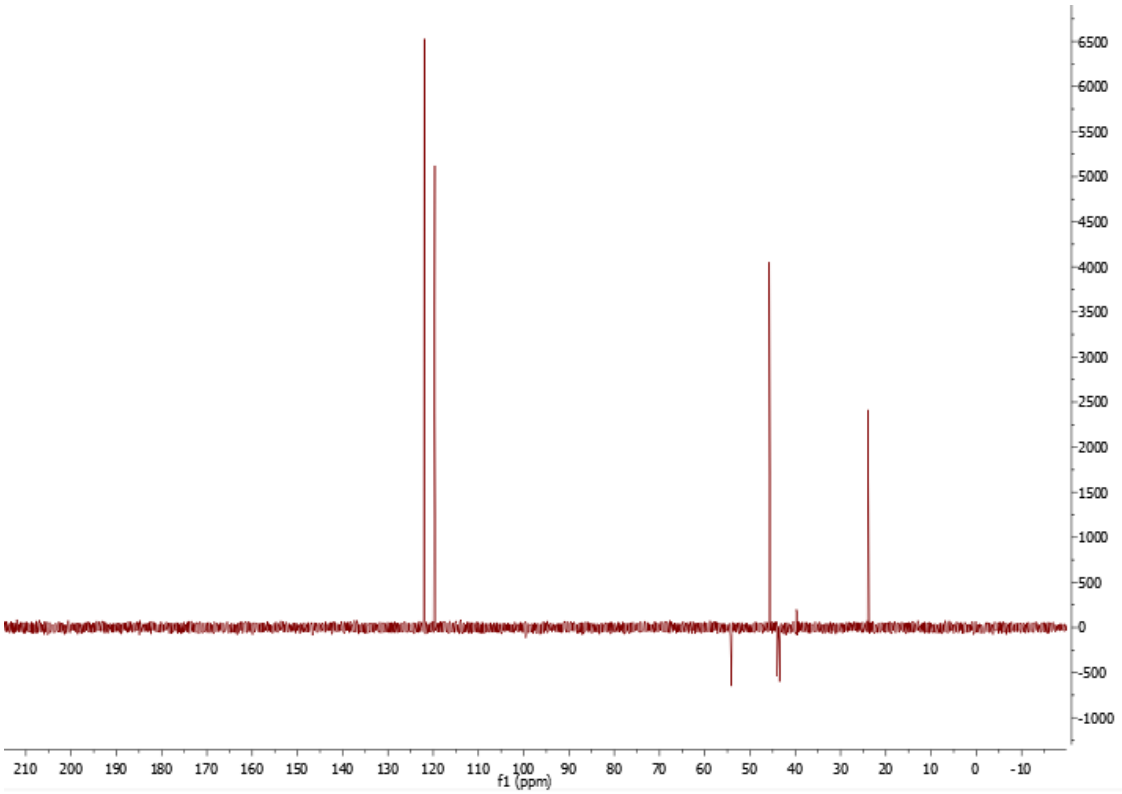


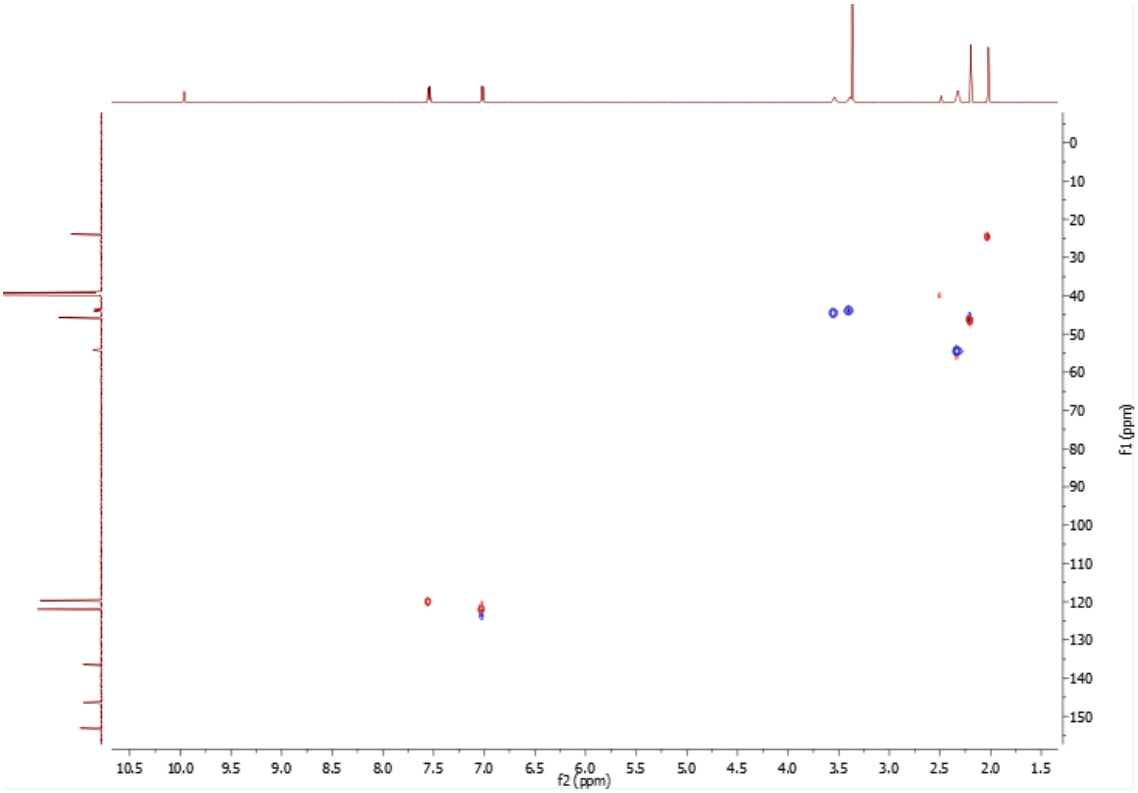
DEPT 90



DEPT 135



HSQC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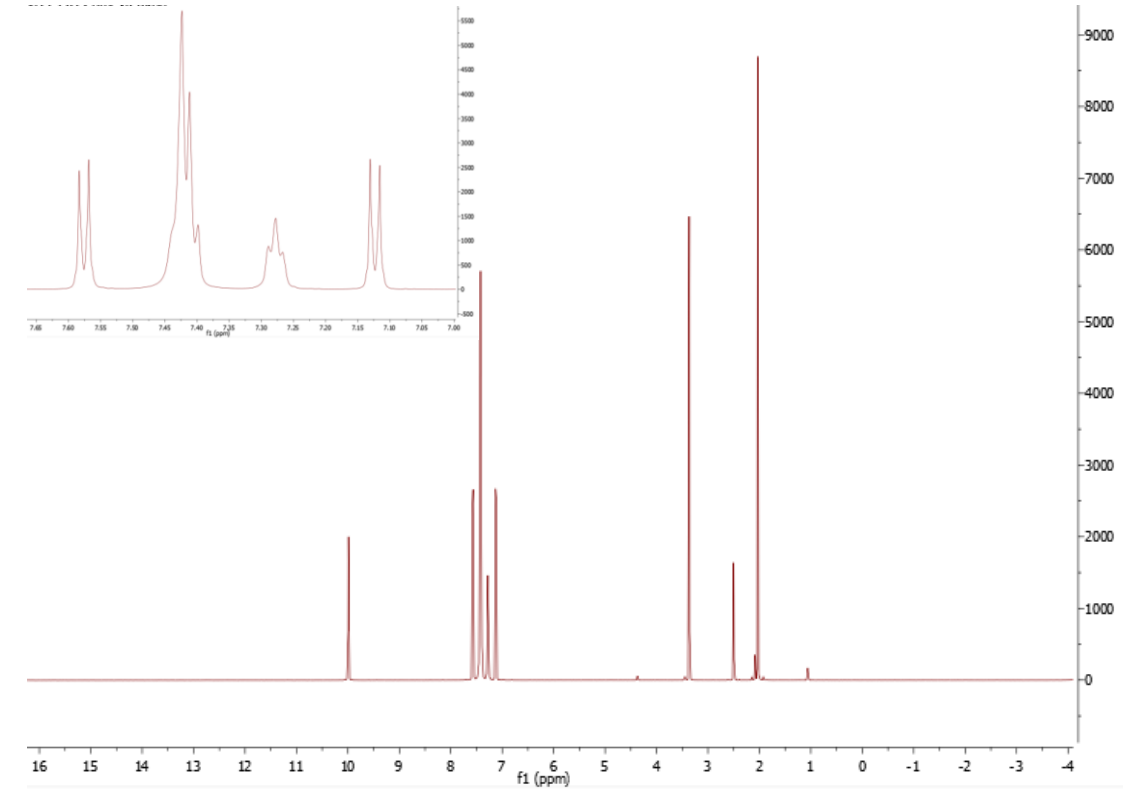
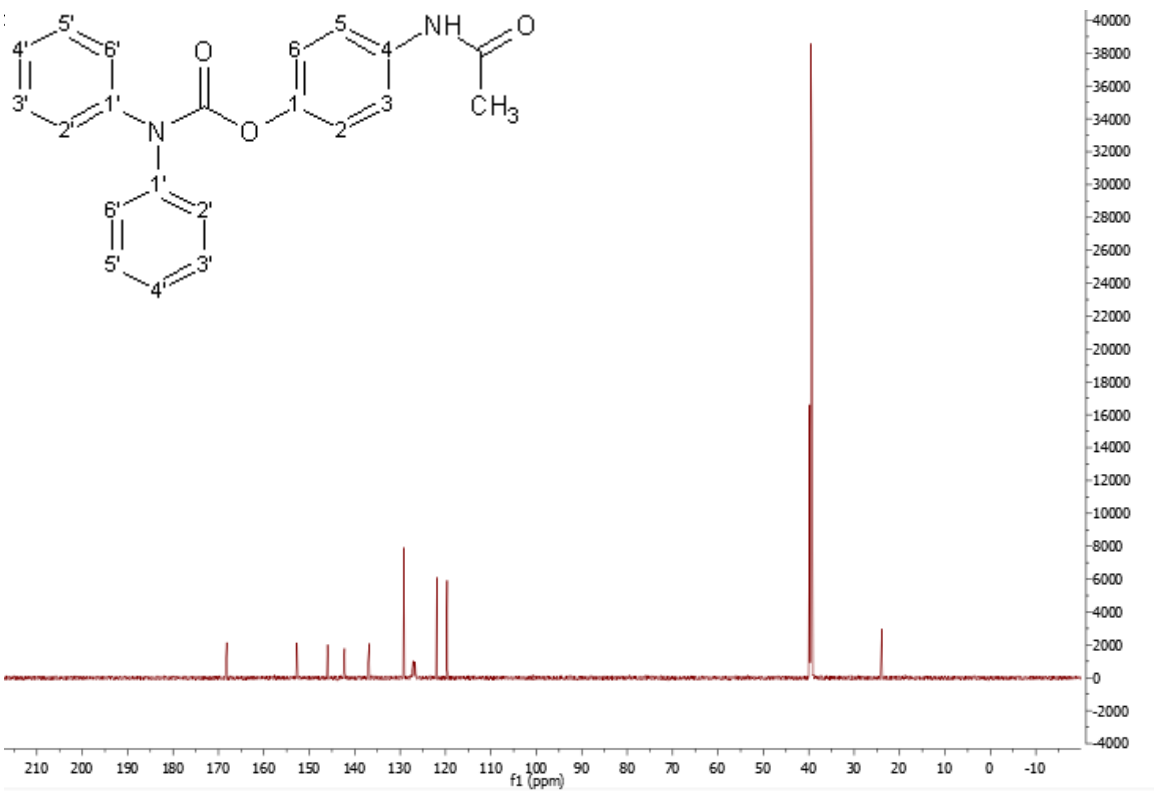
1.5
f2 (ppm)

4-(acetylamino)phenyldiphenylcarbamate (**10e**)

^{1}H-NMR

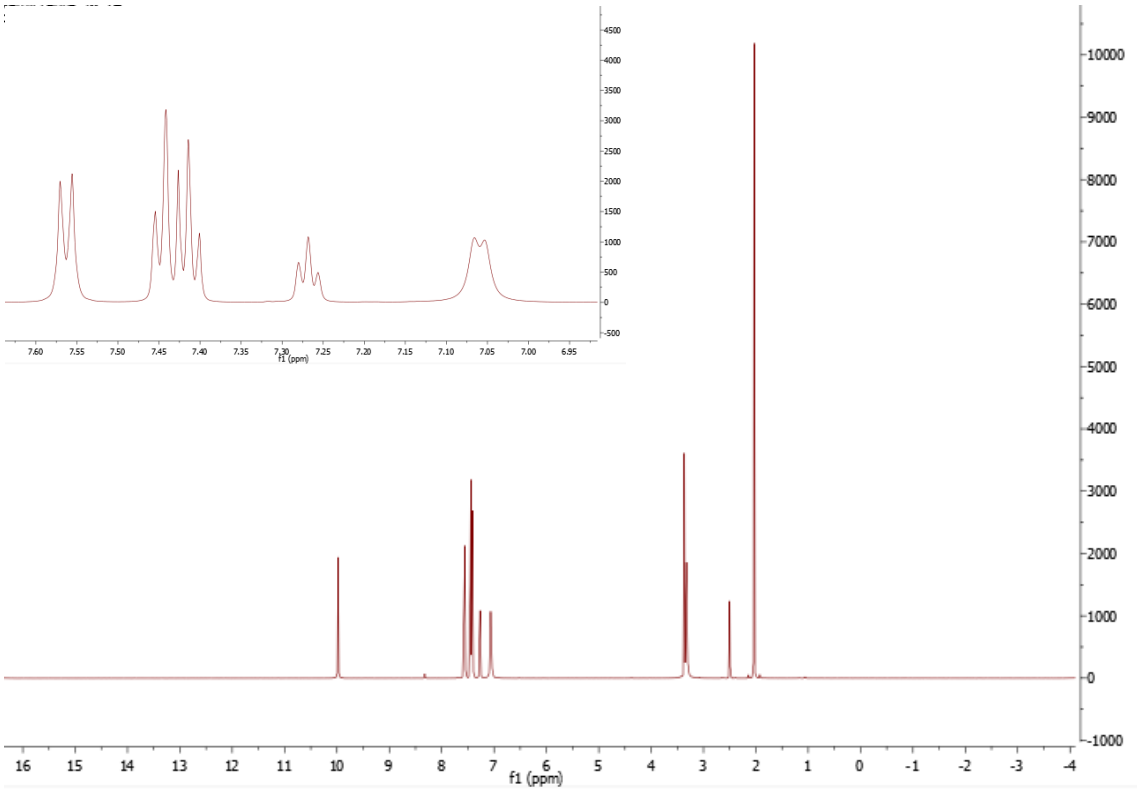


^{13}C-NMR

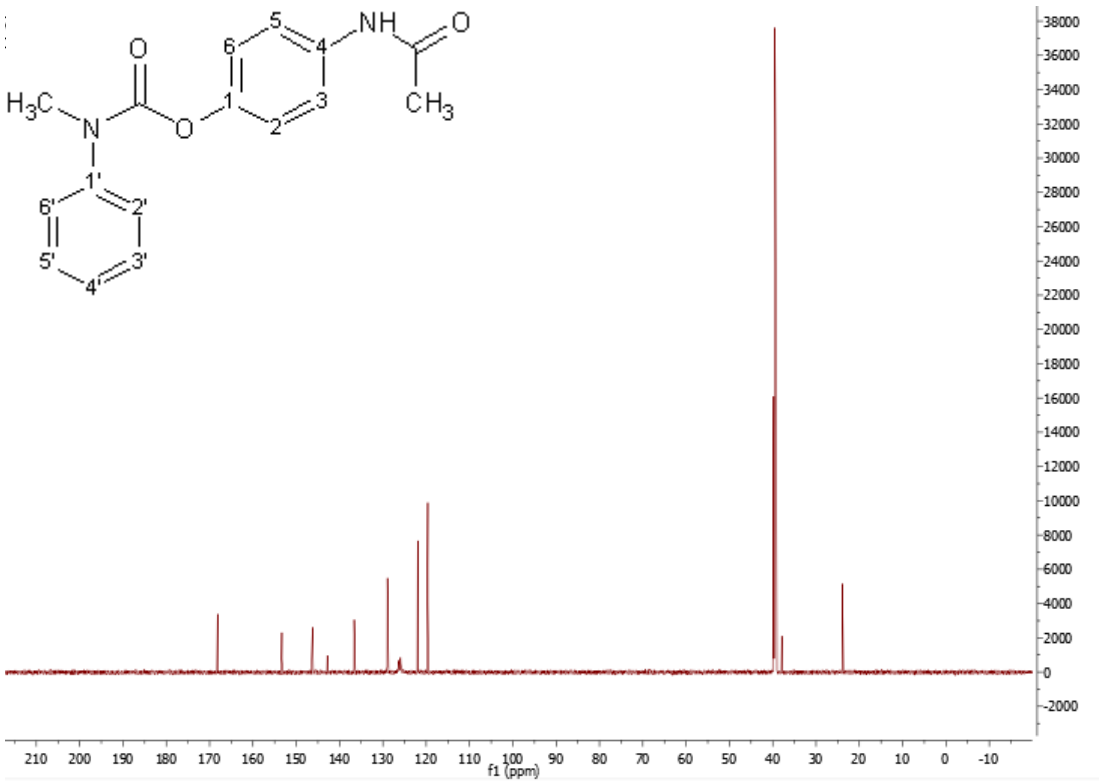


4-(acetylamino)phenyl methyl(phenyl)carbamate (**10f**)

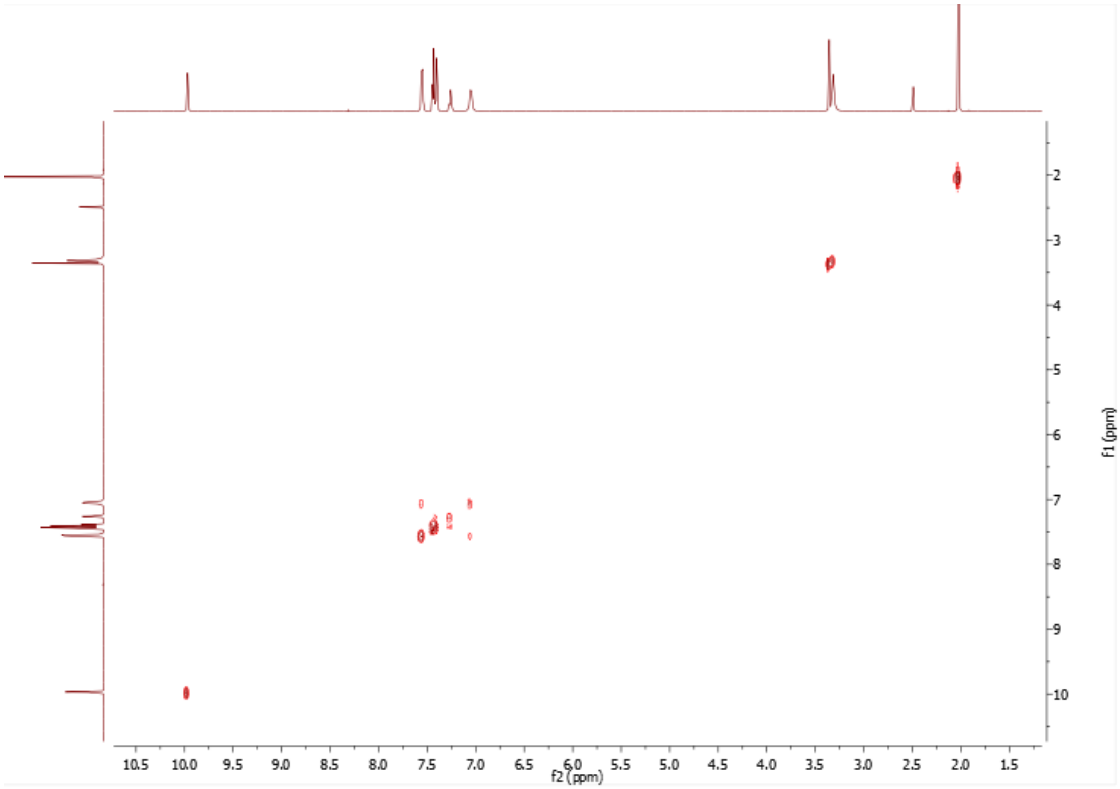
^{1}H-NMR



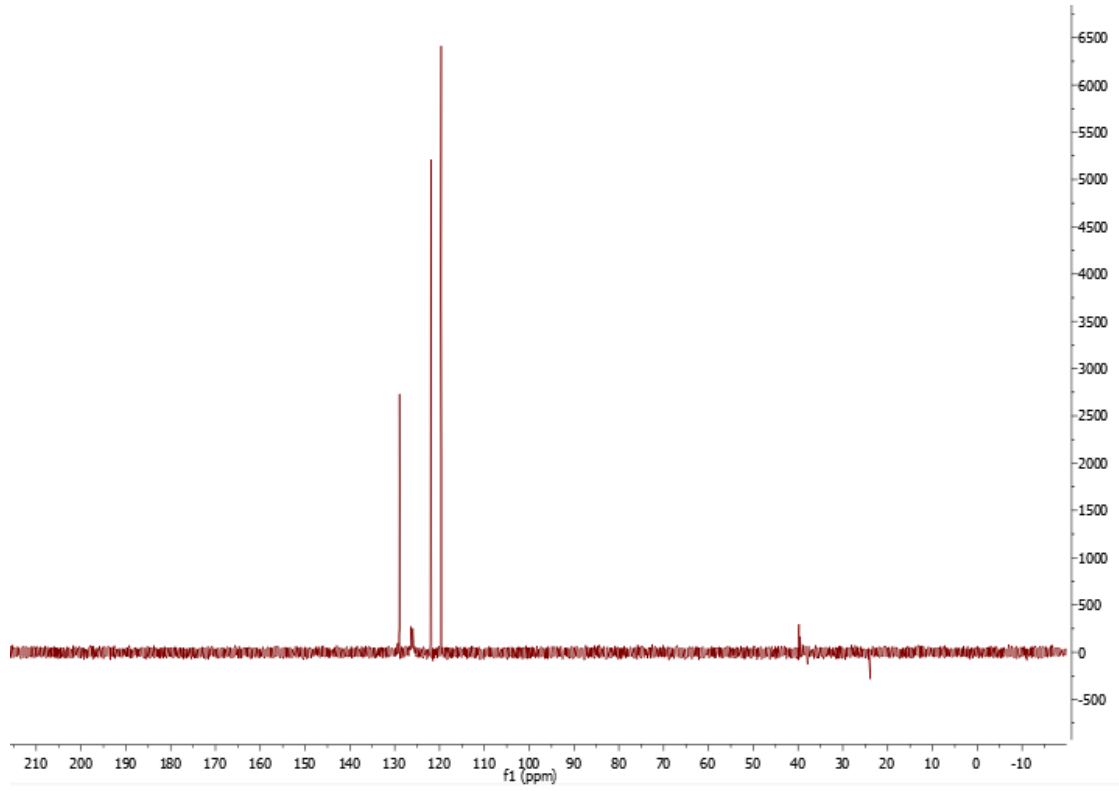
^{13}C-NMR



COSY



DEPT 90



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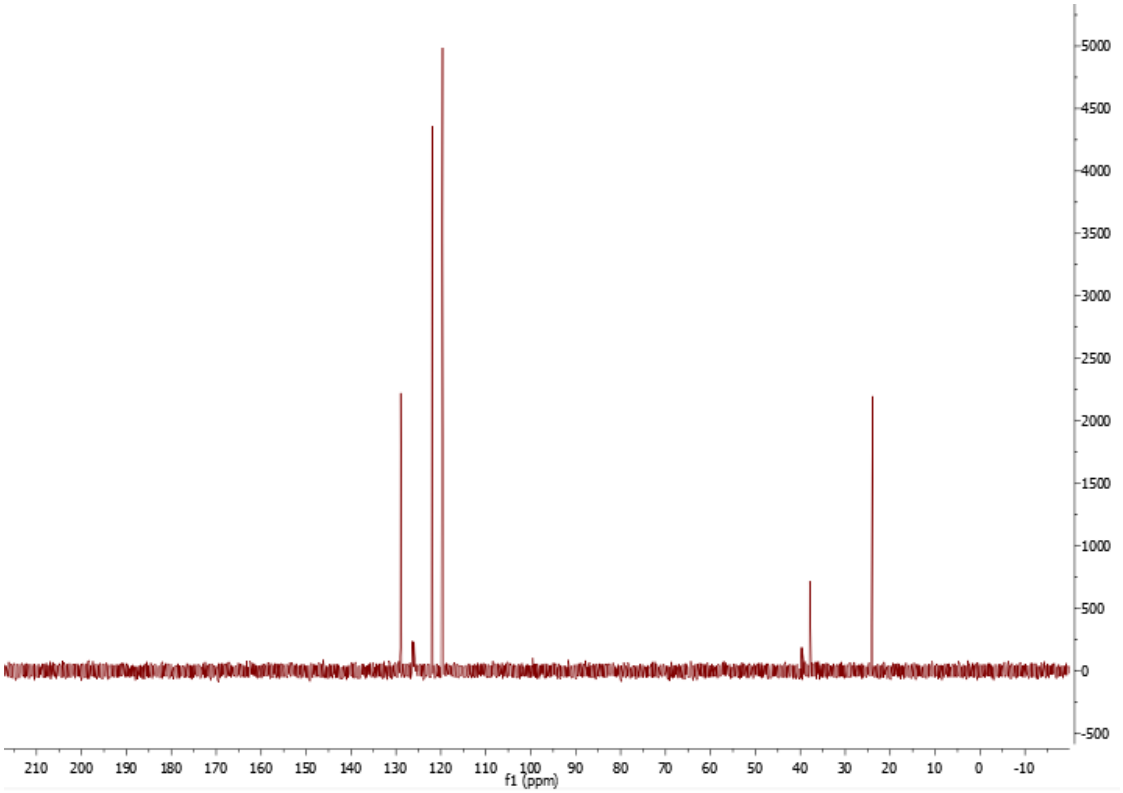

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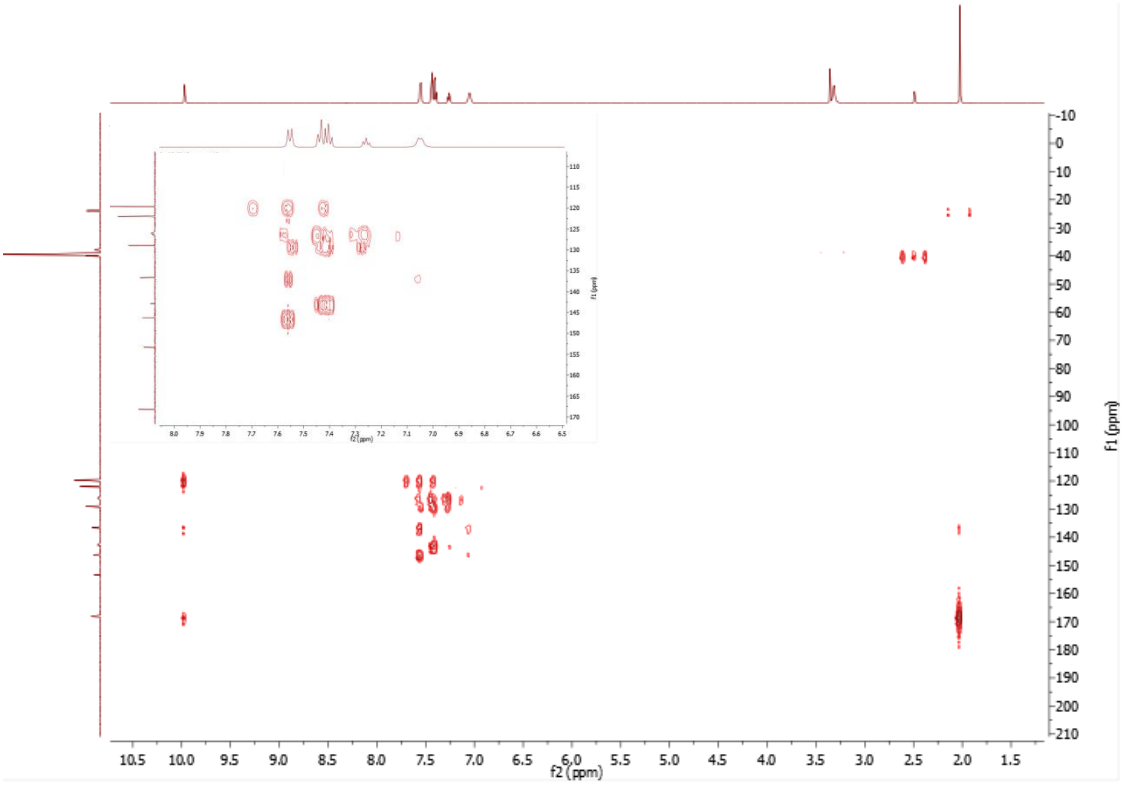
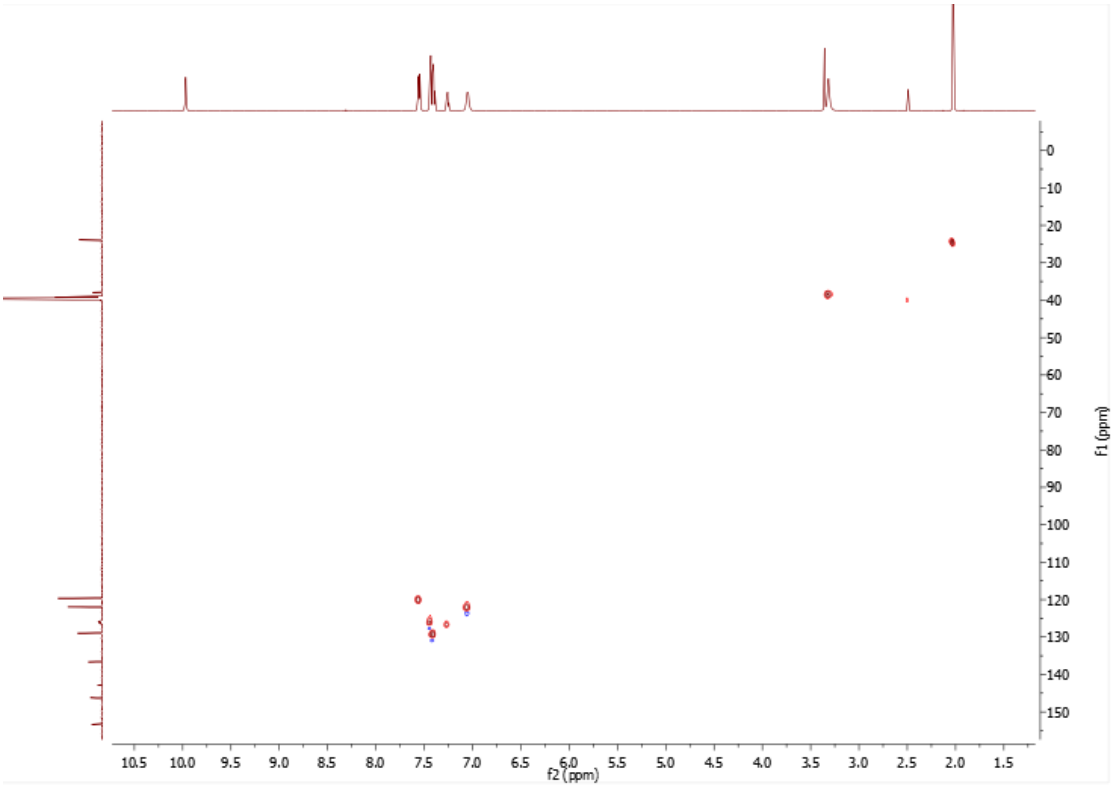


HSQC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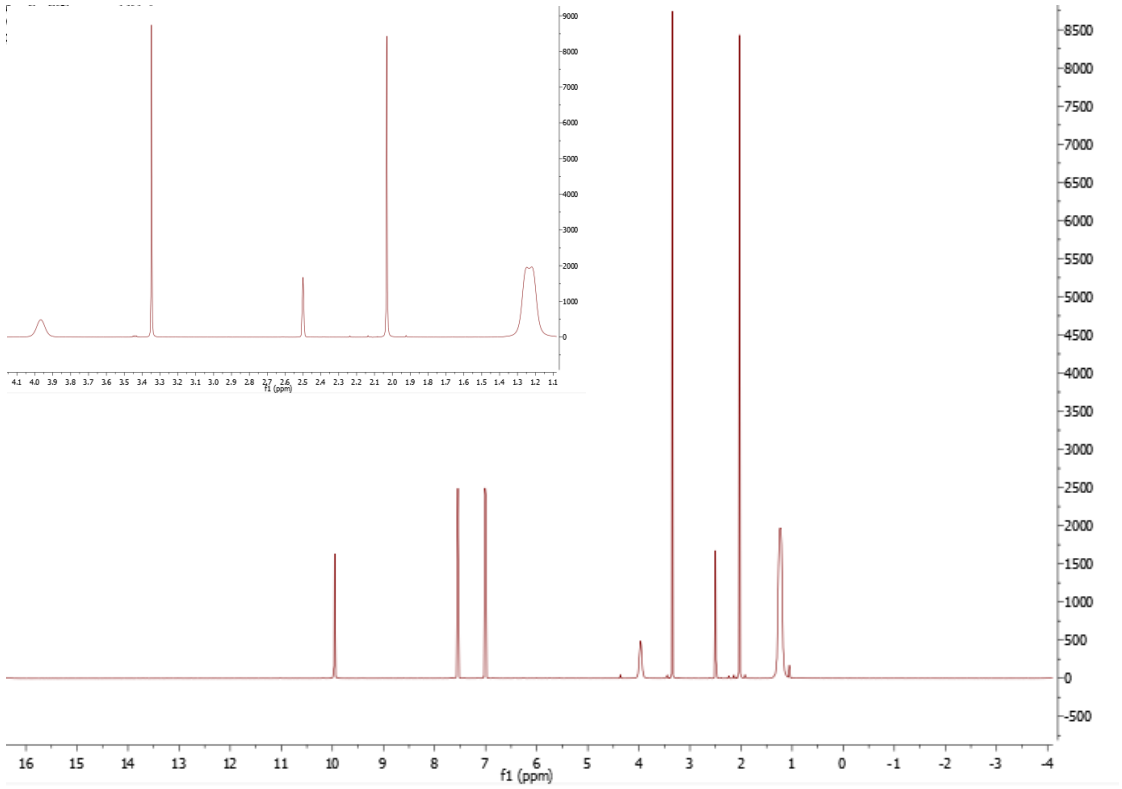
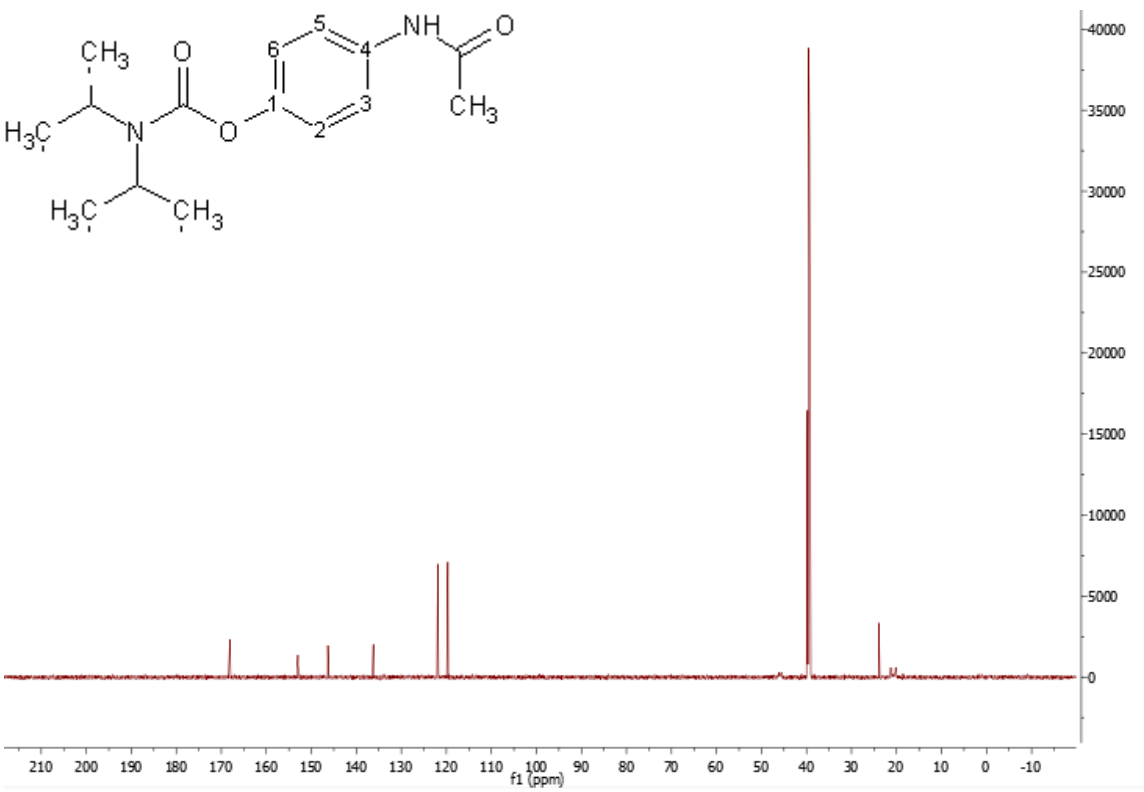
f2 (ppm)

4-(acetylamino)phenyl dipropan-2-ylcarbamate (**10g**)

^{1}H-NMR

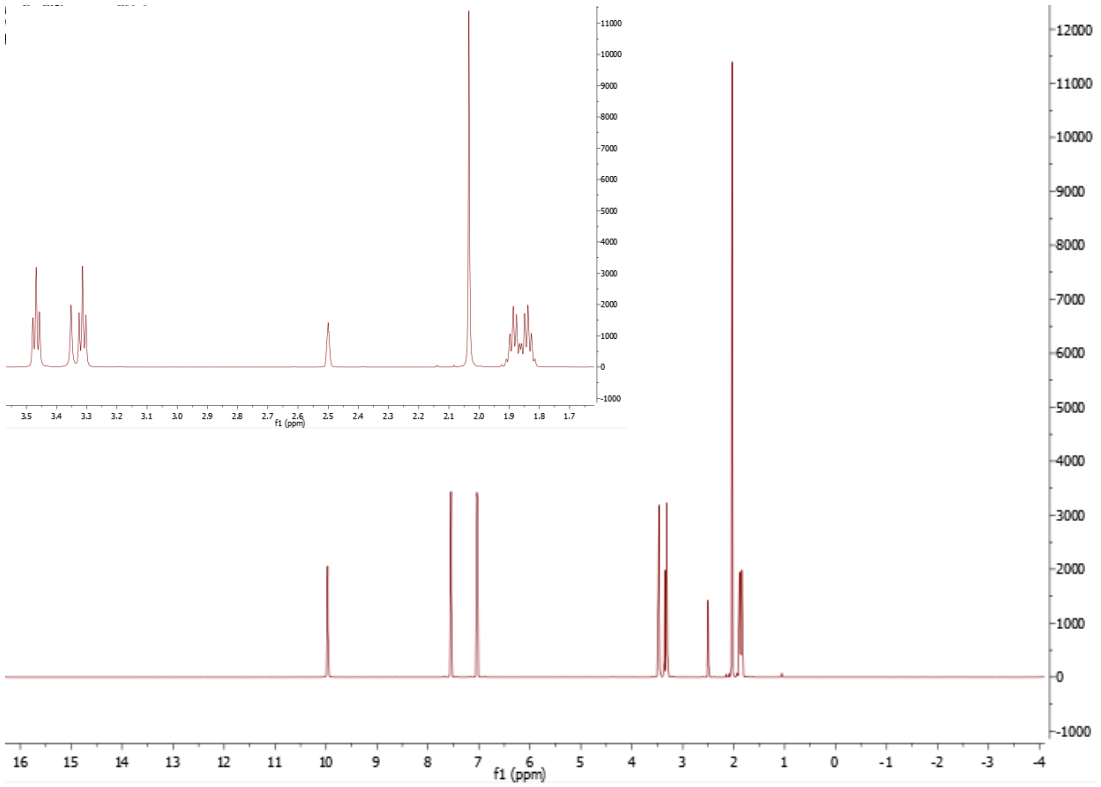


^{13}C-NMR

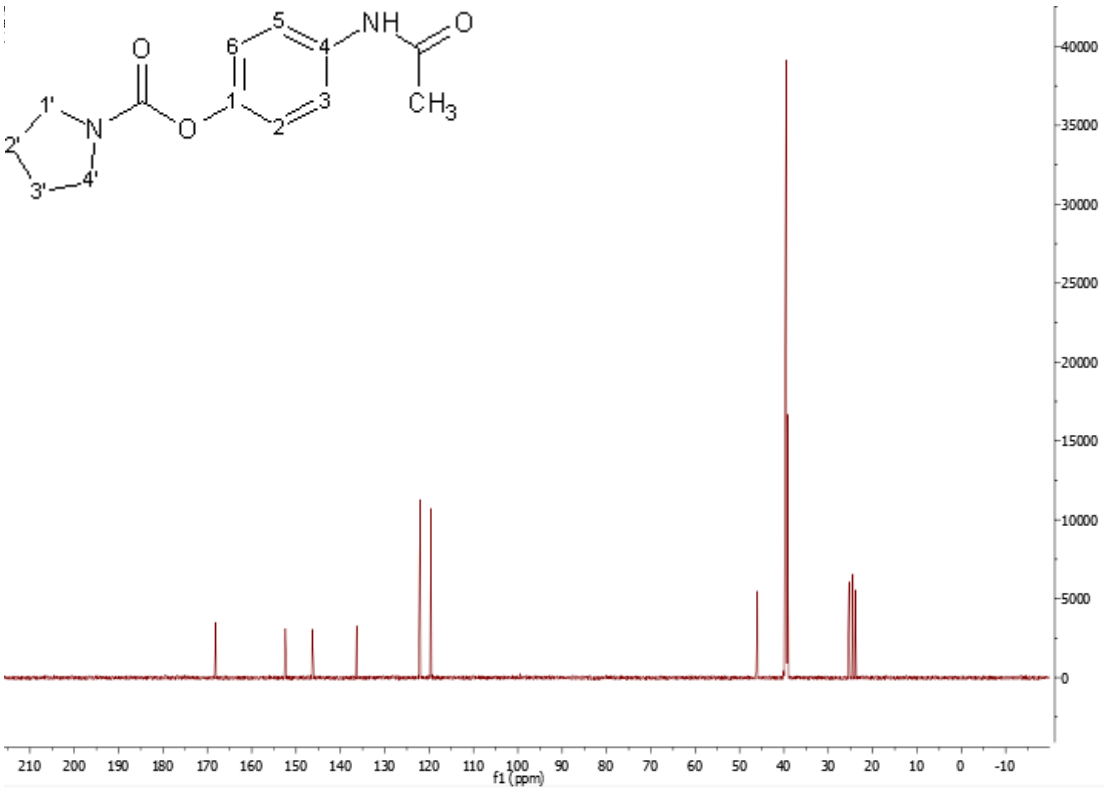


4-(acetylamino)phenyl pyrrolidine-1-carboxylate (**10h**)

^{1}H-NMR

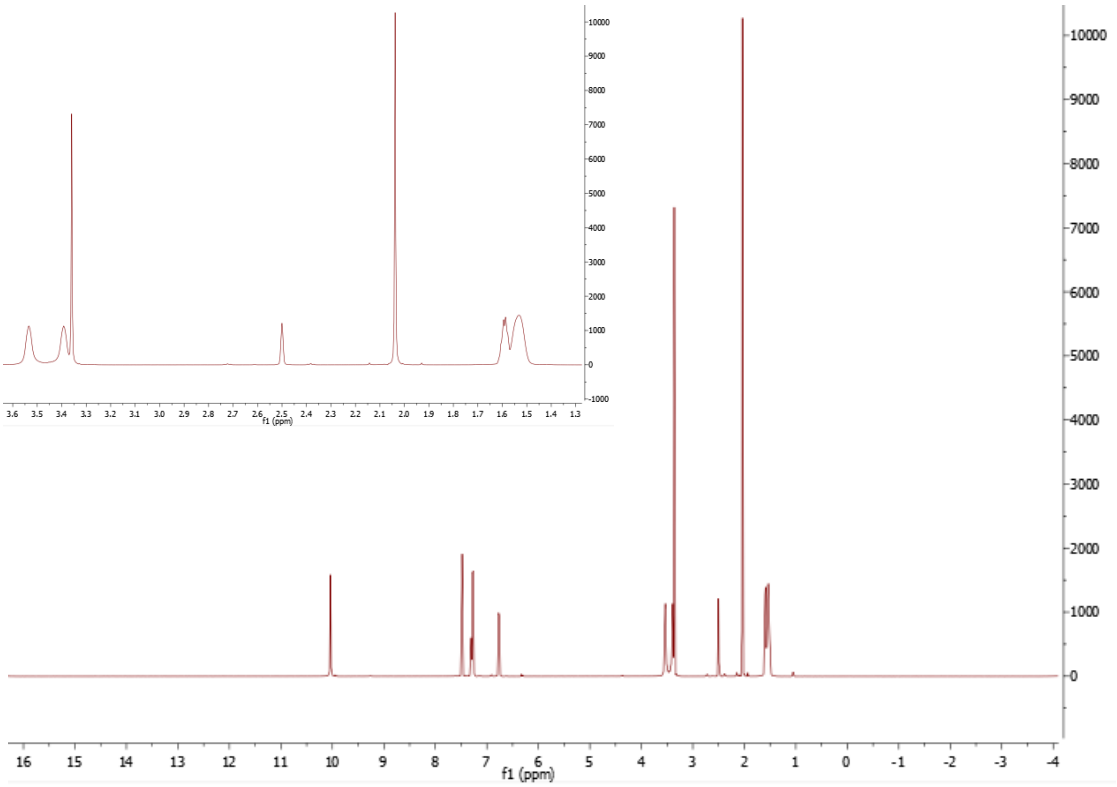


^{13}C-NMR



3-(acetylamino)phenyl piperidine-1-carboxylate (**11a**)

^{1}H-NMR



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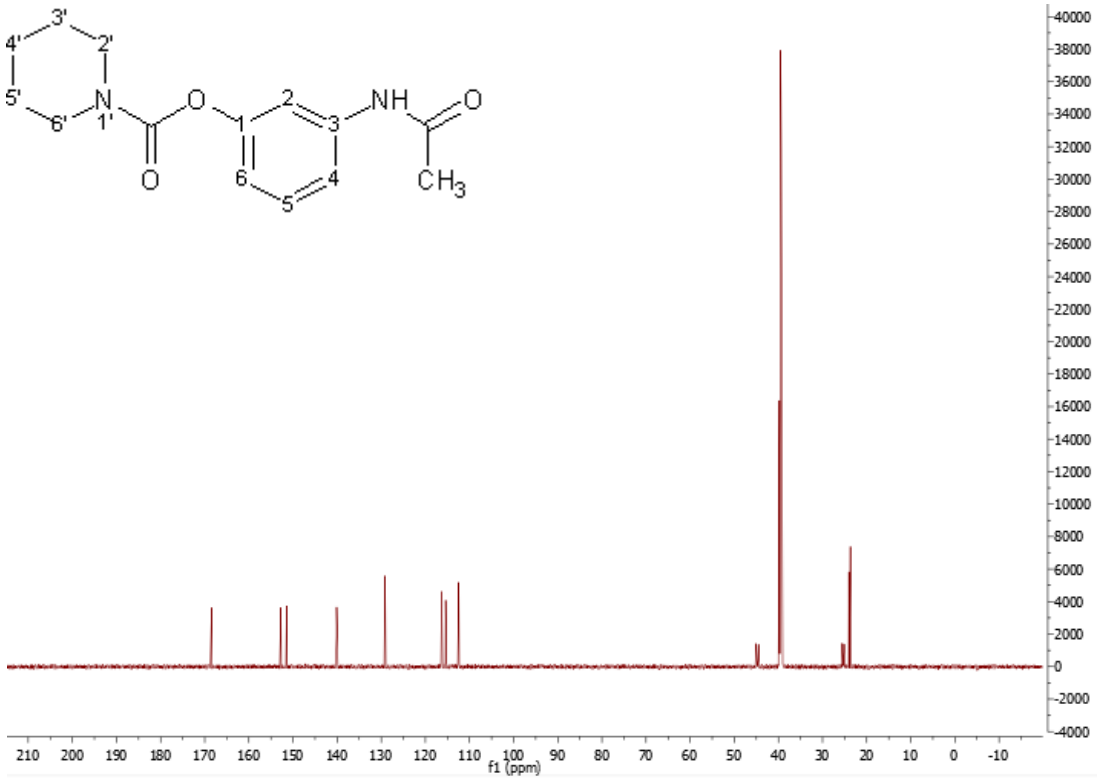
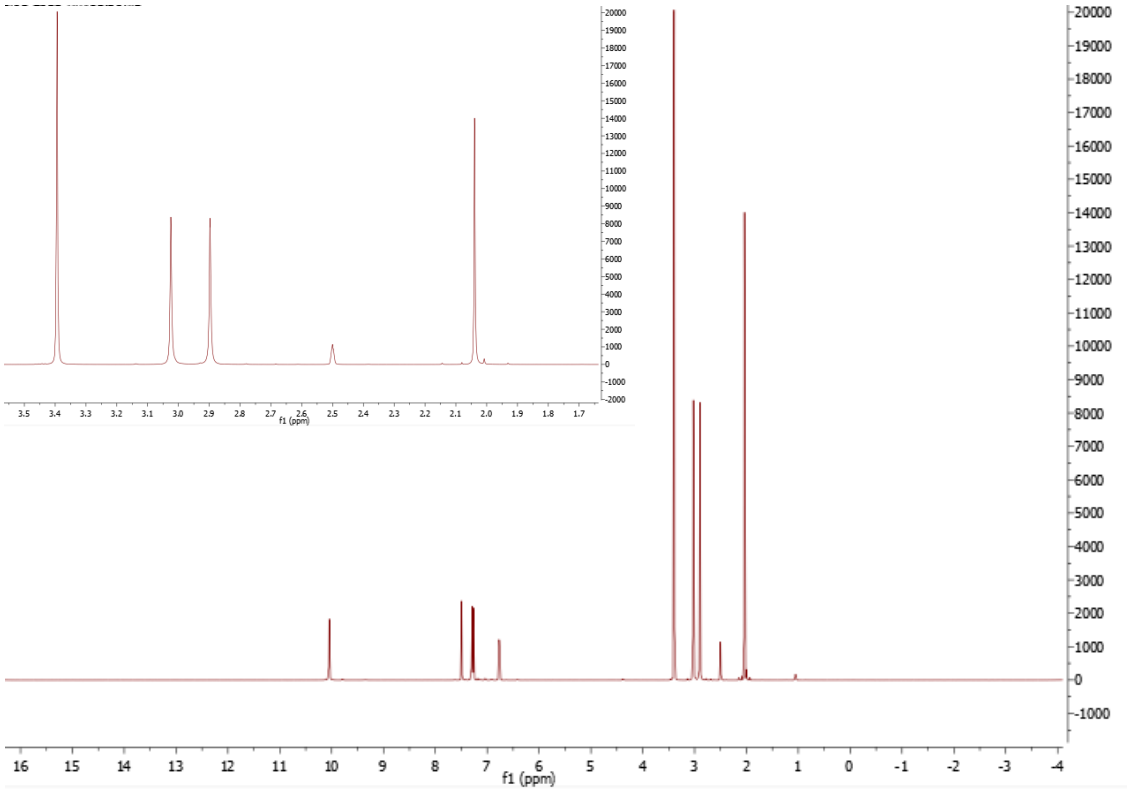
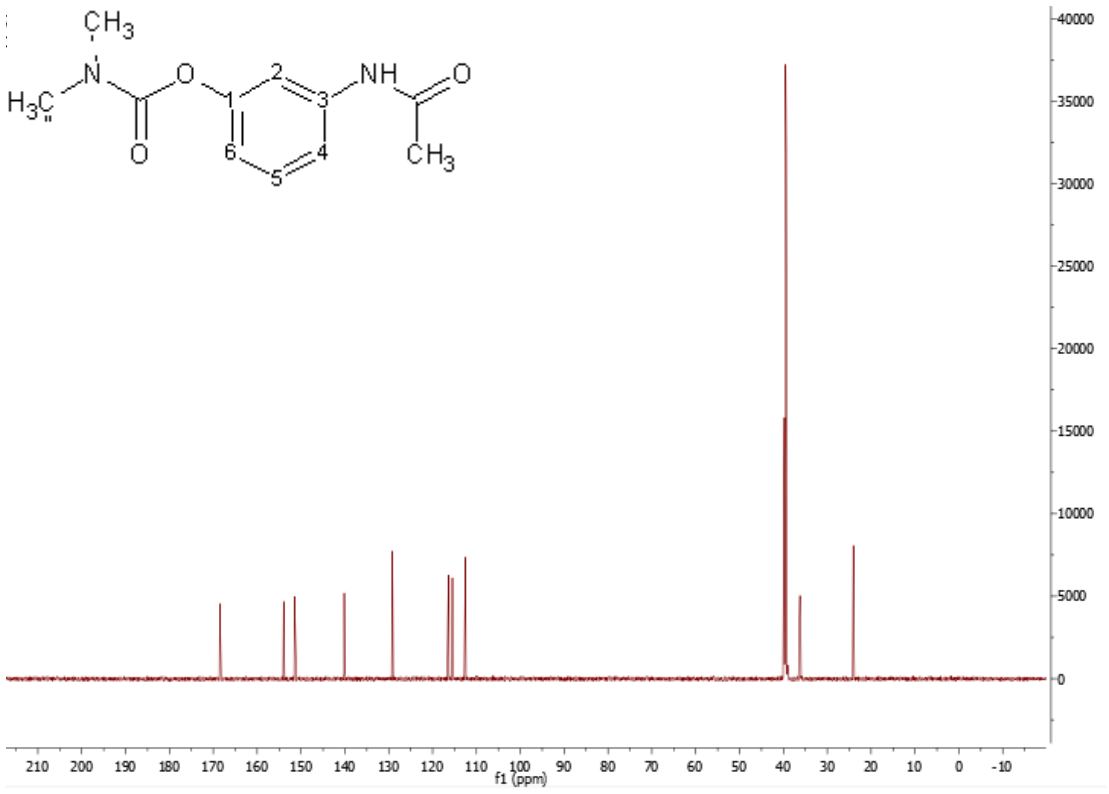


3-(acetylamino)phenyldimethylcarbamate (**11b**)

^{1}H-NMR

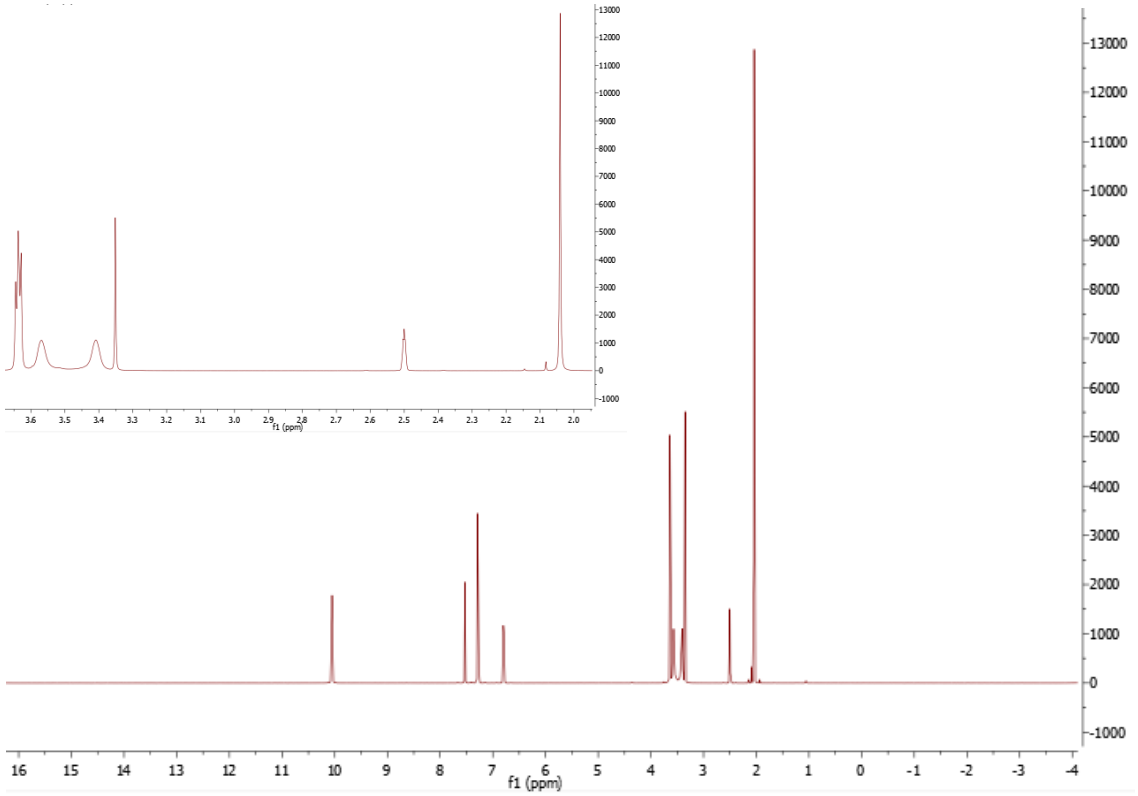


^{13}C-NMR

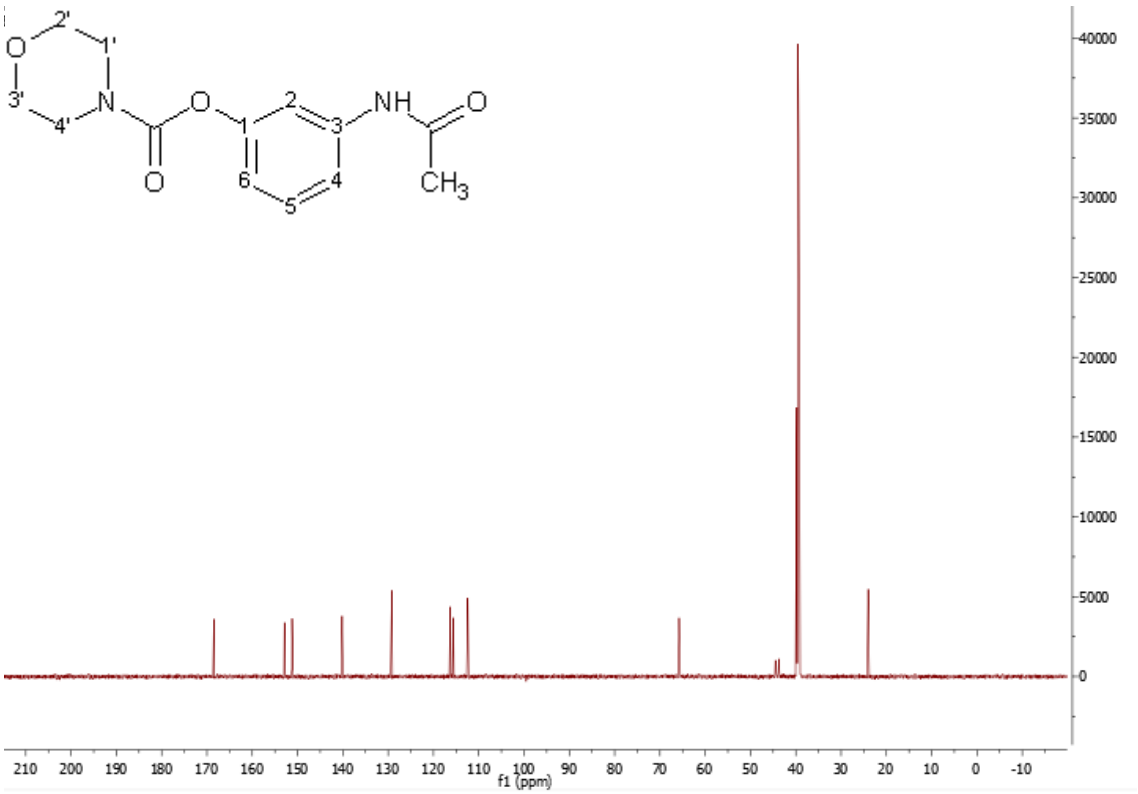


3-(acetylamino)phenyl morpholine-4-carboxylate (**11c**)

^{1}H-NMR

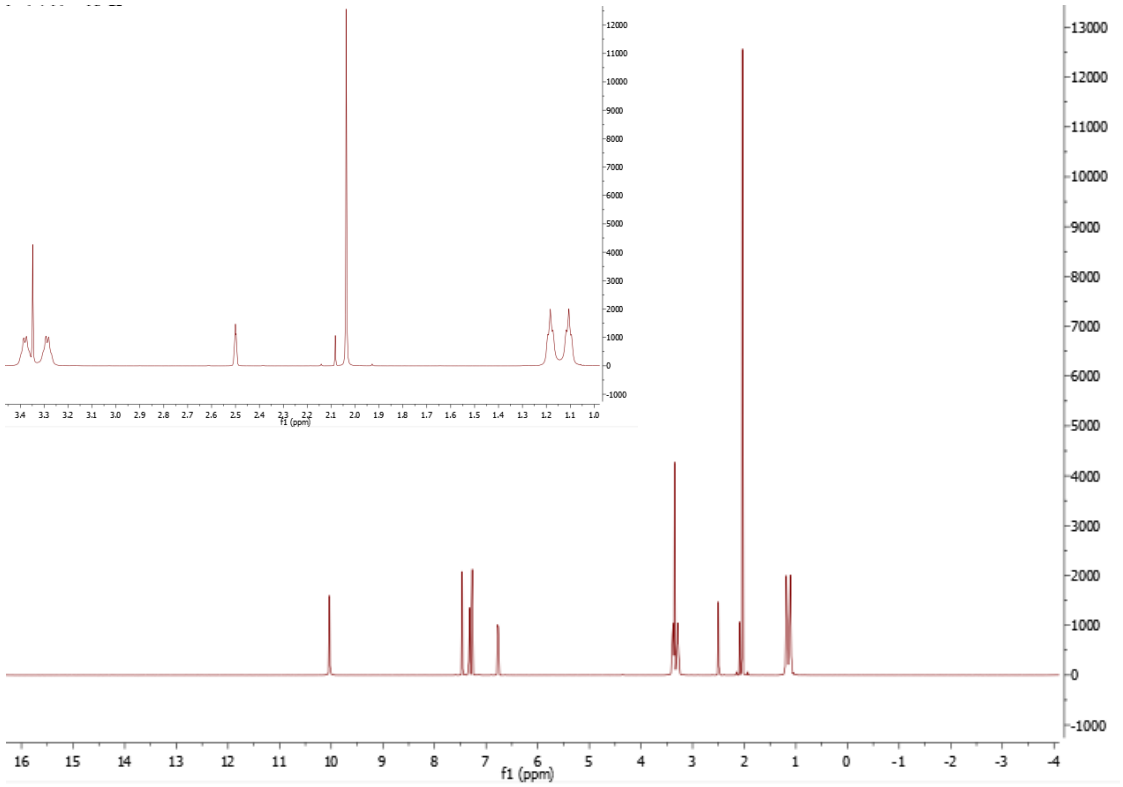


^{13}C-NMR

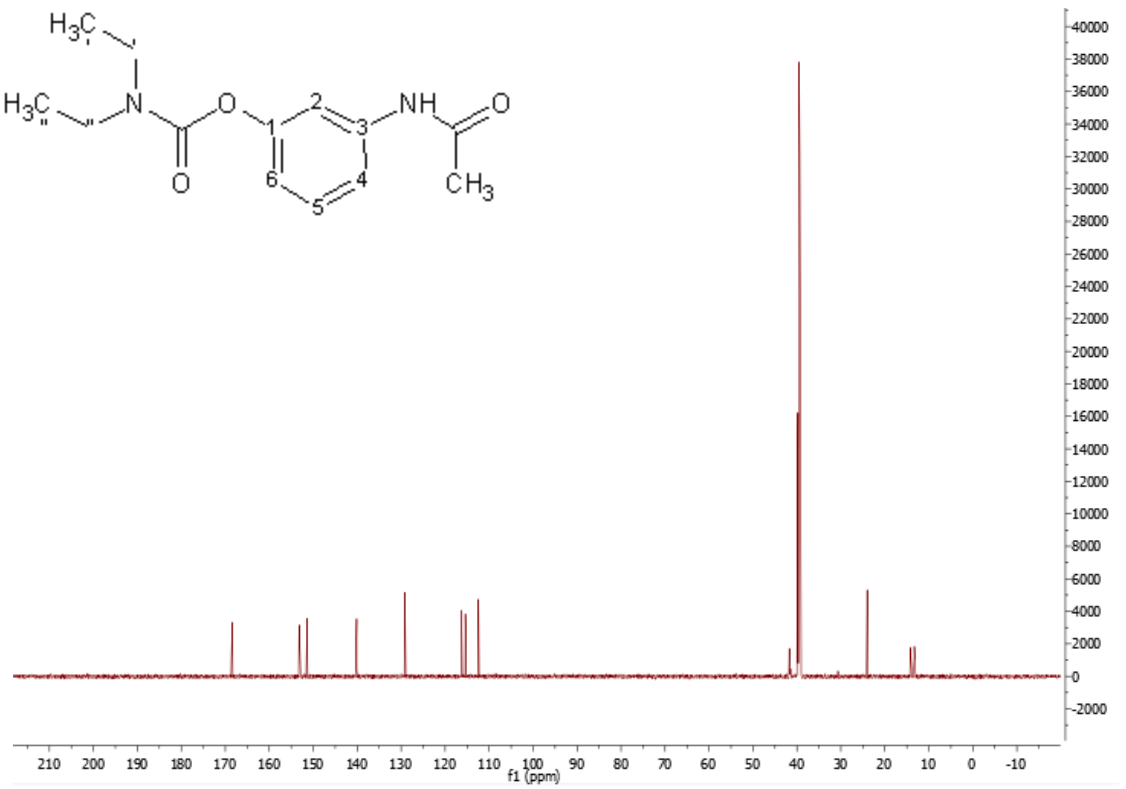


3-(acetylamino)phenyldiethylcarbamate (**11d**)

^{1}H-NMR

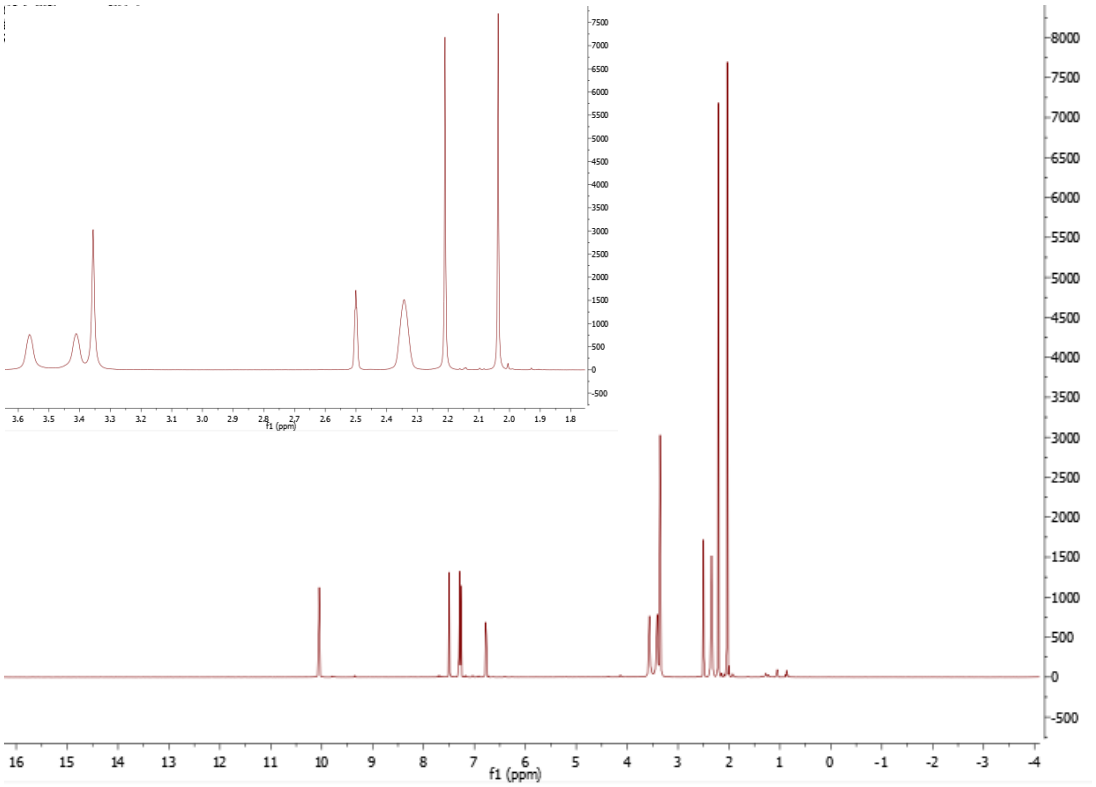


^{13}C-NMR

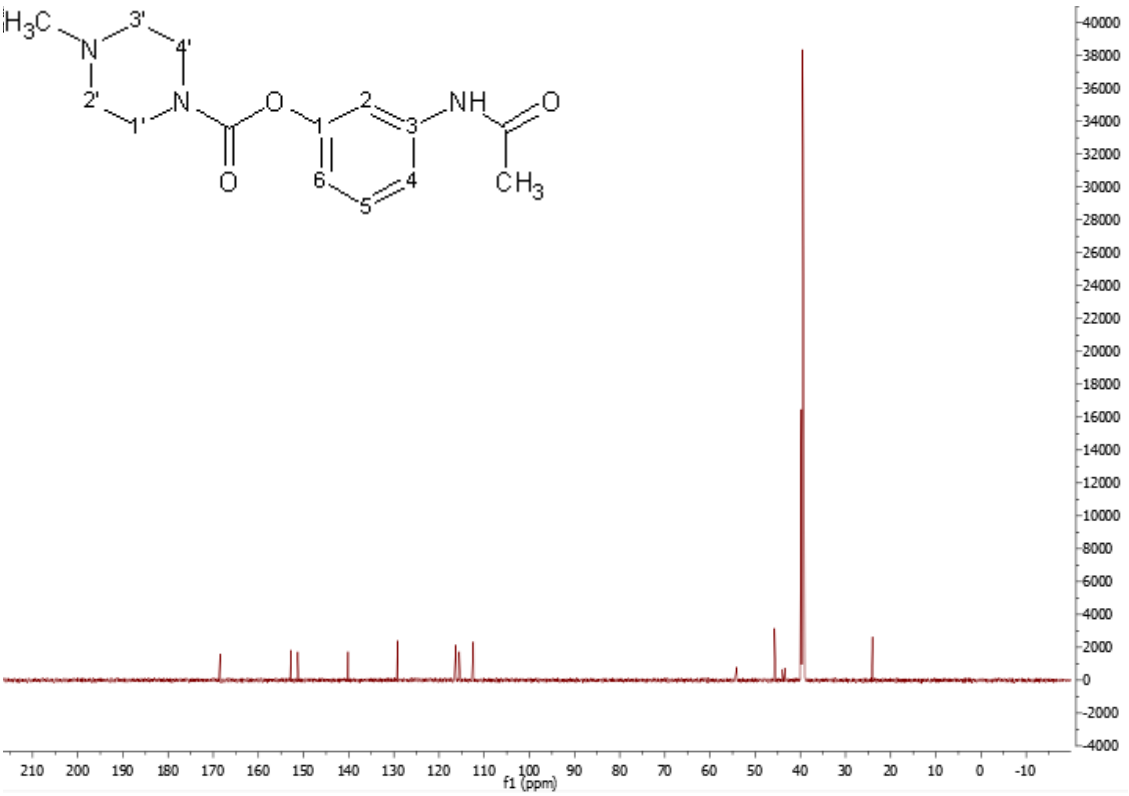


3-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**11e**)

^{1}H-NMR



^{13}C-NMR



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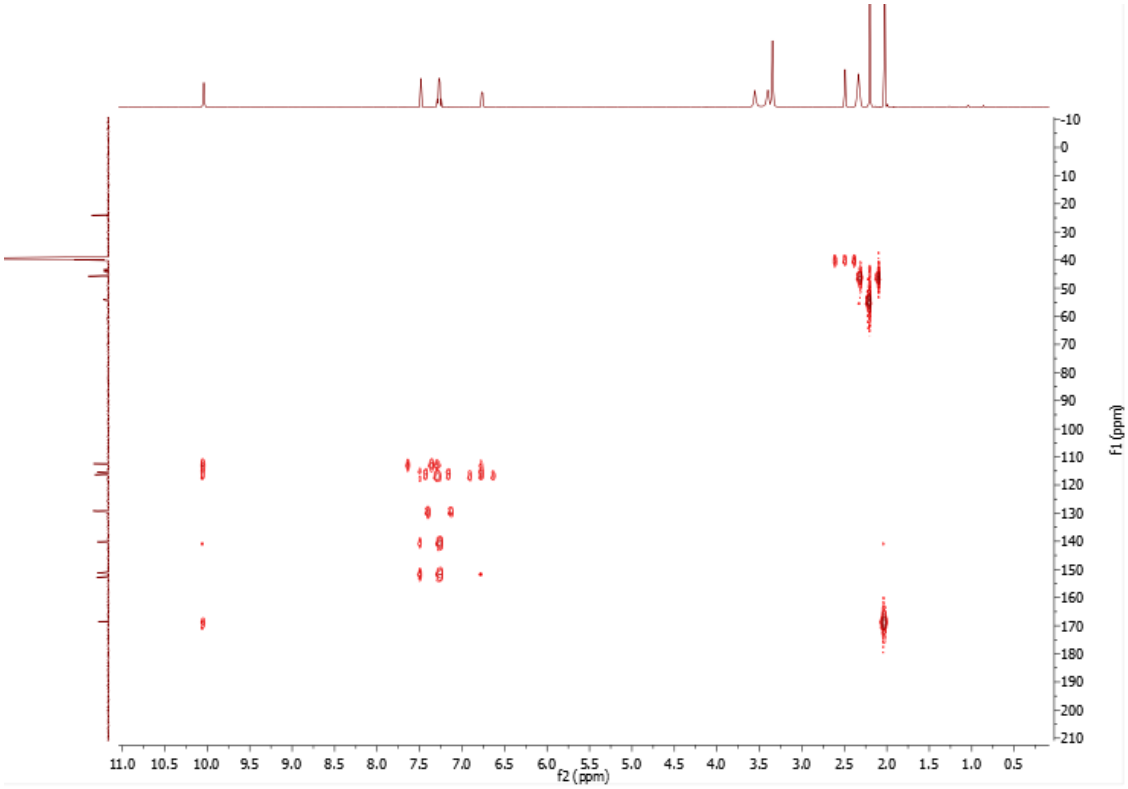
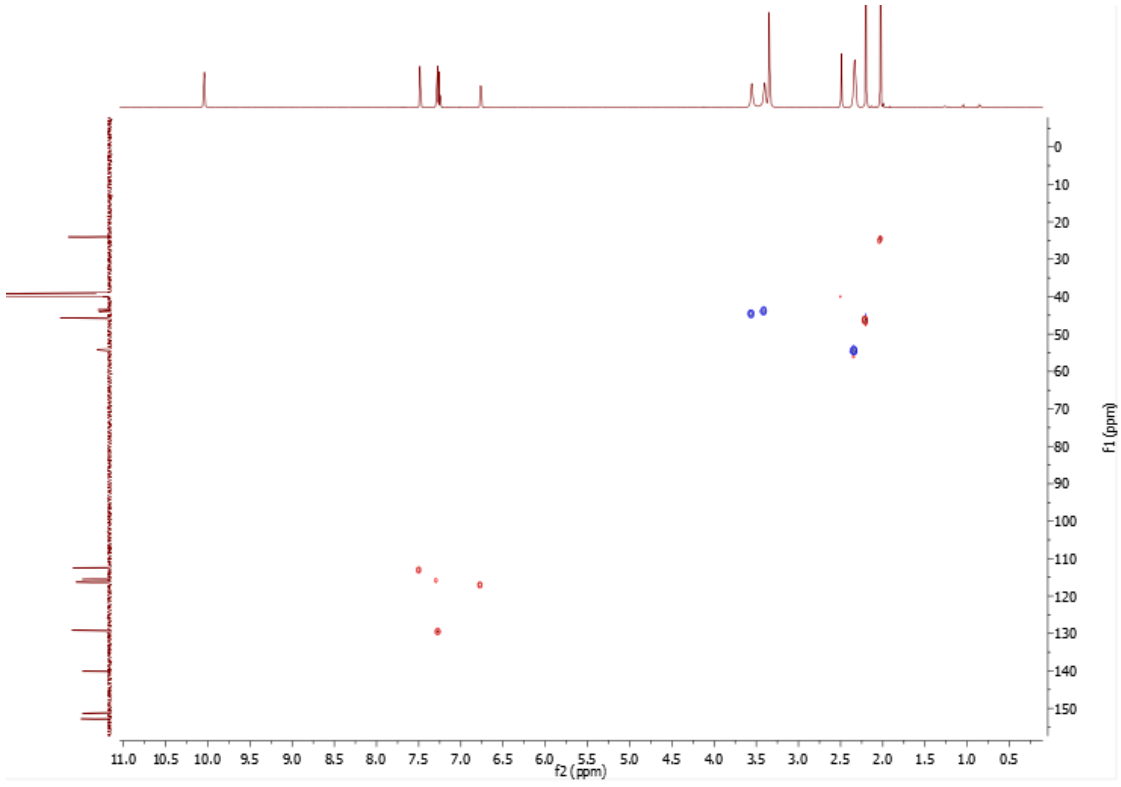
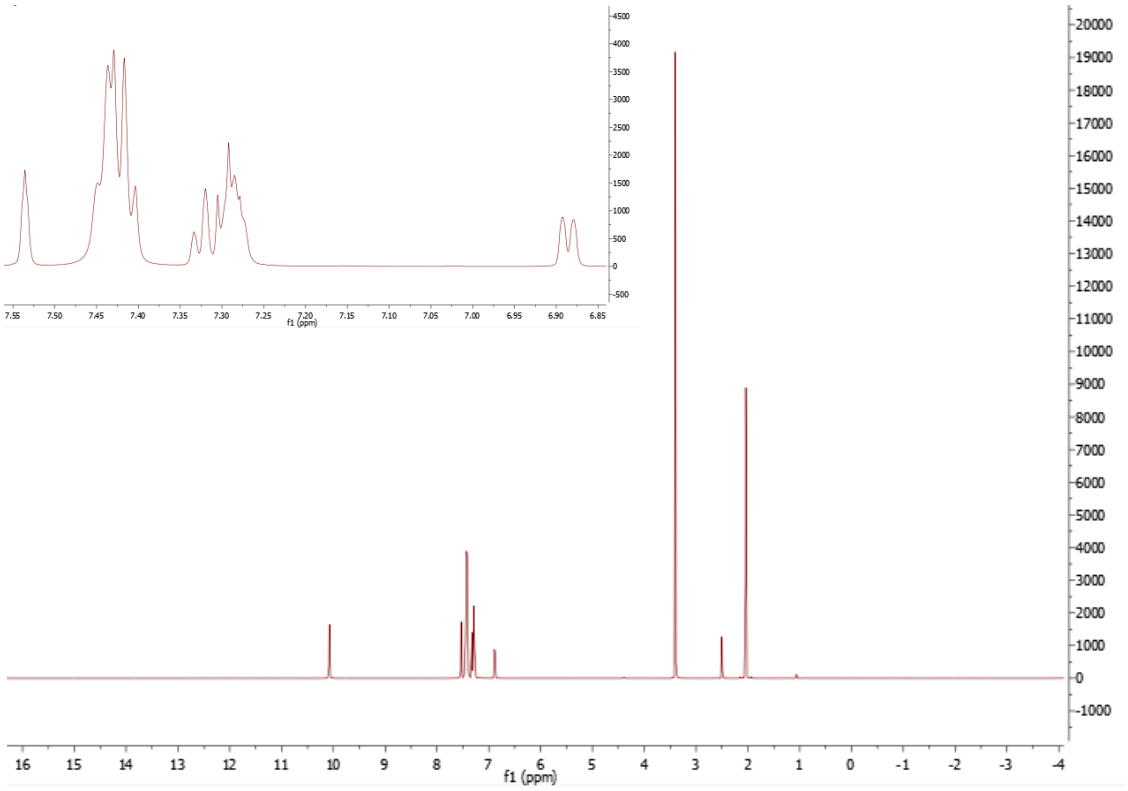


HSQC

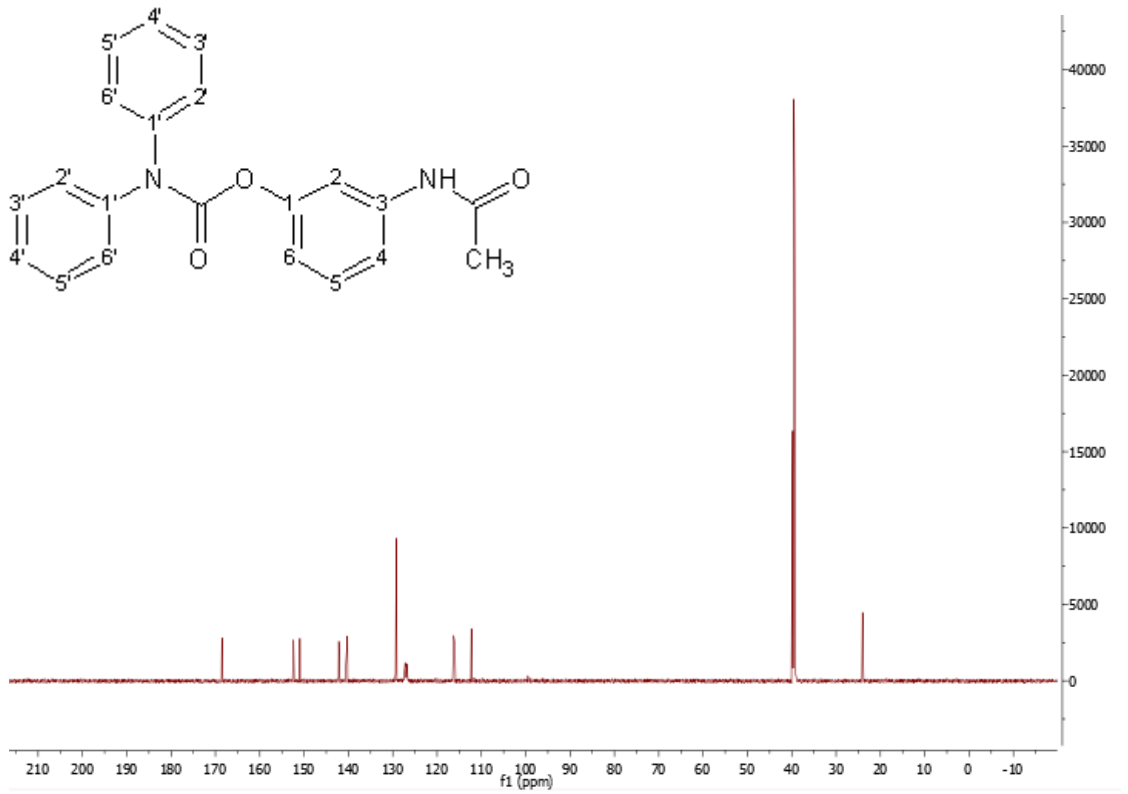


3-(acetylamino)phenyldiphenylcarbamate (**11f**)

^{1}H-NMR

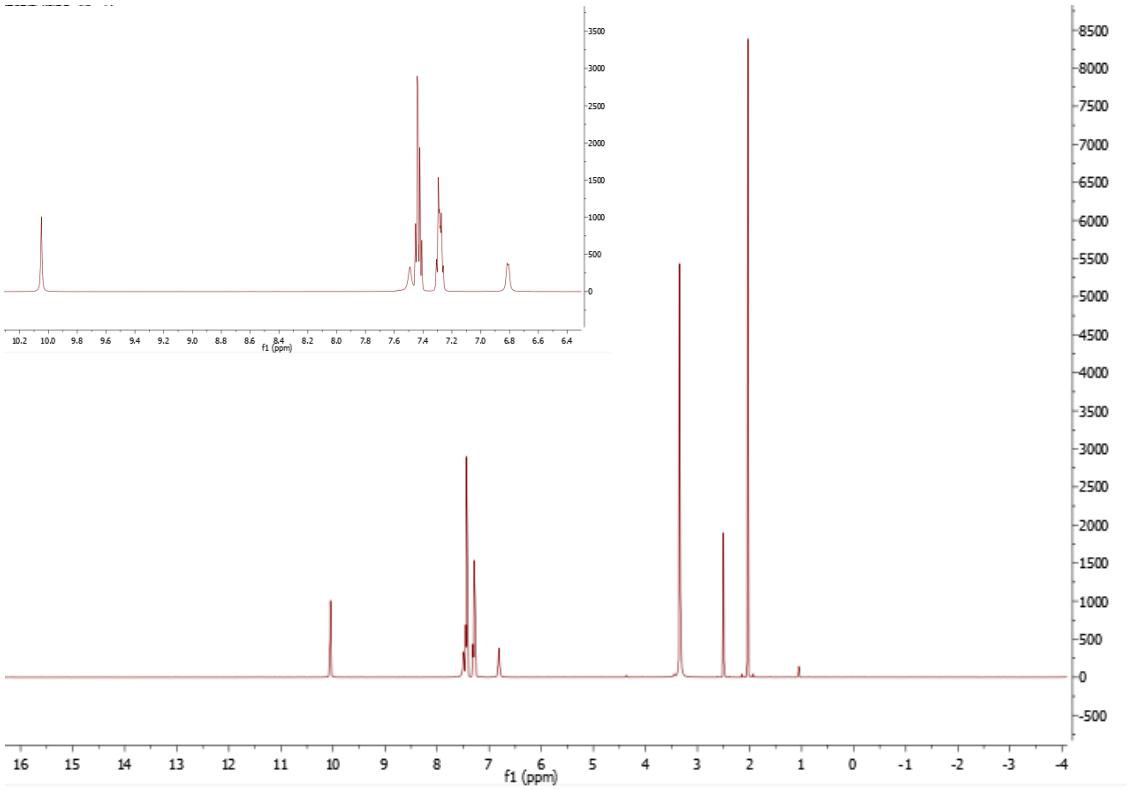


^{13}C-NMR

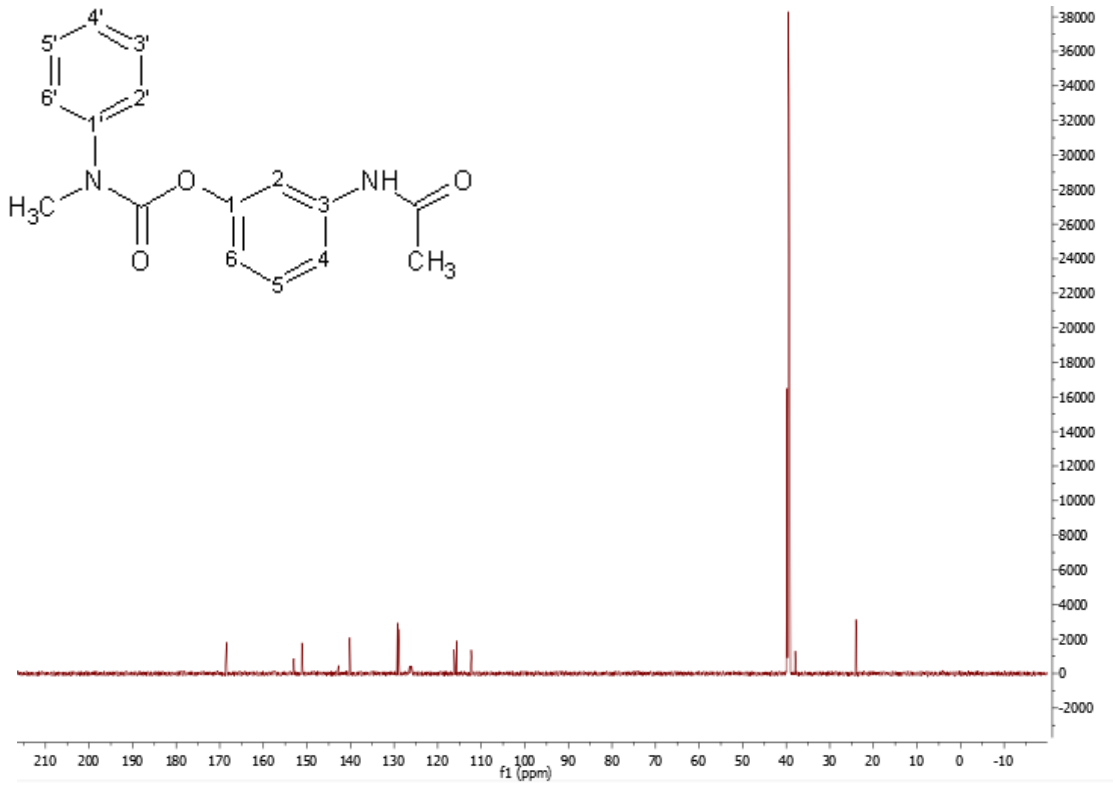


3-(acetylamino)phenyl methyl(phenyl)carbamate (**11g**)

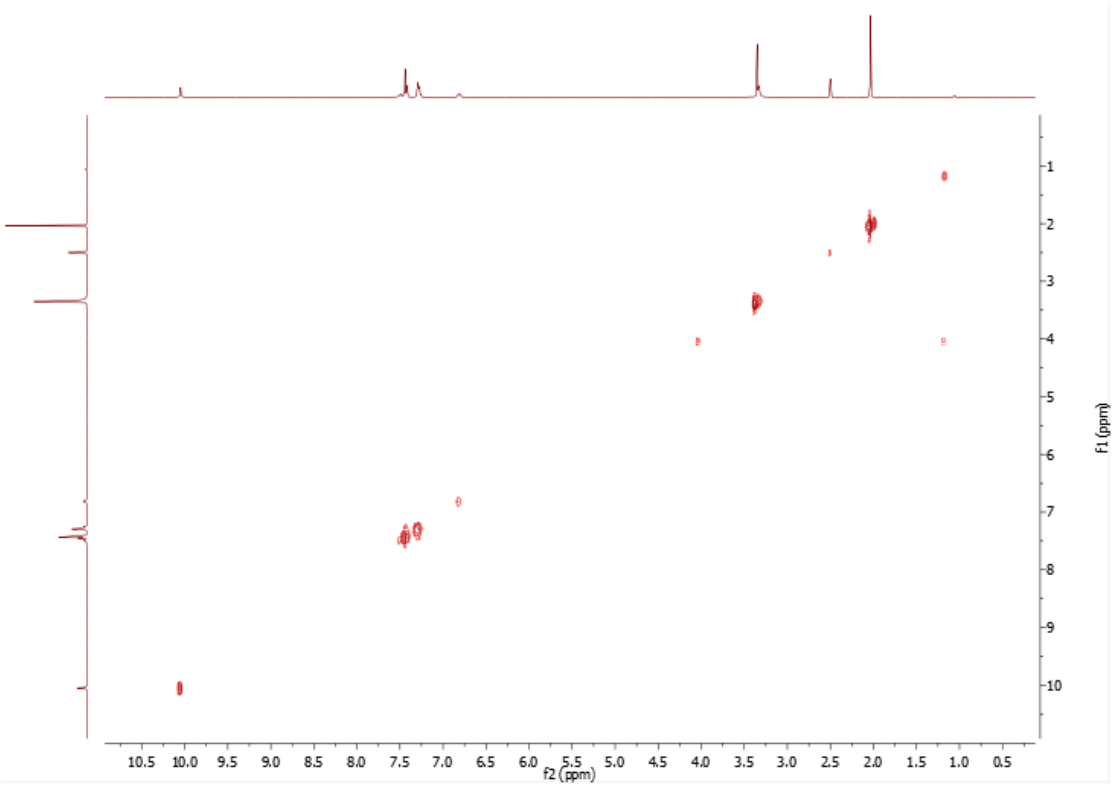
^{1}H-NMR



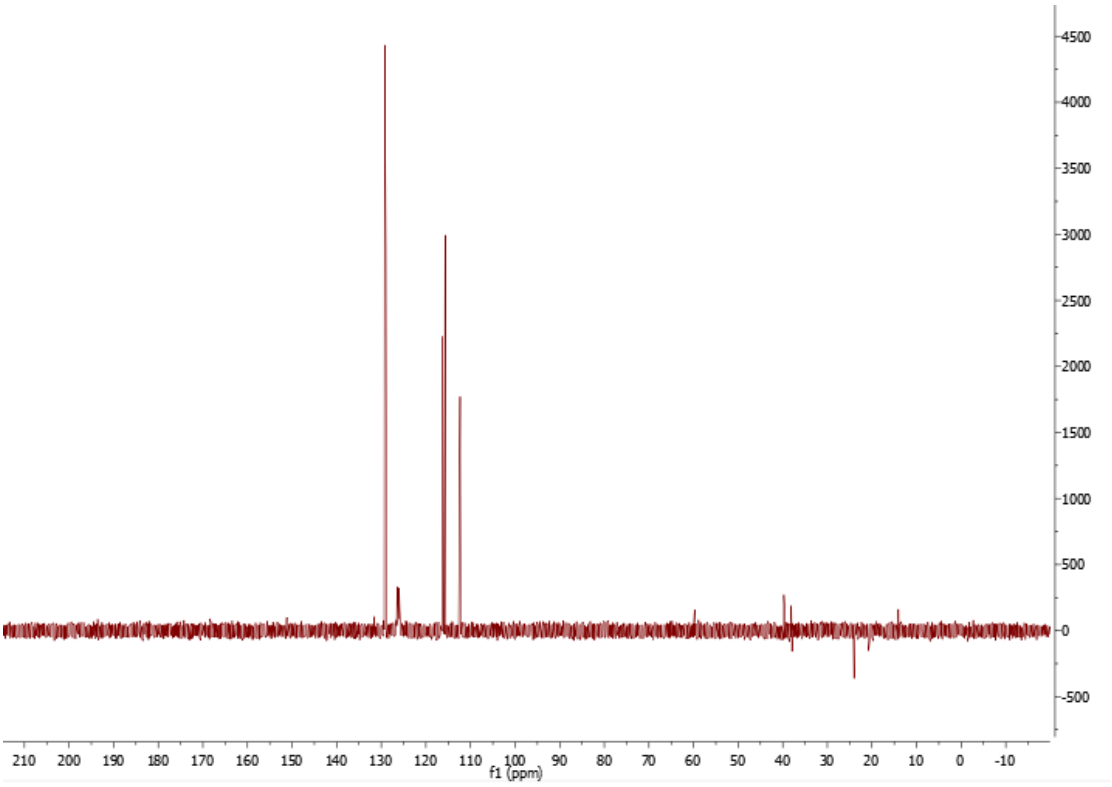
^{13}C-NMR



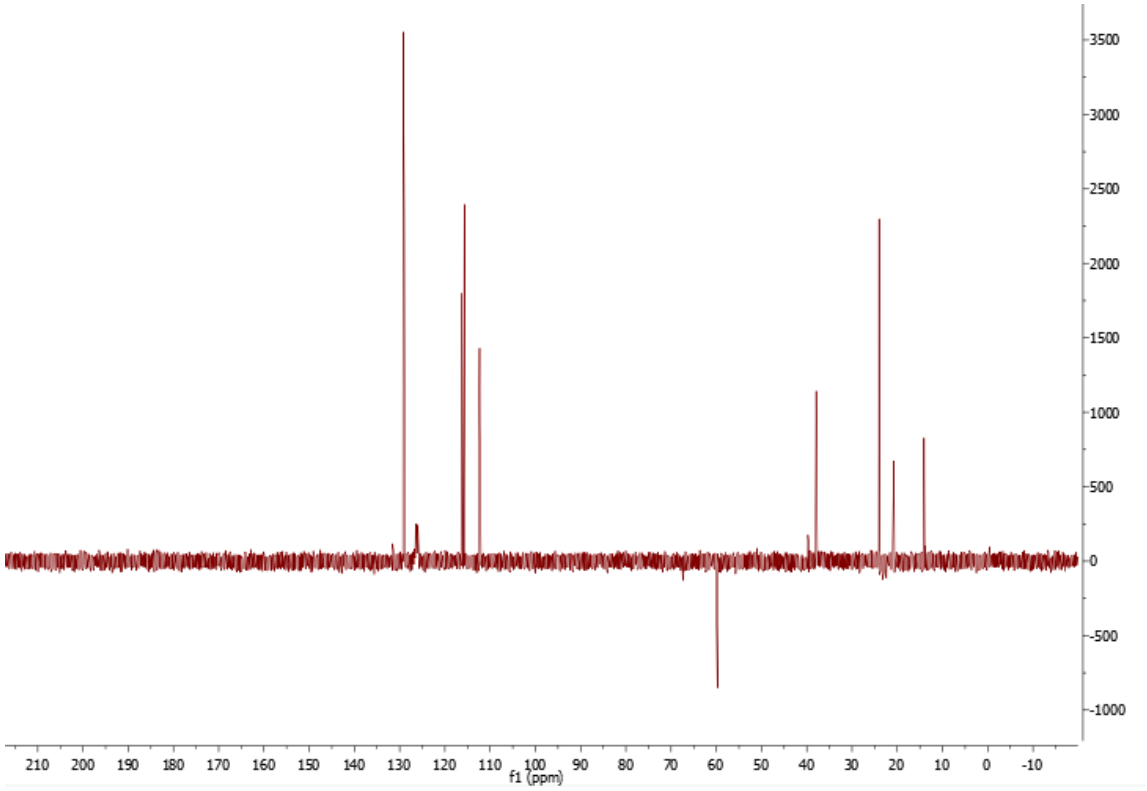
COSY



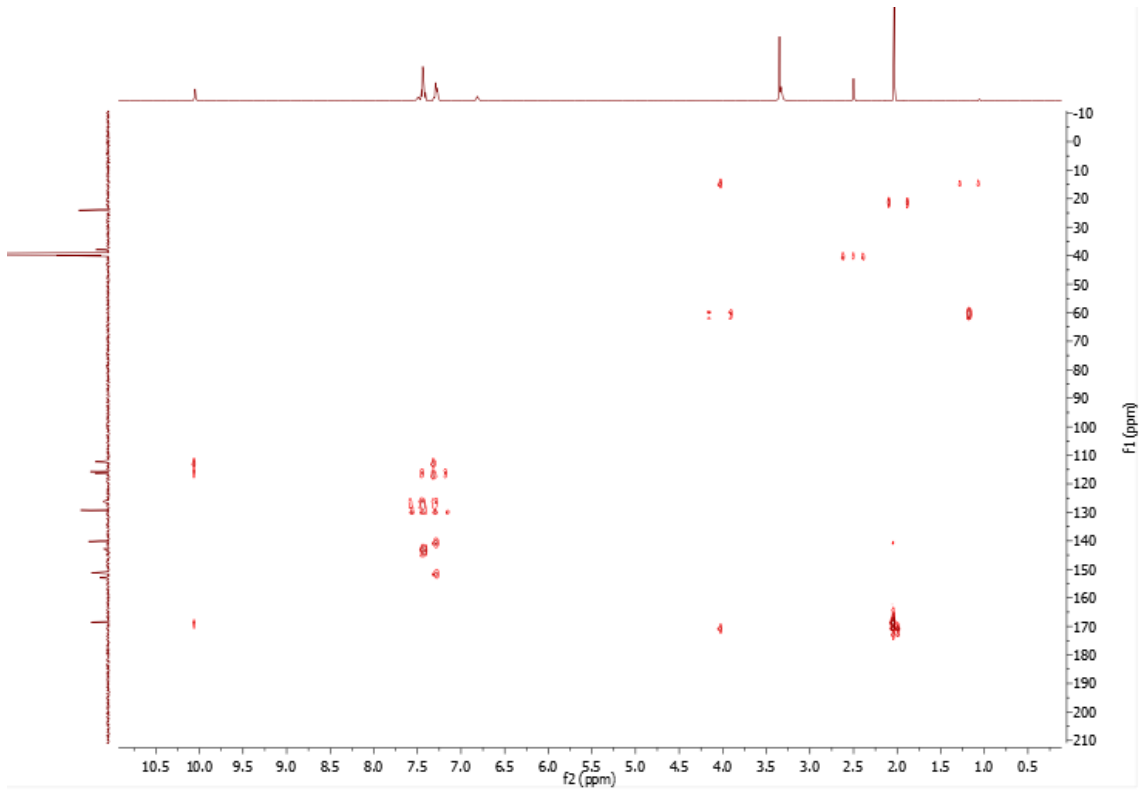
DEPT 95



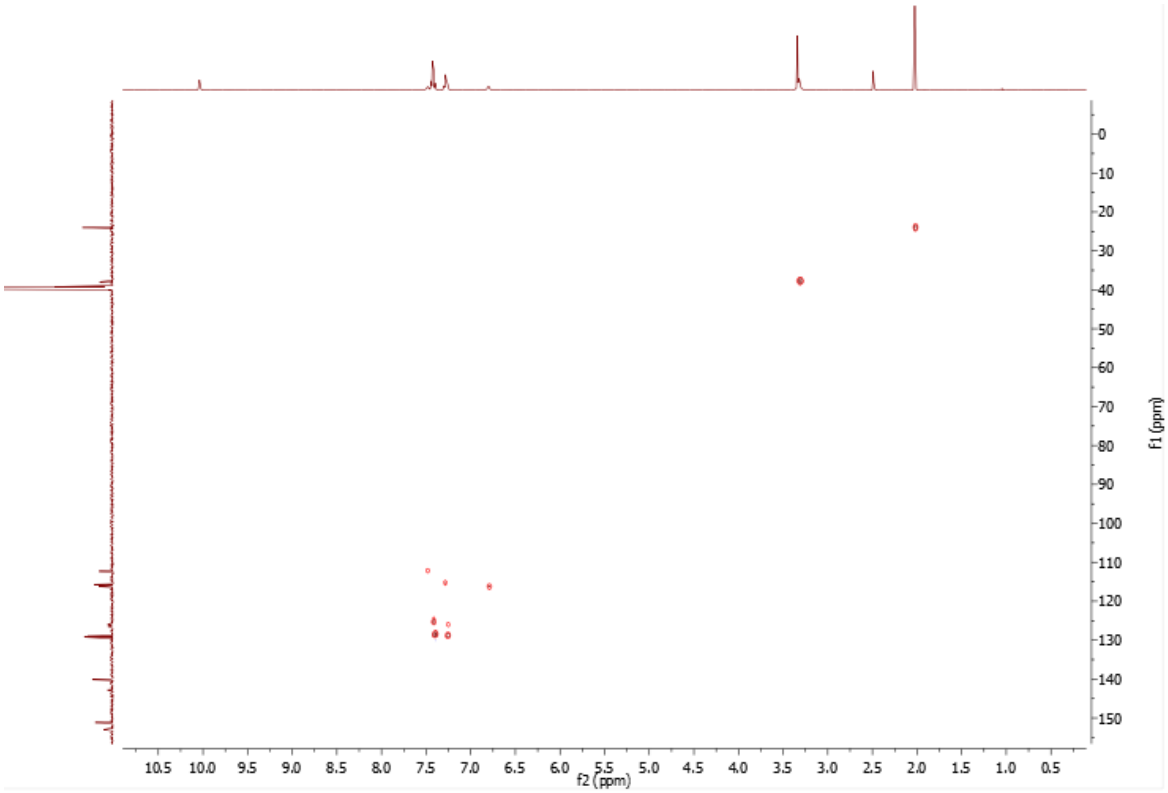
DEPT 135



HMBC

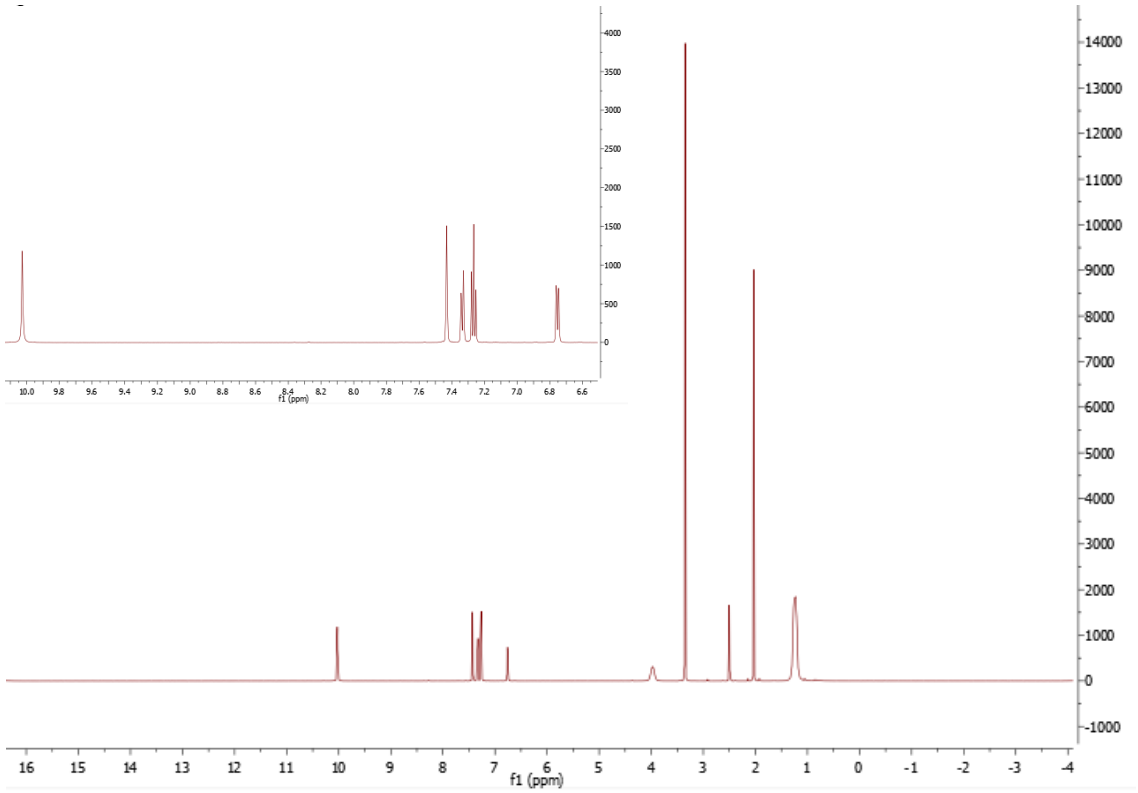


HSQC

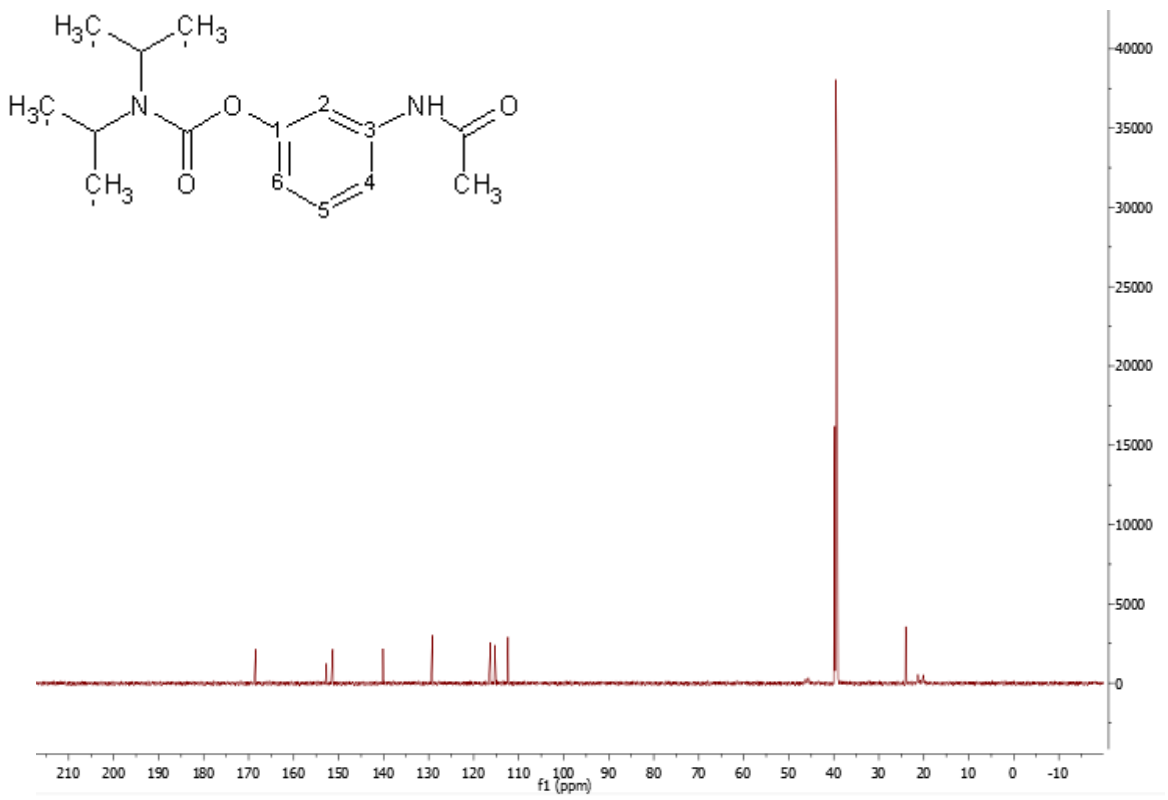

0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
F1 (ppm)
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f2 (ppm)

3-(acetylamino)phenyl dipropan-2-ylcarbamate (**11h**)

^{1}H-NMR

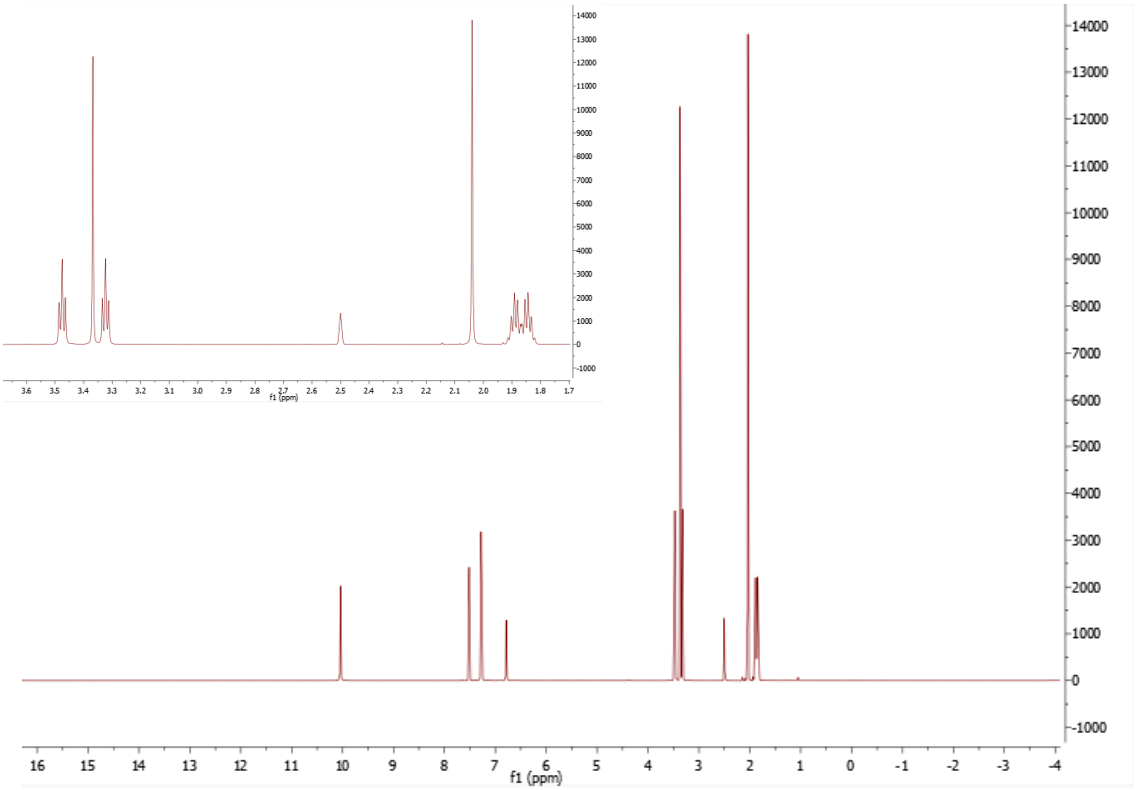


^{13}C-NMR

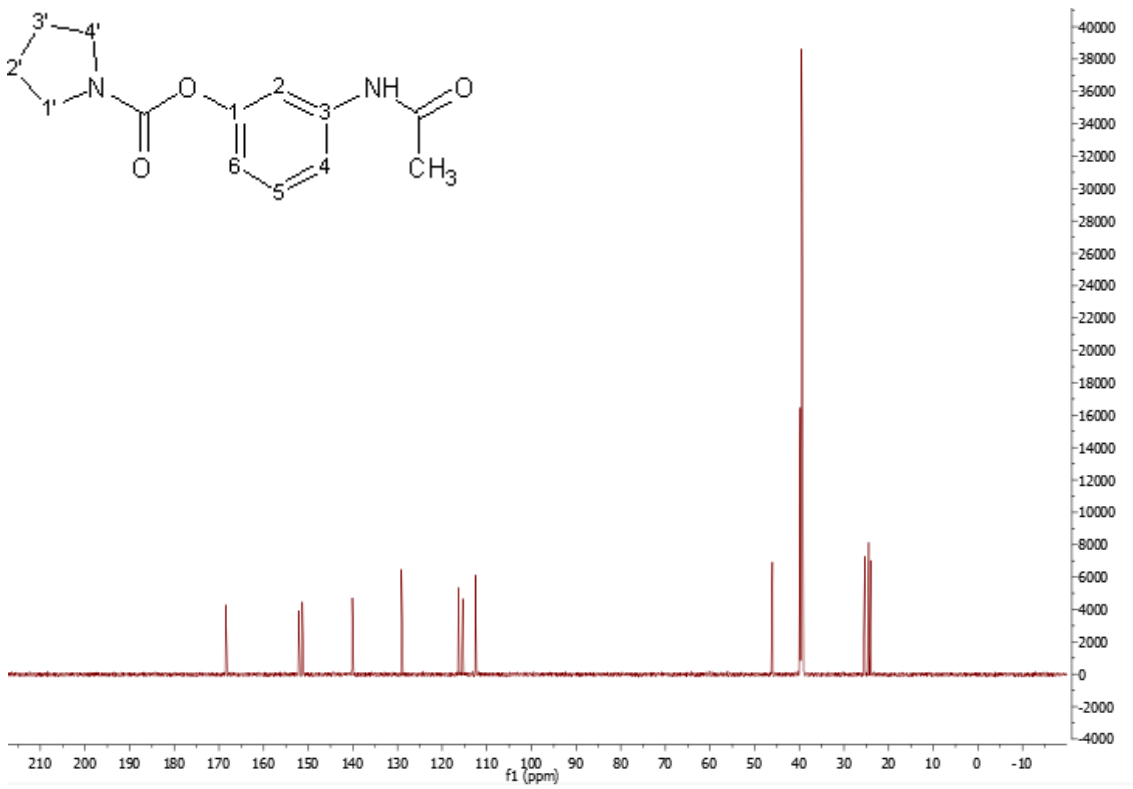


3-(acetylamino)phenyl pyrrolidine-1-carboxylate (**11i**)

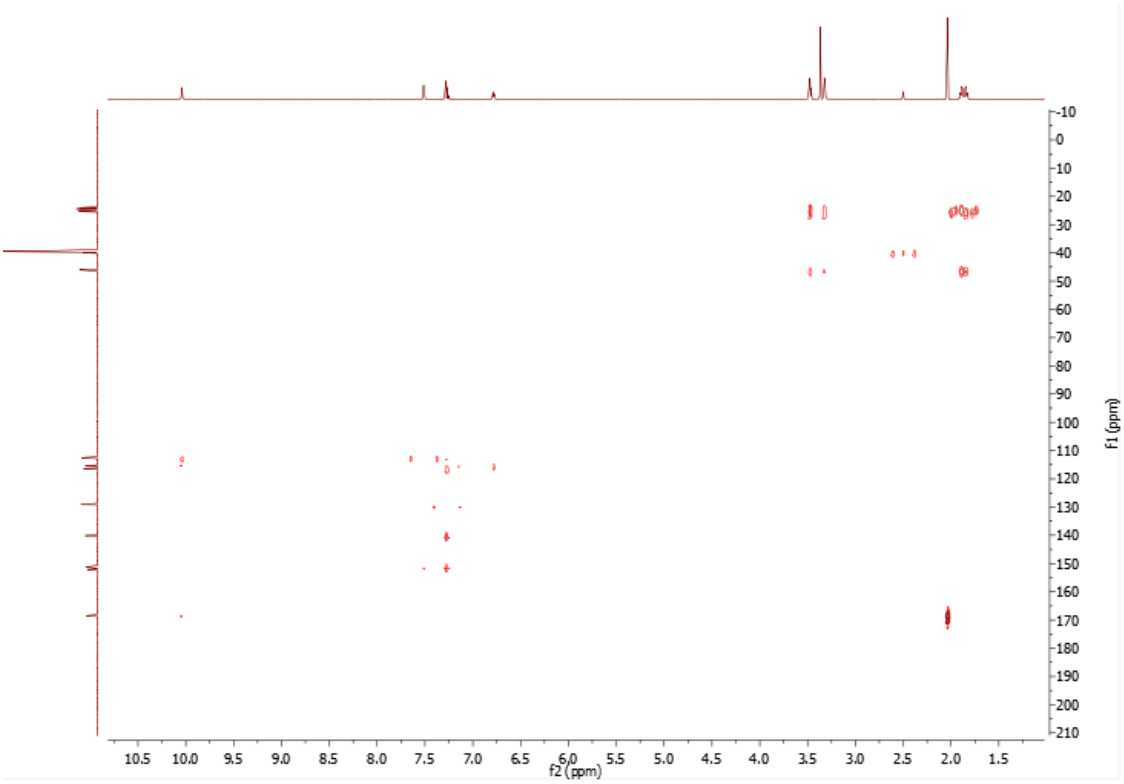
^{1}H-NMR



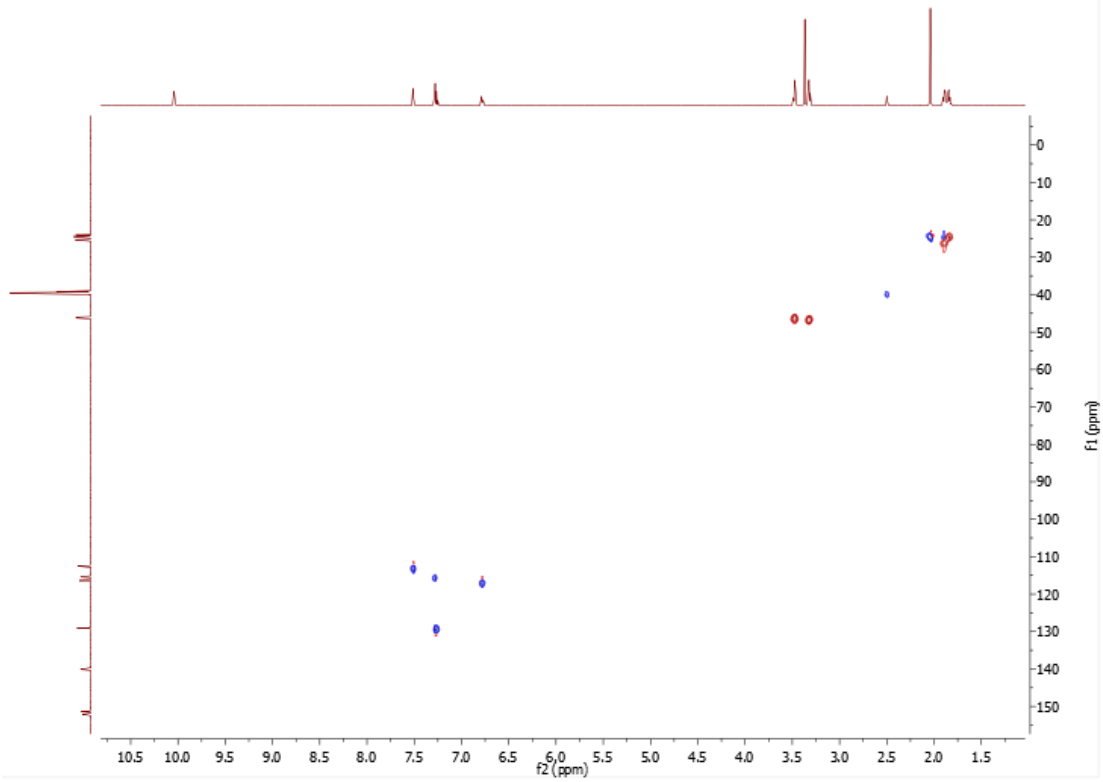
^{13}C-NMR



HMBC

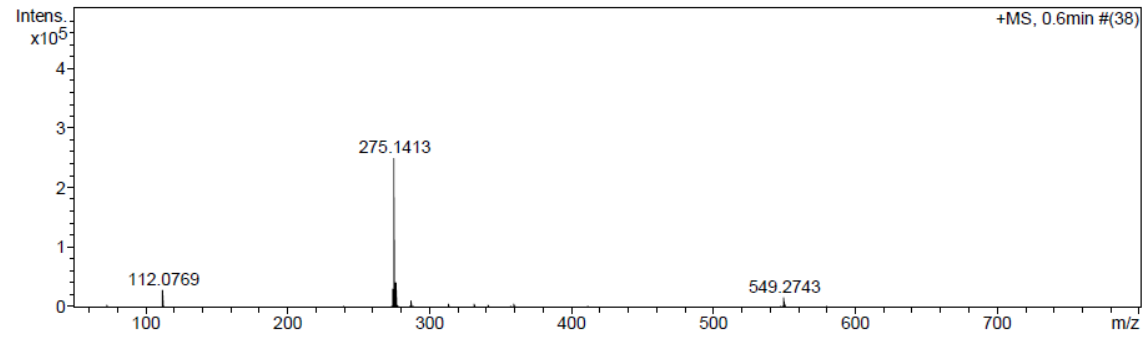


HSQC



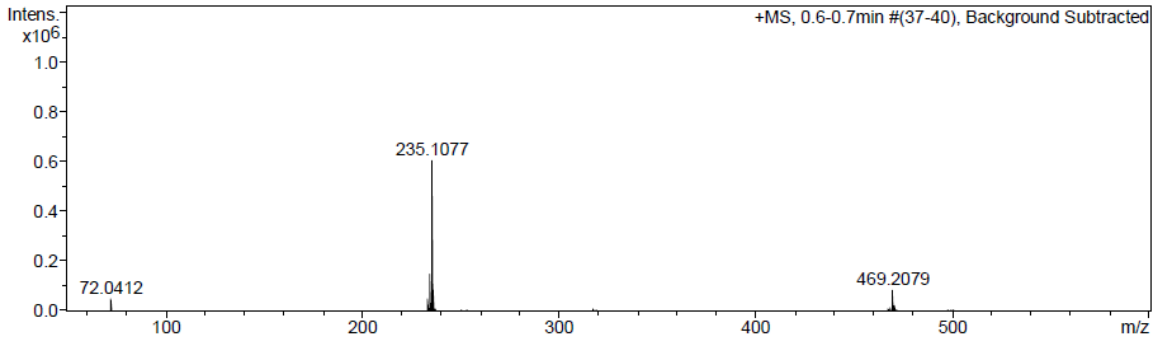
MASS SPECTRA

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl piperidine-1-carboxylate (**8a**)



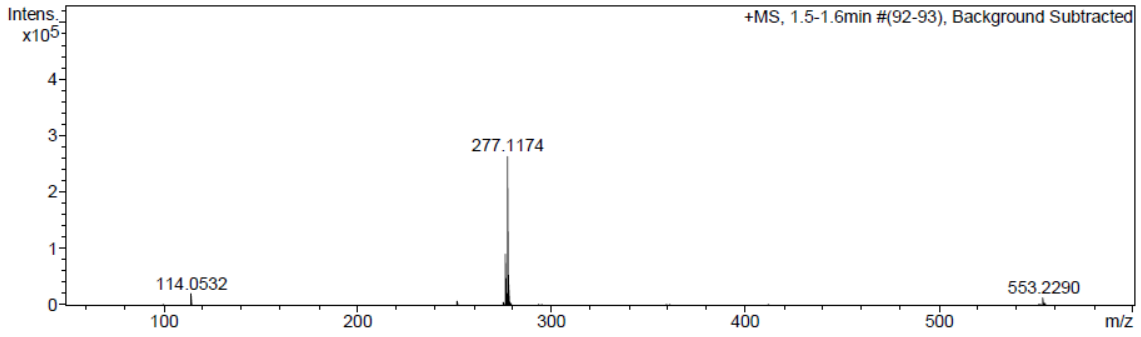
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
275.1413	$C_{15}H_{19}N_2O_3$	100.00	275.1390	-2.2	-8.1	3.4	7.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl dimethylcarbamate (**8b**)



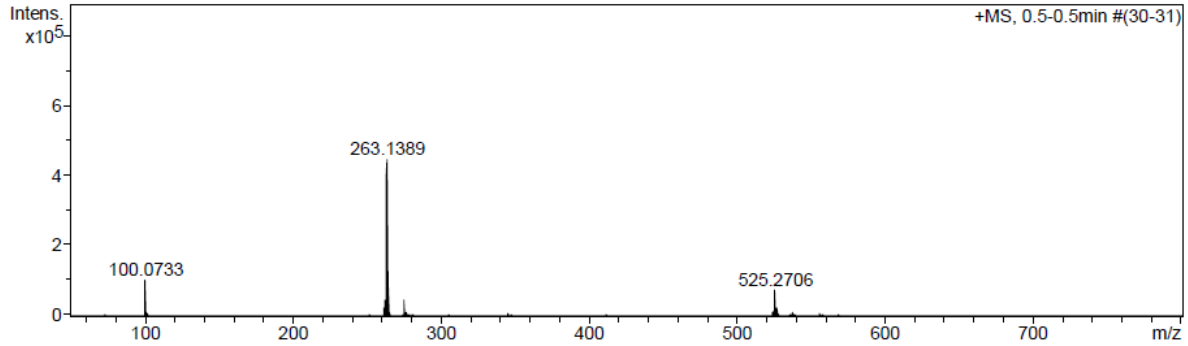
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
235.1077	$C_{12}H_{15}N_2O_3$	10.00	235.1077	0.1	0.2	13.9	6.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**8c**)



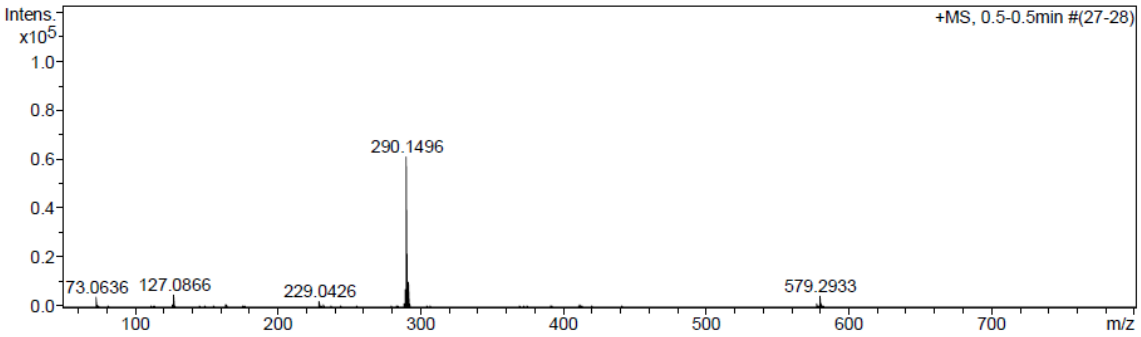
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
277.1174	$C_{14}H_{17}N_2O_4$	100.00	277.1183	0.9	1.3	5.2	7.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diethylcarbamate (**8d**)



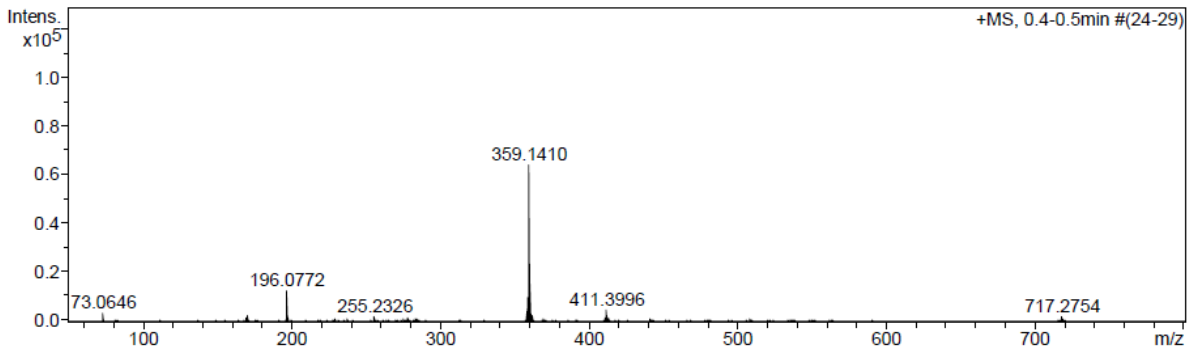
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
263.1389	$C_{14}H_{19}N_2O_3$	100.00	263.1390	0.1	0.4	11.0	6.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl 4-methylpiperazine-1-carboxylate (**8e**)



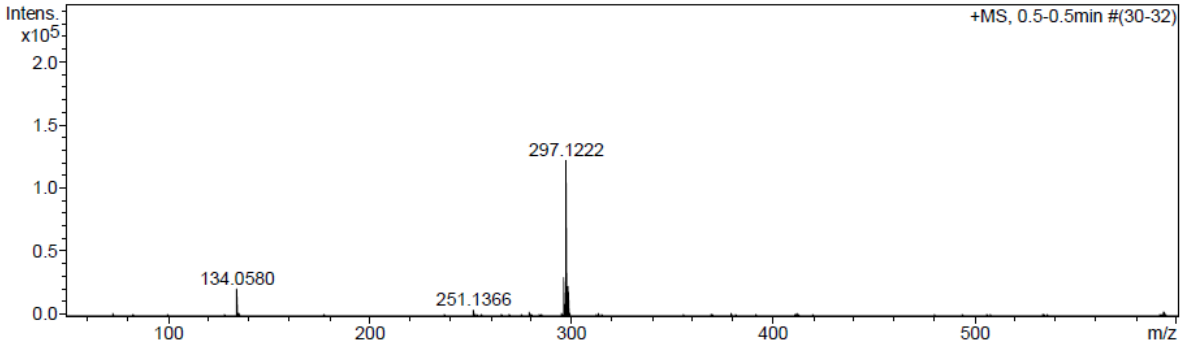
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
290.1496	$C_{15}H_{19}N_2O_3$	100.00	290.1489	0.3	1.2	3.1	7.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diphenylcarbamate (**8f**)



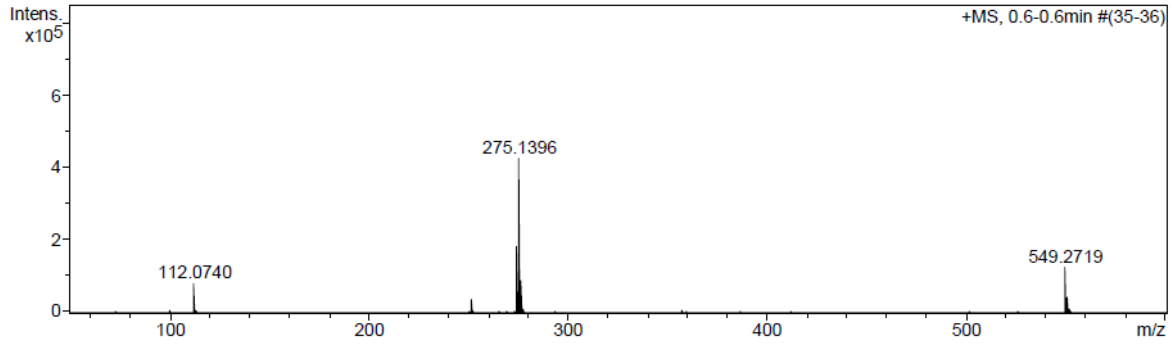
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
359.1410	$C_{22}H_{19}N_2O_3$	100.00	359.1390	-2.0	-5.5	5.4	14.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-7-yl methyl(phenyl)carbamate (**8g**)



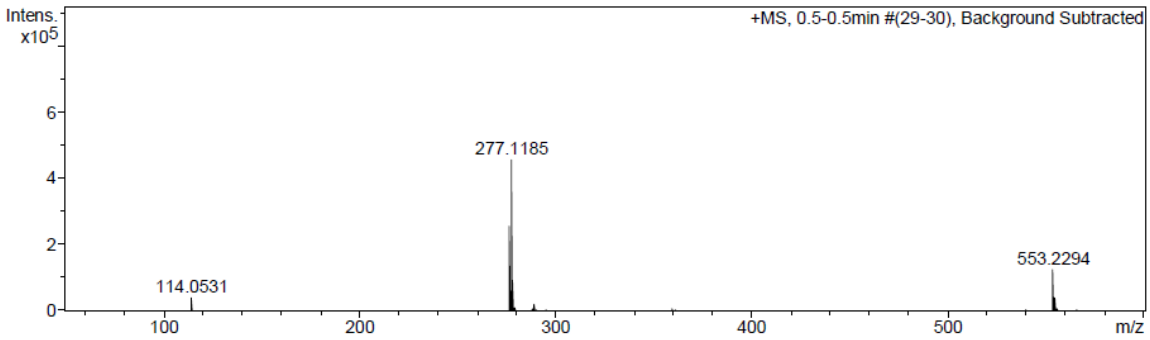
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
297.1222	$C_{17}H_{17}N_2O_3$	100.00	297.1234	1.2	4.0	4.3	10.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl piperidine-1-carboxylate (**9a**)



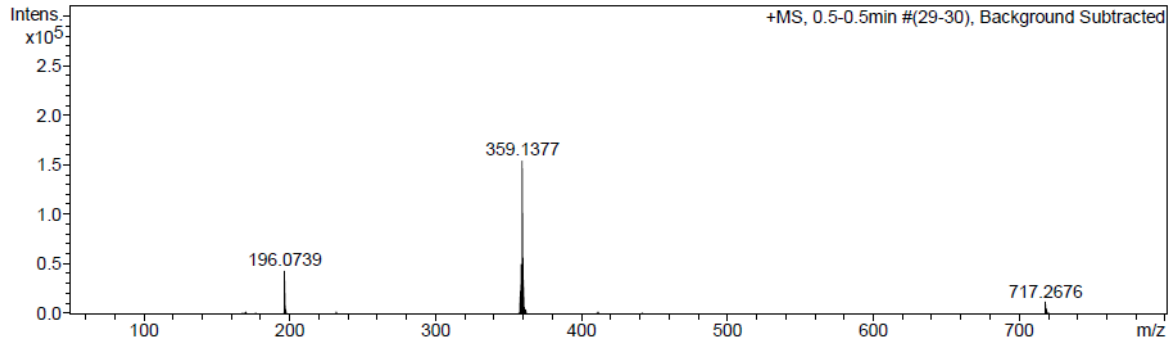
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e- Conf
275.1396	C15H19N2O3	100.00	275.1390	-0.5	-0.2	23.2	7.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (9b)



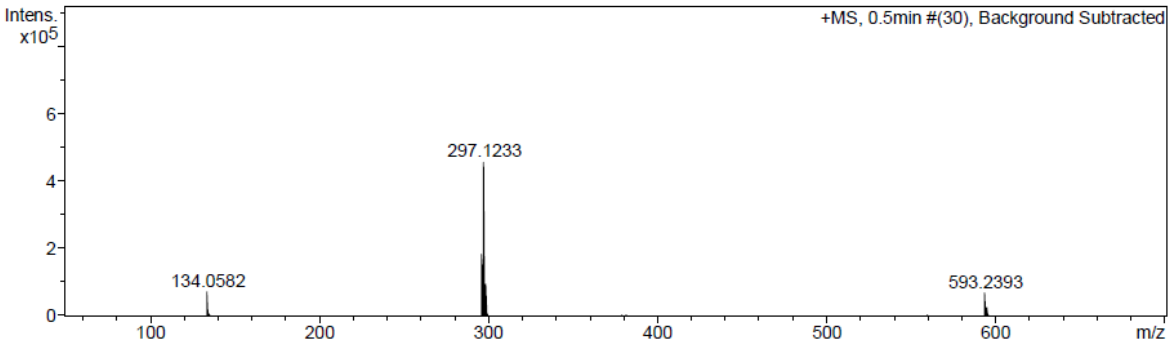
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
277.1185	$C_{14}H_{17}N_2O_4$	100.00	277.1183	-0.2	-0.7	25.1	7.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl diphenylcarbamate (**9c**)



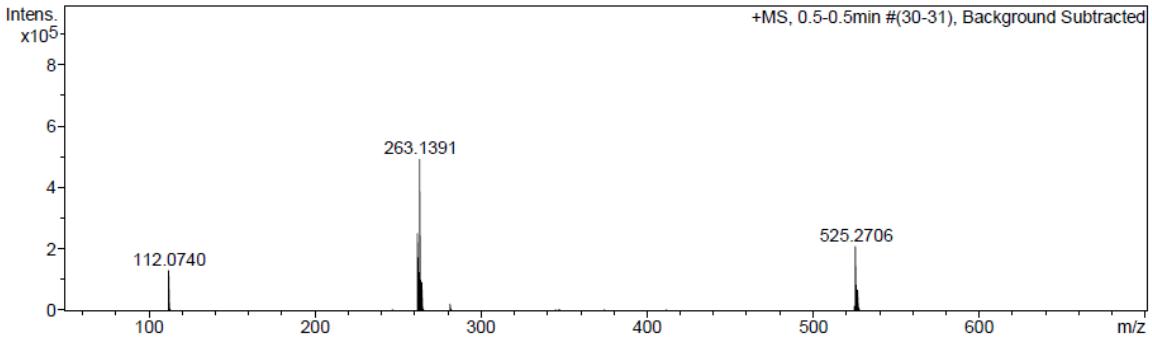
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
359.1377	$C_{22}H_{19}N_2O_3$	100.00	359.1390	1.4	3.8	13.7	14.5	ok	even

2-oxo-1,2,3,4-tetrahydroquinolin-6-yl methyl(phenyl)carbamate (**9d**)



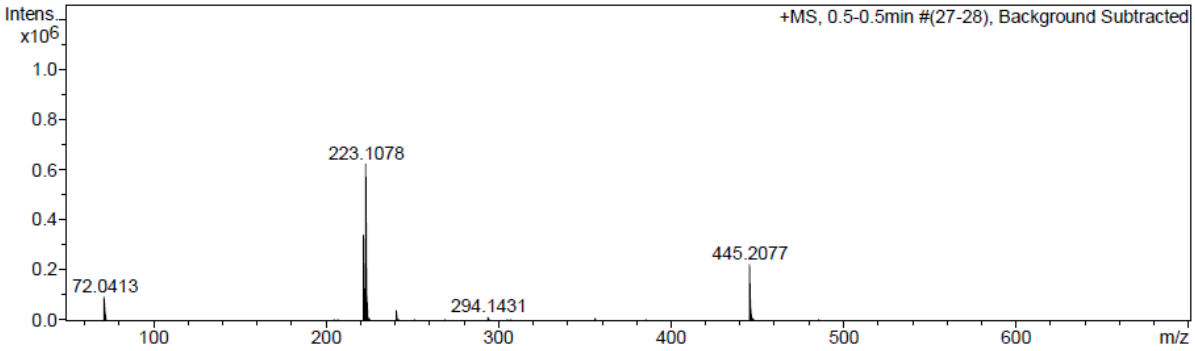
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
297.1233	$C_{17}H_{17}N_2O_3$	100.00	279.1234	0.1	0.2	9.9	10.5	ok	even

4-(acetylamino)phenyl piperidine-1-carboxylate (**10a**)



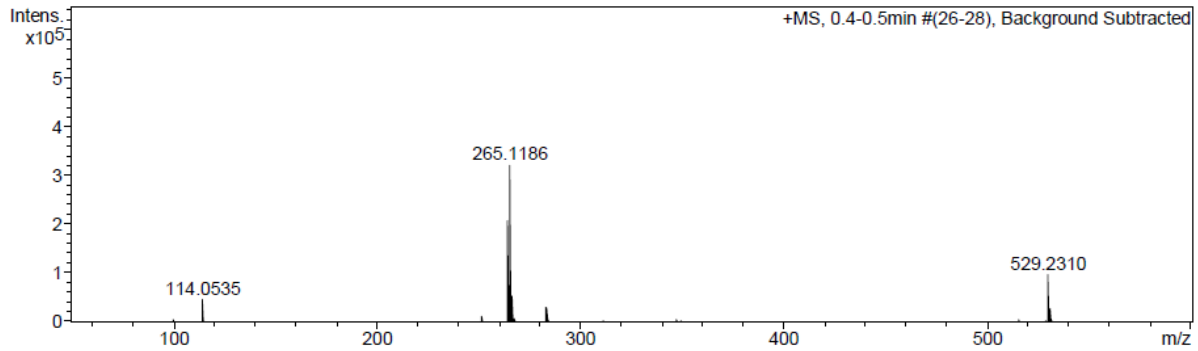
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
263.1391	$C_{14}H_{19}N_2O_3$	100.00	263.1390	-0.1	-0.5	18.1	6.5	ok	even

4-(acetylamino)phenyldimethylcarbamate (**10b**)



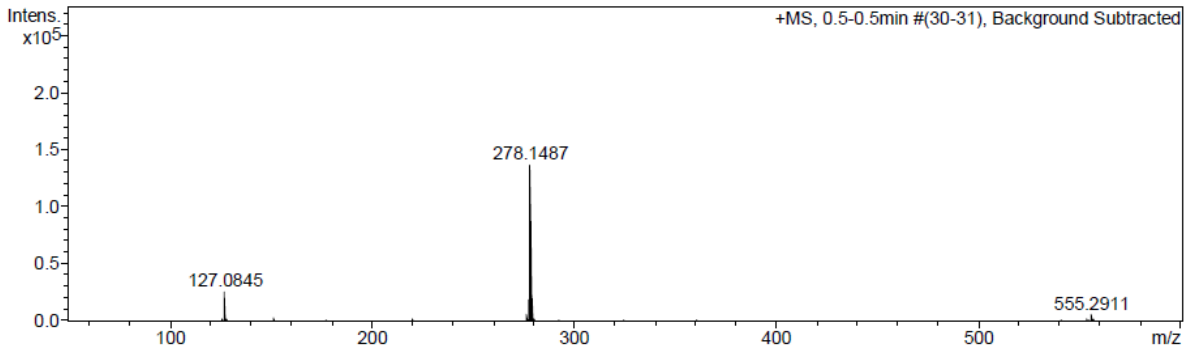
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
223.1078	$C_{11}H_{15}N_2O_3$	100.00	223.1077	-0.1	-0.4	19.7	5.5	ok	even

4-(acetylamino)phenyl morpholine-4-carboxylate (**10c**)



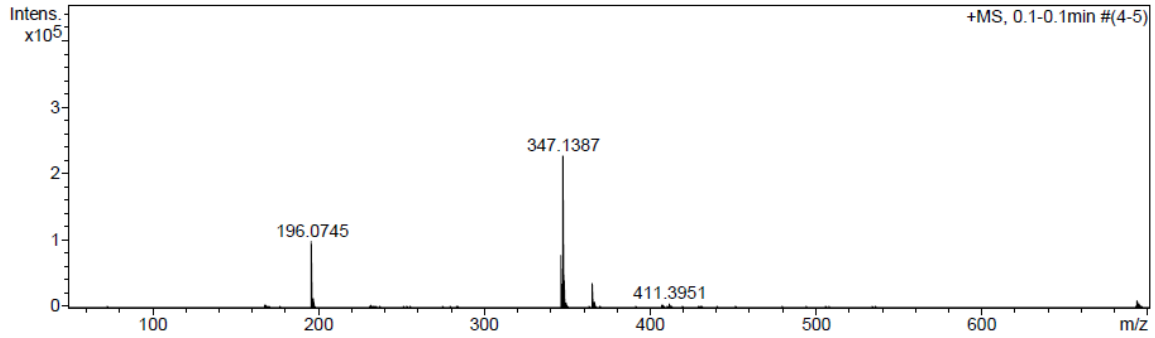
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
265.1186	$C_{13}H_{17}N_2O_4$	100.00	265.1183	-0.3	-1.2	12.2	6.5	ok	even

4-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**10d**)



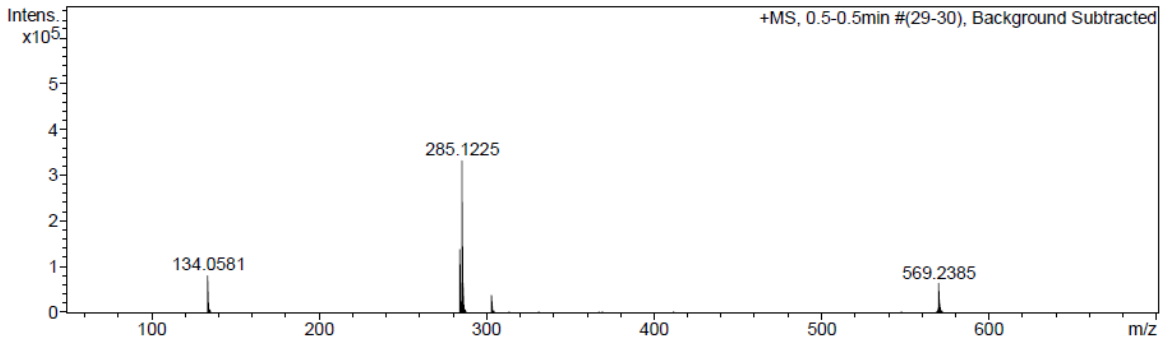
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
278.1487	$C_{14}H_{20}N_3O_3$	100.00	278.1499	1.2	4.3	1.1	6.5	ok	even

4-(acetylamino)phenyldiphenylcarbamate (**10e**)



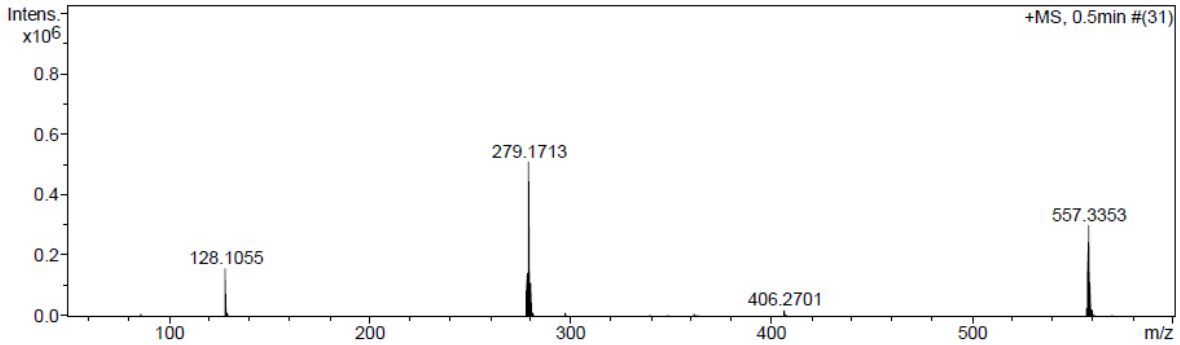
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
347.1387	$C_{21}H_{19}N_2O_3$	100.00	347.1390	0.3	1.0	12.1	13.5	ok	even

4-(acetylamino)phenyl methyl(phenyl)carbamate (**10f**)



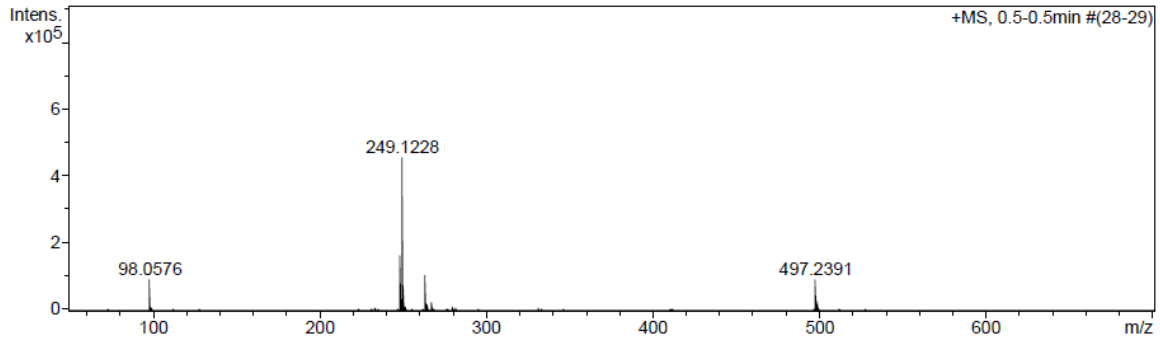
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
265.1225	$C_{16}H_{17}N_2O_3$	100.00	285.1234	0.9	3.0	7.1	9.5	ok	even

4-(acetylamino)phenyl dipropan-2-ylcarbamate (**10g**)



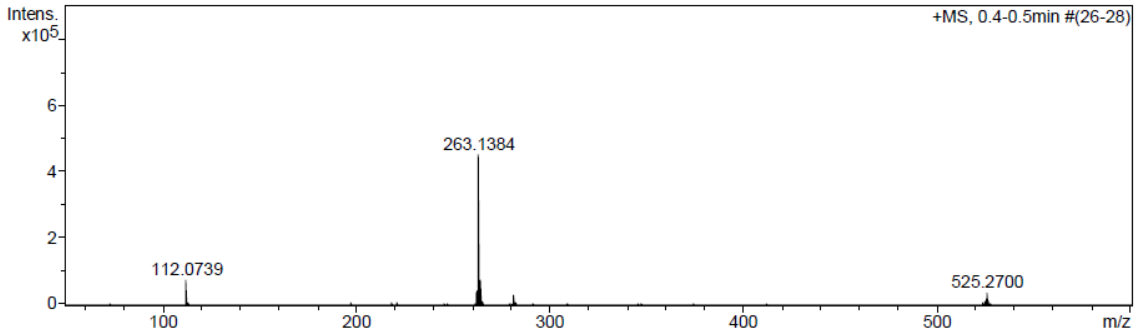
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
279.1713	$C_{15}H_{23}N_2O_3$	100.00	279.1703	-1.0	-3.4	27.5	5.5	ok	even

4-(acetylamino)phenyl pyrrolidine-1-carboxylate (**10h**)



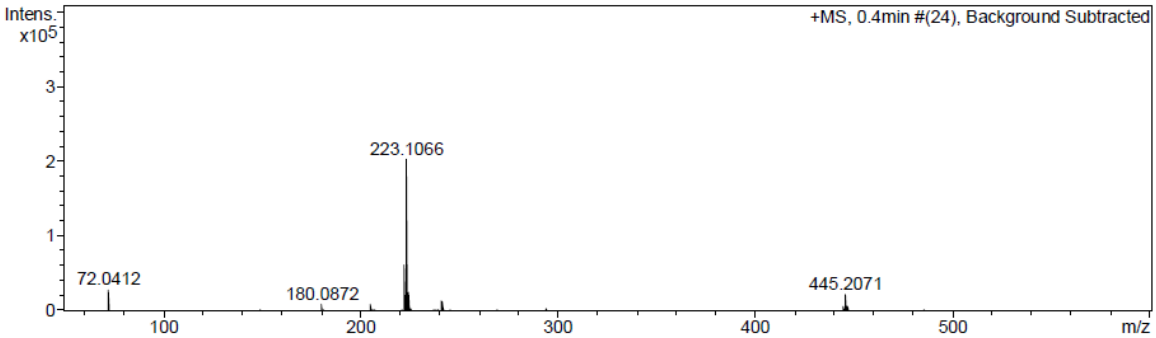
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
249.1228	$C_{13}H_{17}N_2O_3$	100.00	249.1234	0.6	2.4	5.0	6.5	ok	even

3-(acetylamino)phenyl piperidine-1-carboxylate (**11a**)



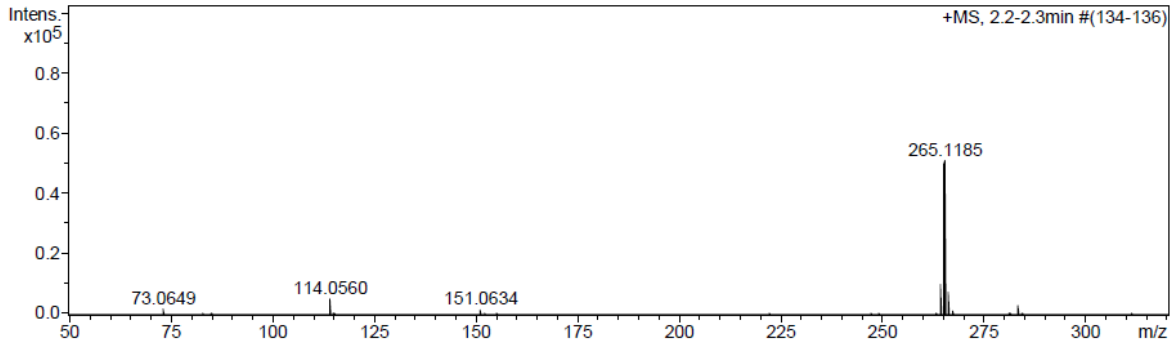
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
263.1384	$C_{14}H_{19}N_2O_3$	100.00	263.1390	0.6	2.2	1.4	6.5	ok	even

3-(acetylamino)phenyldimethylcarbamate (**11b**)



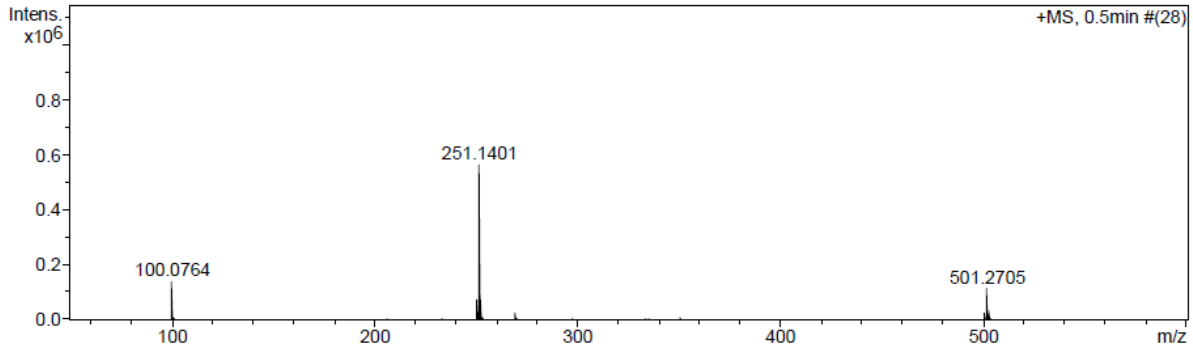
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
223.1066	$C_{11}H_{15}N_2O_3$	100.00	223.1077	1.2	5.2	2.6	5.5	ok	even

3-(acetylamino)phenyl morpholine-4-carboxylate (**11c**)



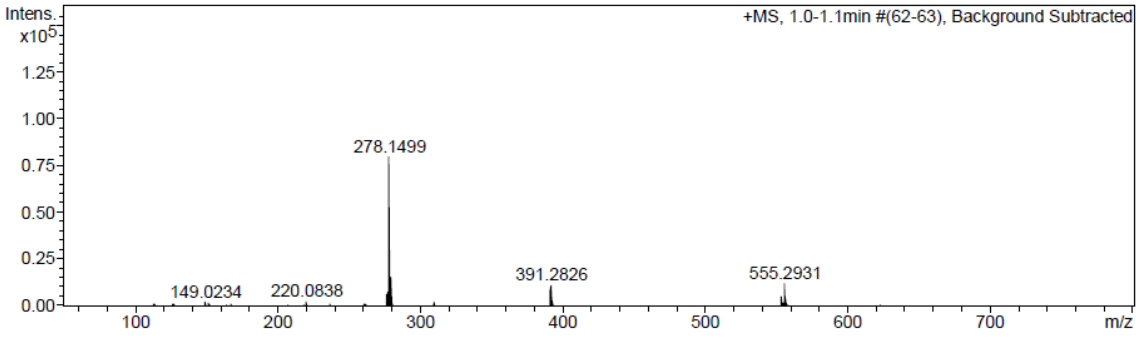
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
265.1185	$C_{13}H_{17}N_2O_3$	100.00	265.1183	-0.2	-0.7	0.7	6.5	ok	even

3-(acetylamino)phenyldiethylcarbamate (**11d**)



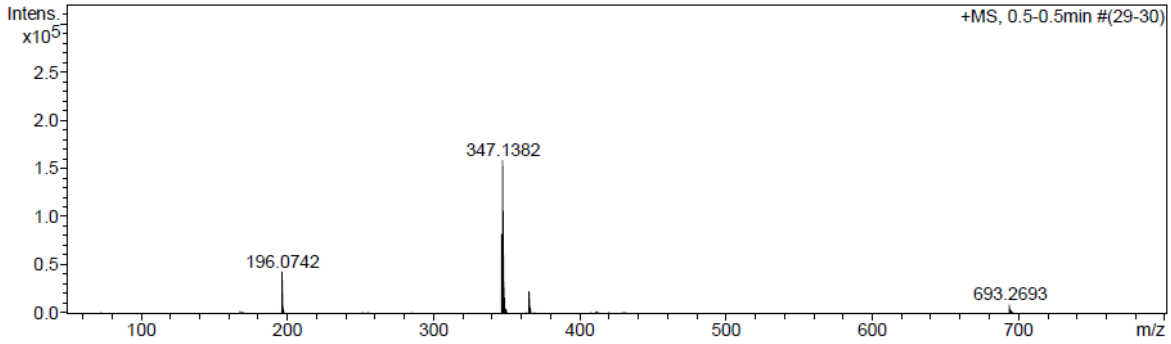
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
251.1401	$C_{13}H_{19}N_2O_3$	100.00	251.1390	-1.0	-4.1	7.7	5.5	ok	Even

3-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**11e**)



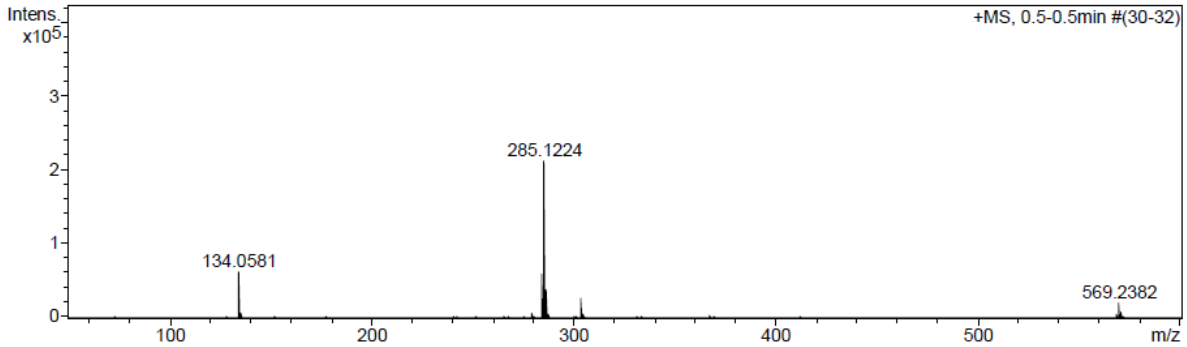
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
278.1499	$C_{14}H_{20}N_3O_3$	100.00	278.1499	0.0	0.0	19.8	6.5	ok	even

3-(acetylamino)phenyldiphenylcarbamate (**11f**)



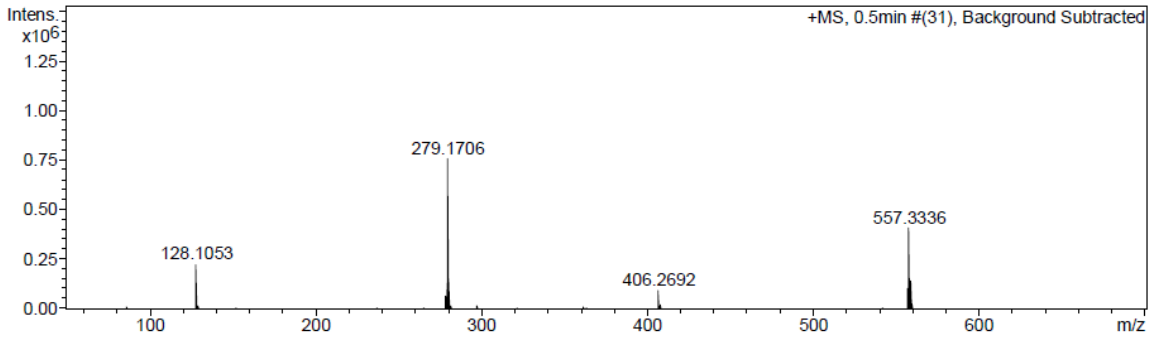
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
347.1382	$C_{21}H_{19}N_2O_3$	100.00	347.1390	0.9	2.5	14.1	13.5	ok	Even

3-(acetylamino)phenyl methyl(phenyl)carbamate (**11g**)



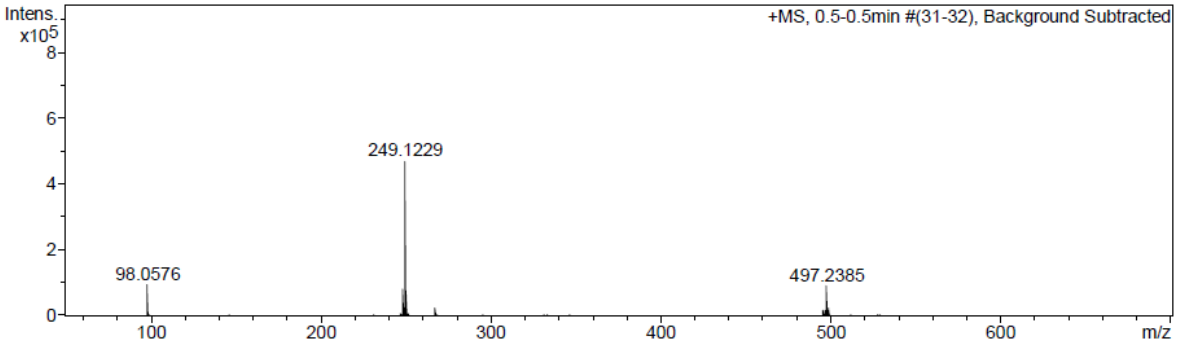
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
285.1224	$C_{16}H_{17}N_2O_3$	100.00	285.1234	1.0	3.4	0.5	9.5	ok	Even

3-(acetylamino)phenyl dipropan-2-ylcarbamate (**11h**)



Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
279.1706	$C_{15}H_{23}N_2O_3$	100.00	279.1703	-0.3	-0.1	21.0	5.5	ok	even

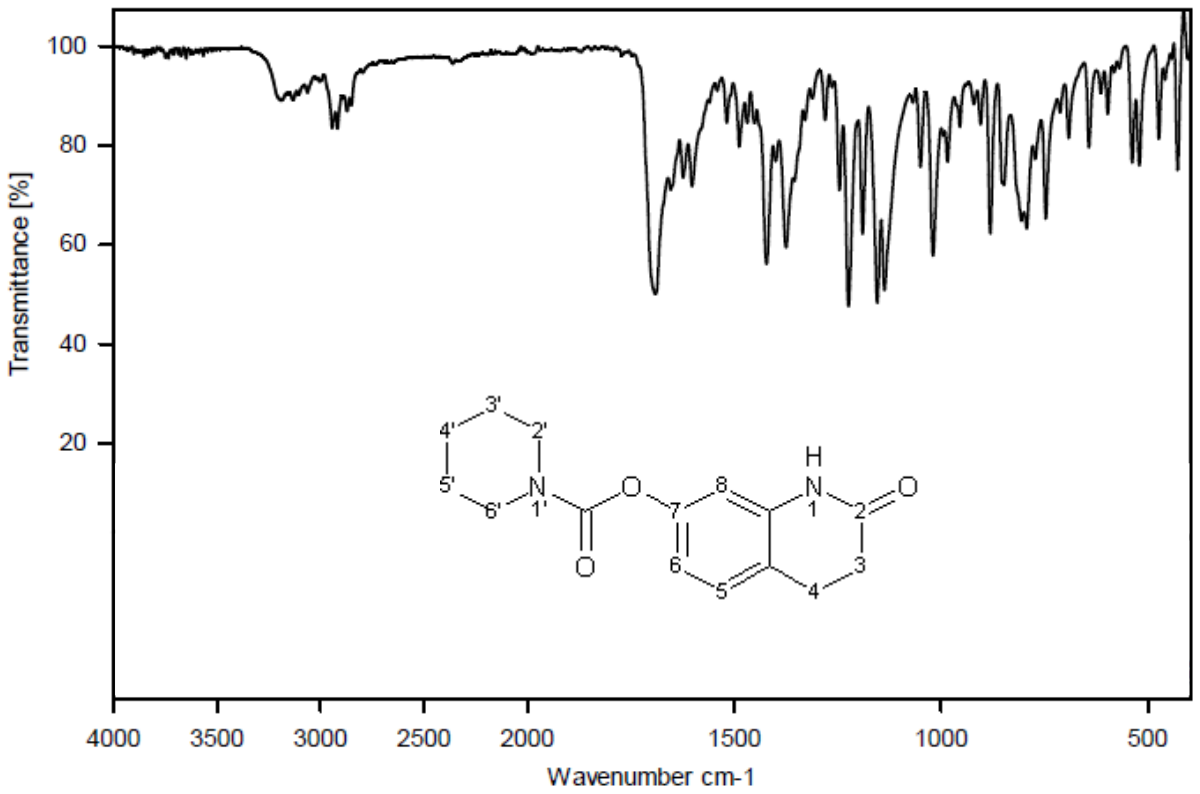
3-(acetylamino)phenyl pyrrolidine-1-carboxylate (**11i**)



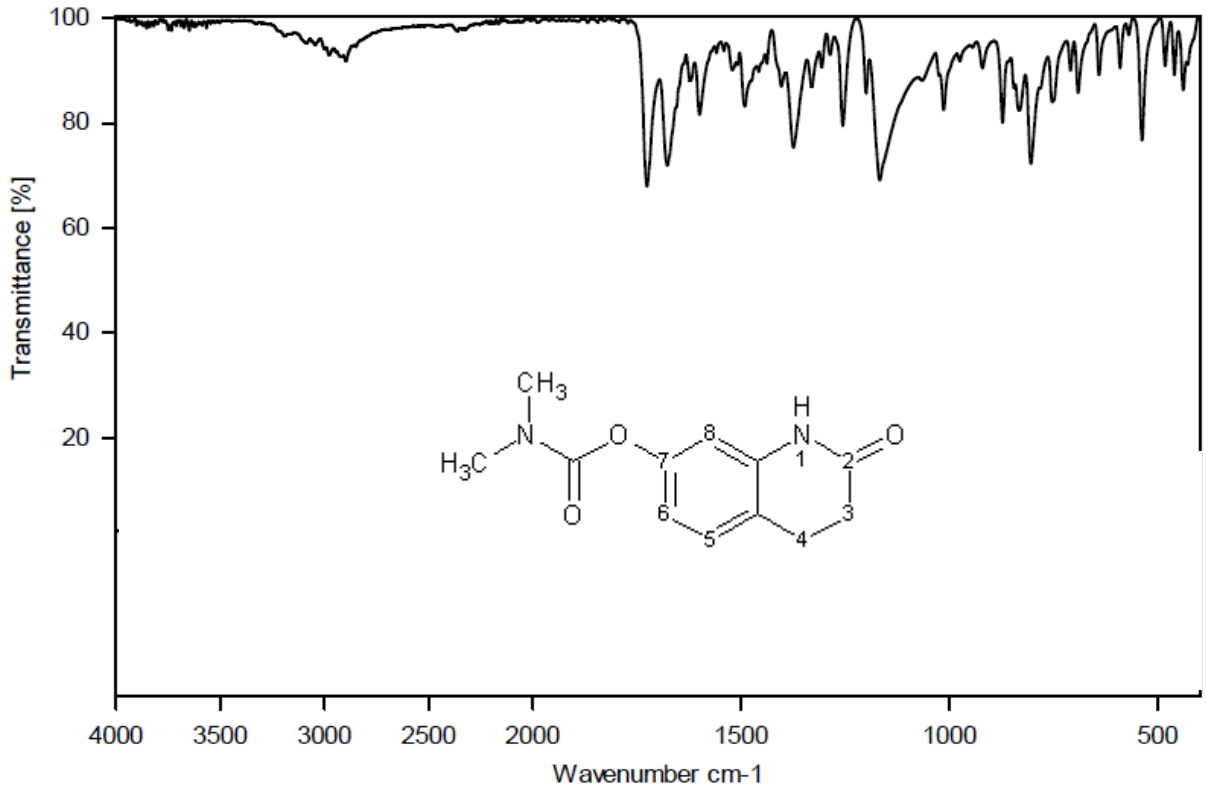
Meas. m/z	Formula	Score	m/z	Err [mDa]	Err [ppm]	mSigma	rdb	N-Rule	e^- Conf
249.1229	$C_{13}H_{17}N_2O_3$	100.00	249.1234	0.4	1.8	8.1	6.5	ok	even

INFRA-RED SPECTRA

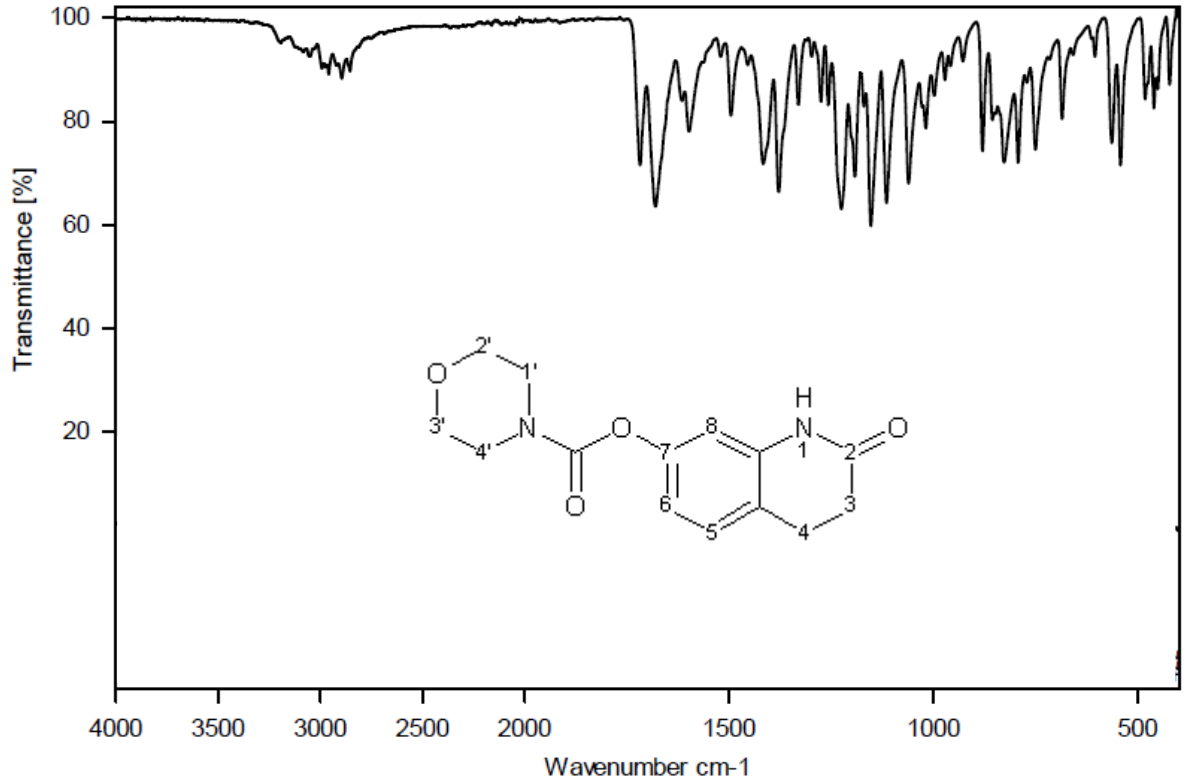
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl piperidine-1-carboxylate (**8a**)



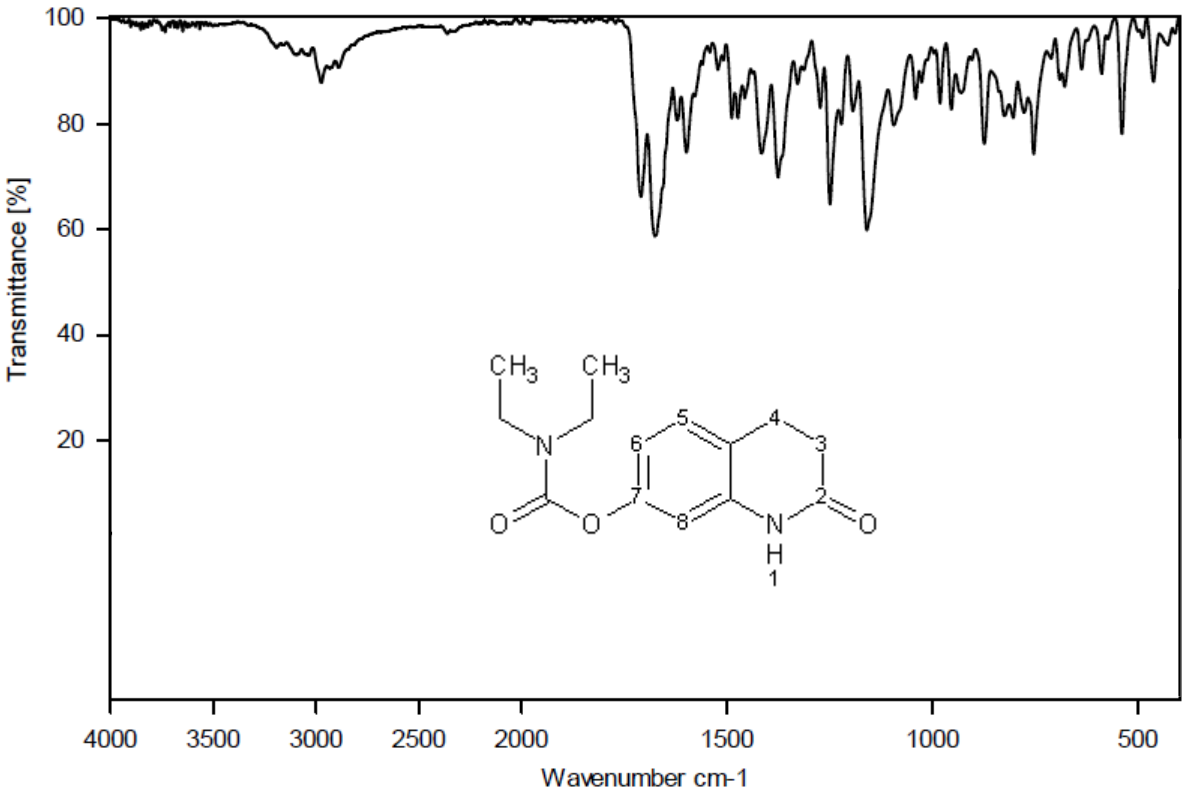
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl dimethylcarbamate (**8b**)



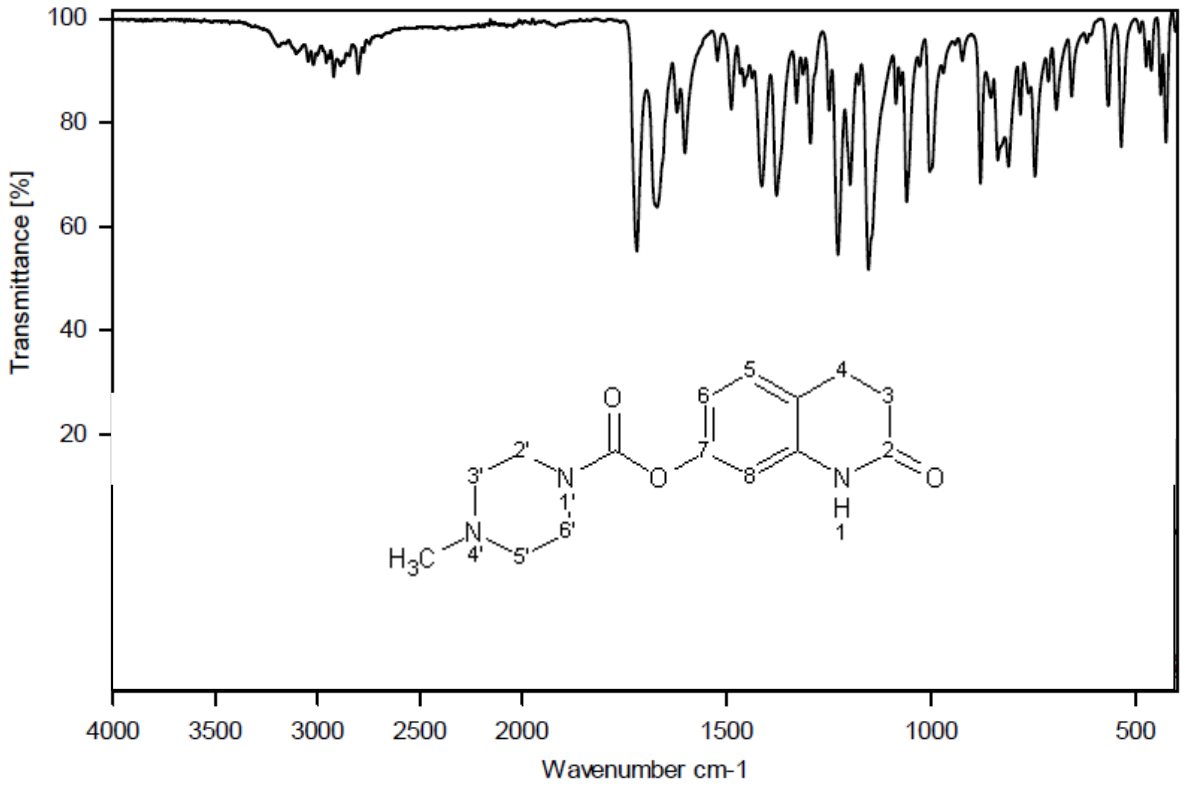
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**8c**)



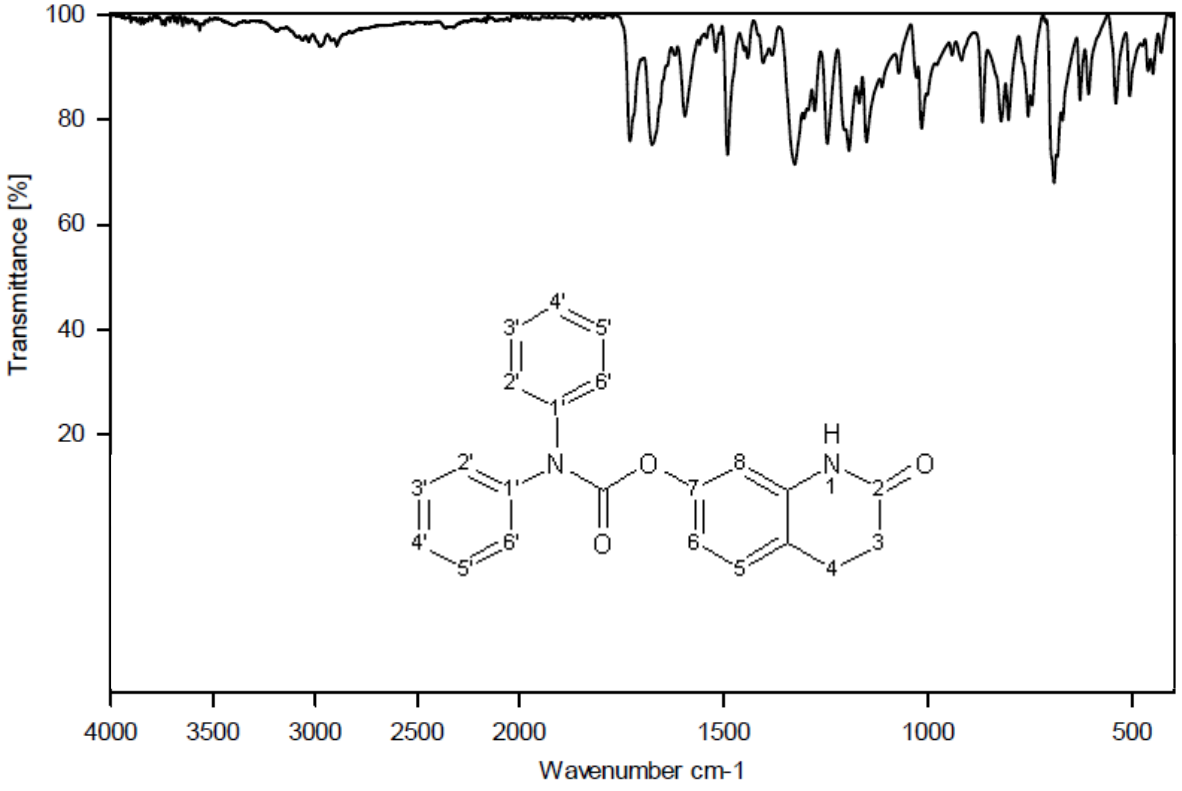
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diethylcarbamate (**8d**)



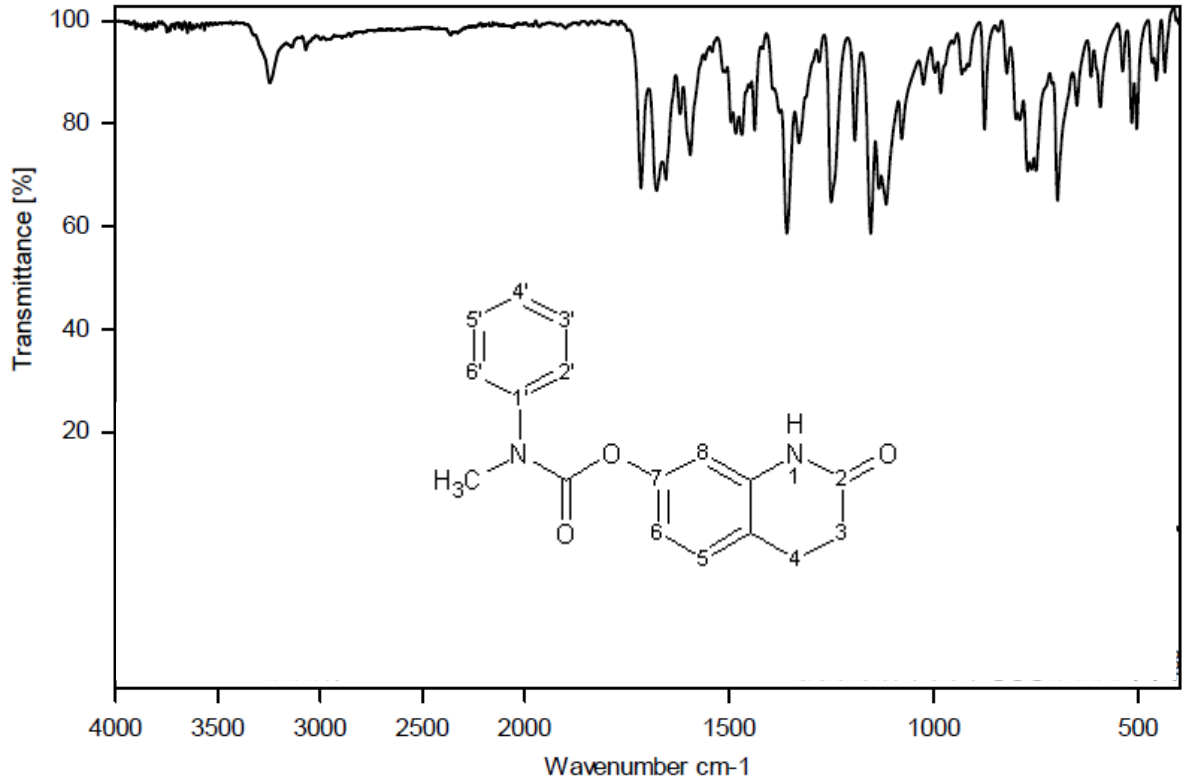
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl 4-methylpiperazine-1-carboxylate (**8e**)



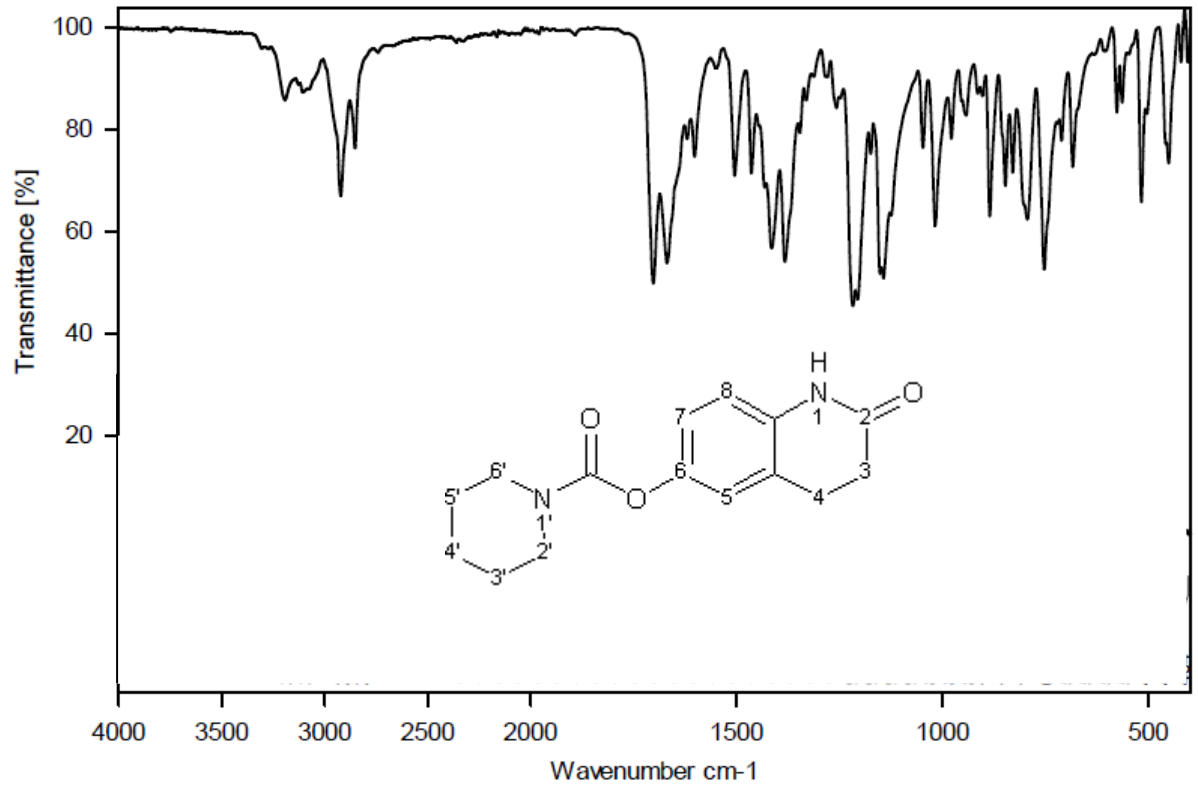
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diphenylcarbamate (**8f**)



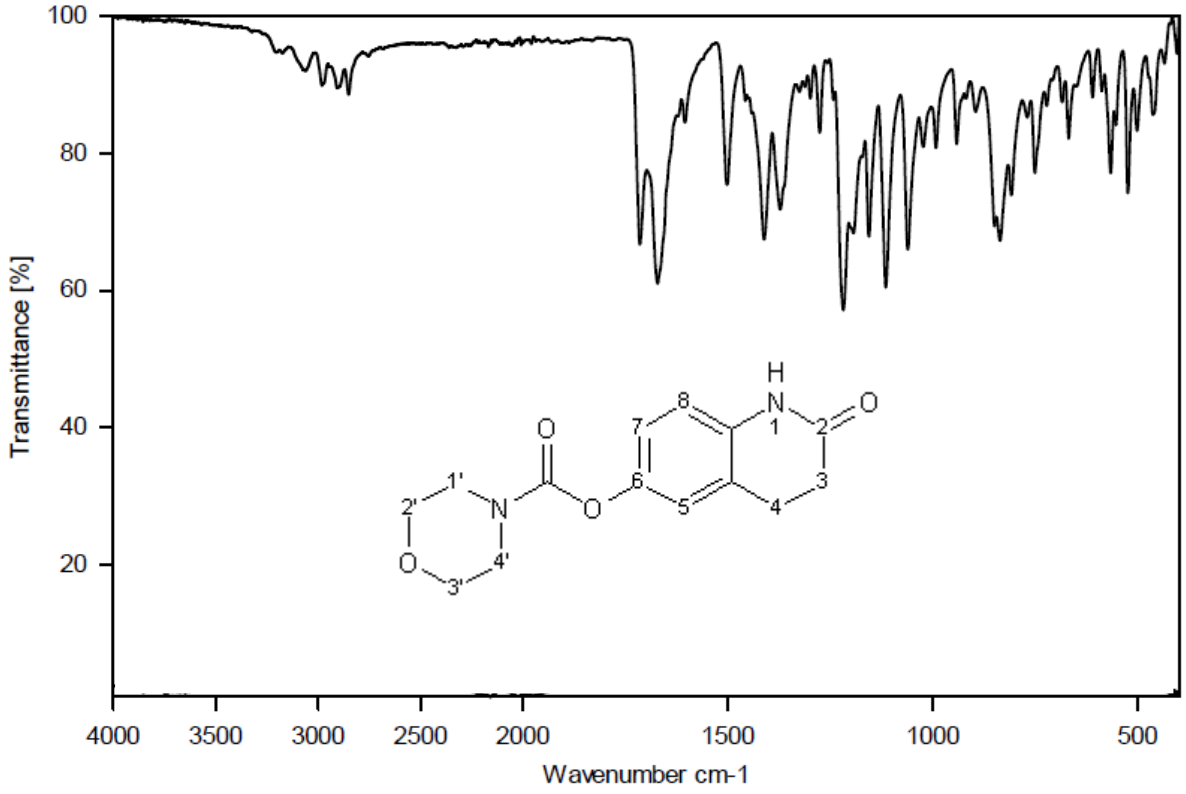
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl methyl(phenyl)carbamate (**8g**)



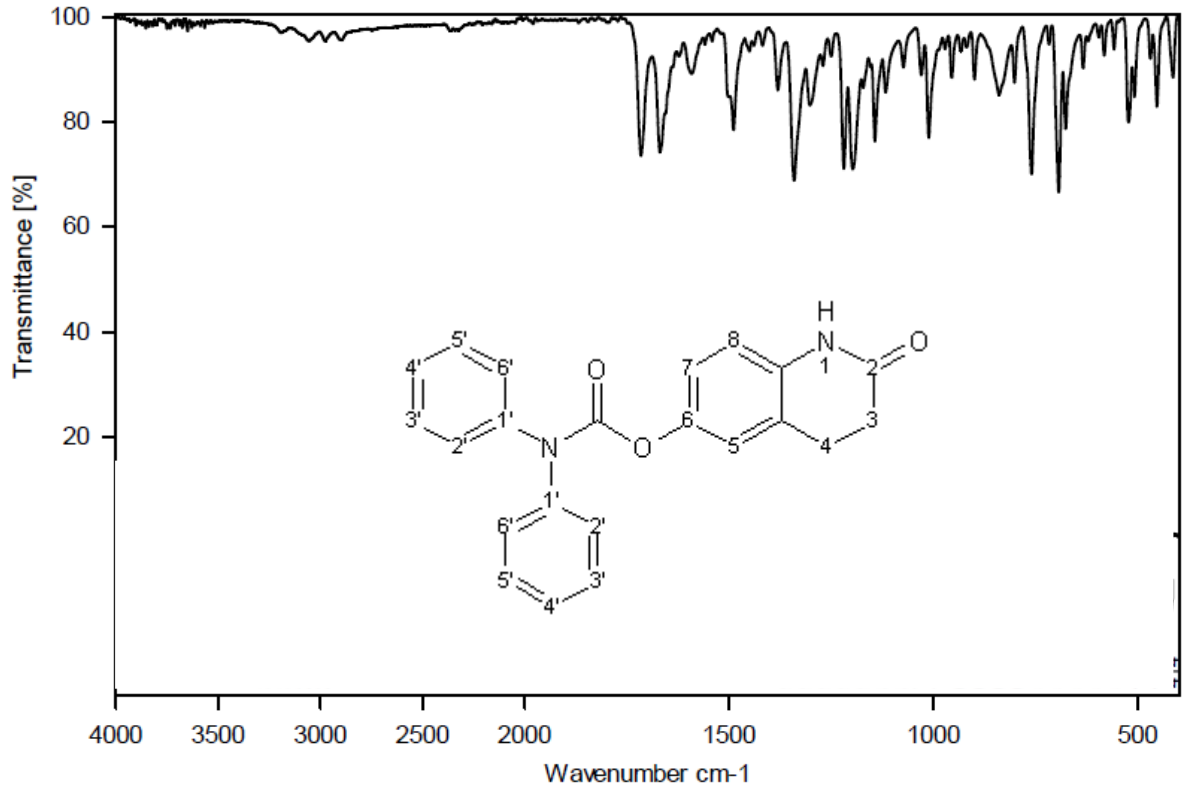
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl piperidine-1-carboxylate (**9a**)



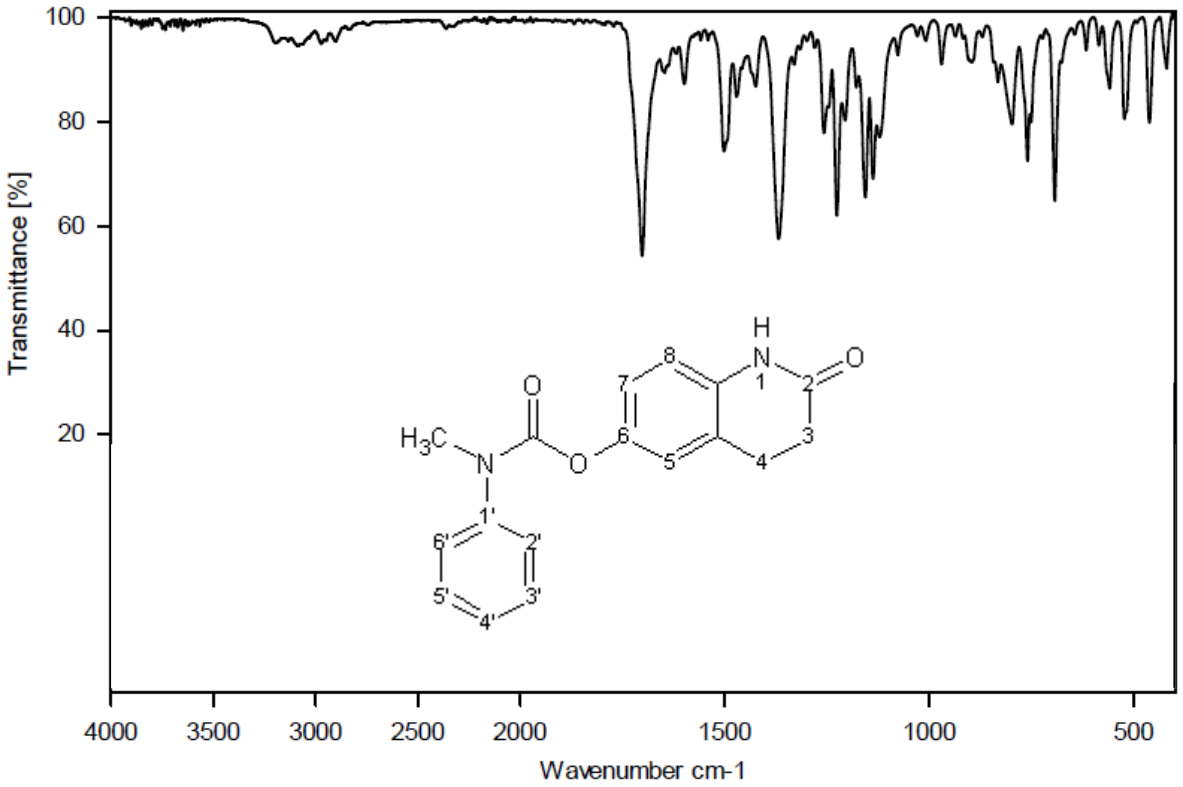
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**9b**)



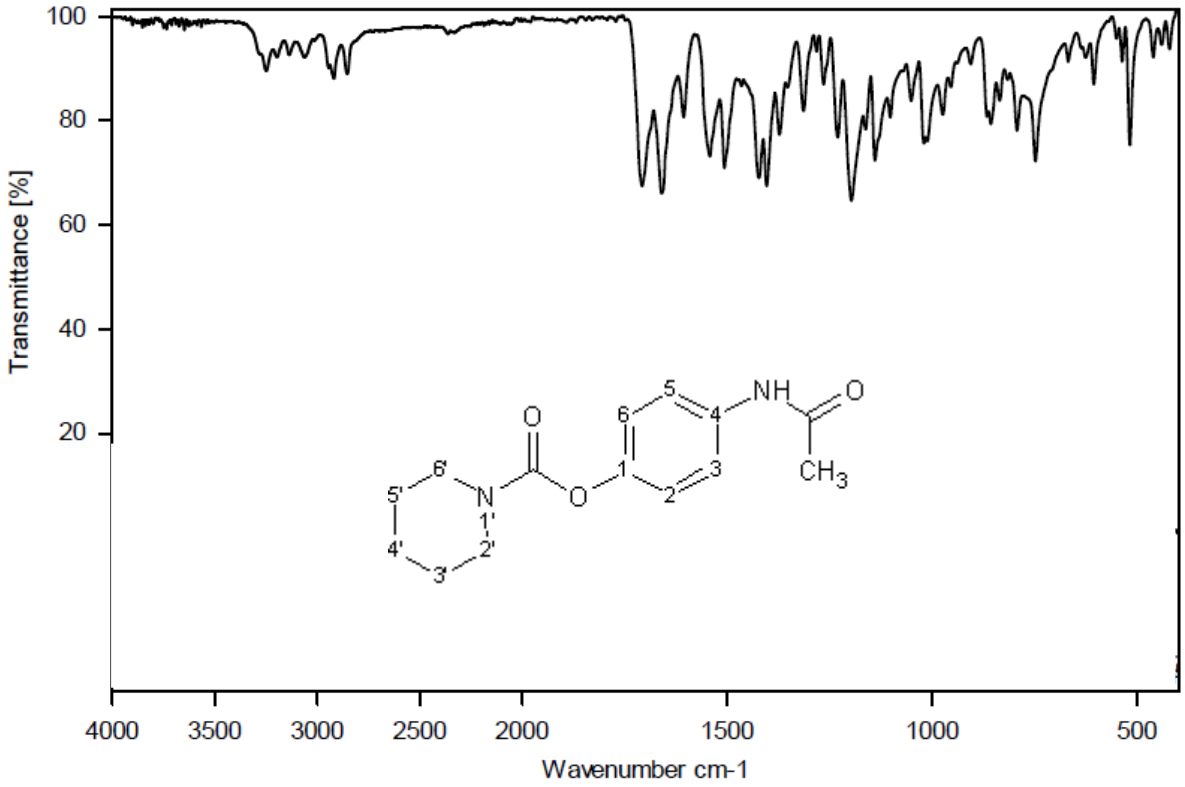
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl diphenylcarbamate (**9c**)



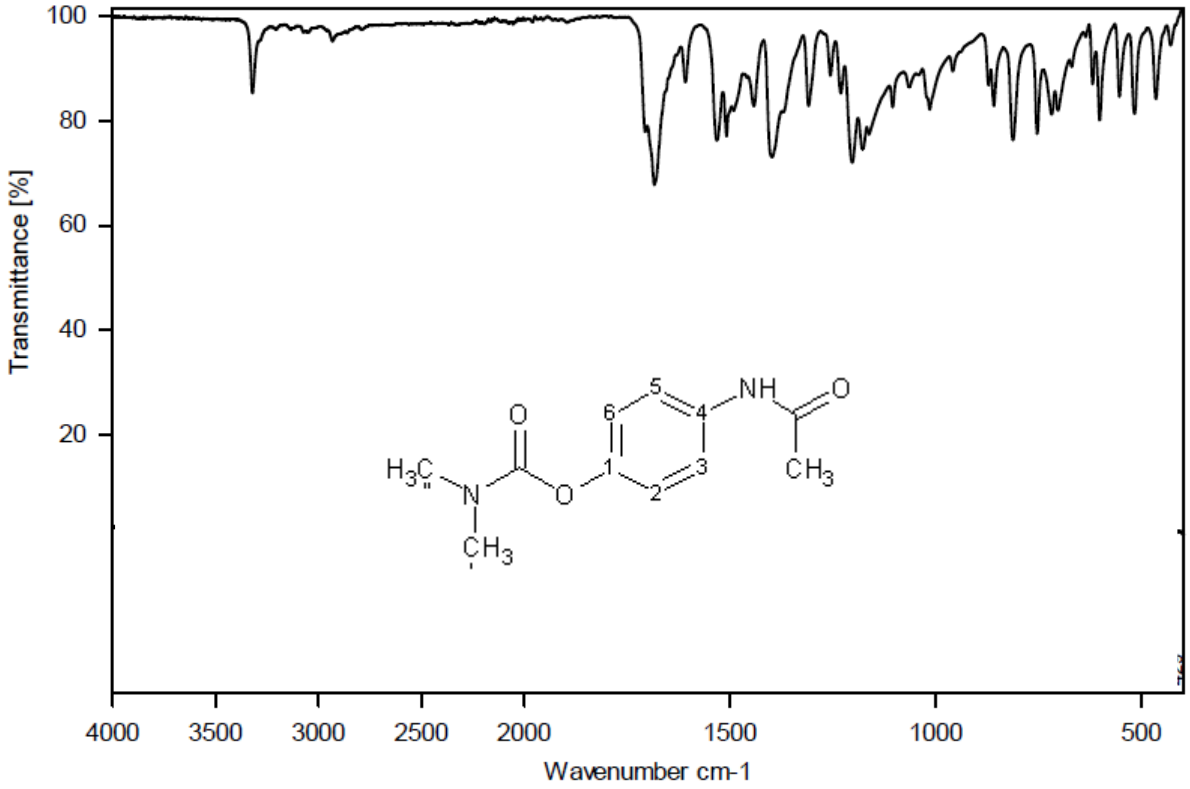
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl methyl(phenyl)carbamate (**9d**)



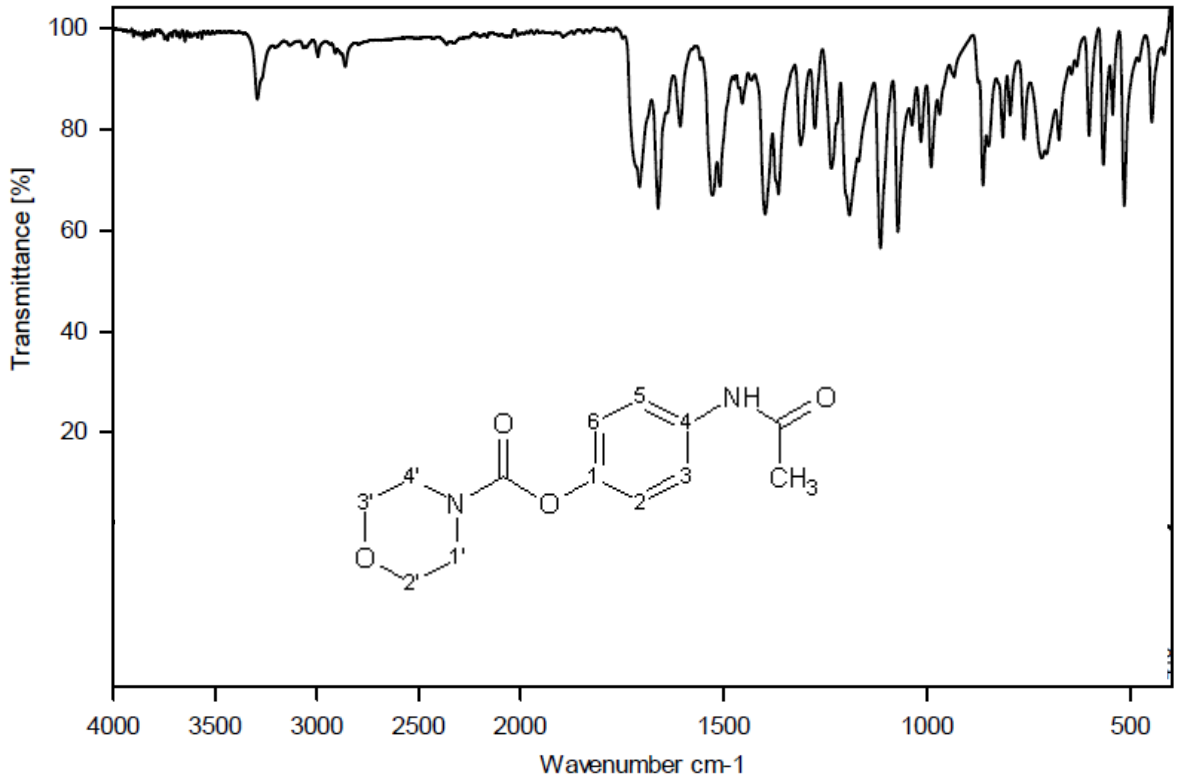
4-(acetylamino)phenyl piperidine-1-carboxylate (**10a**)



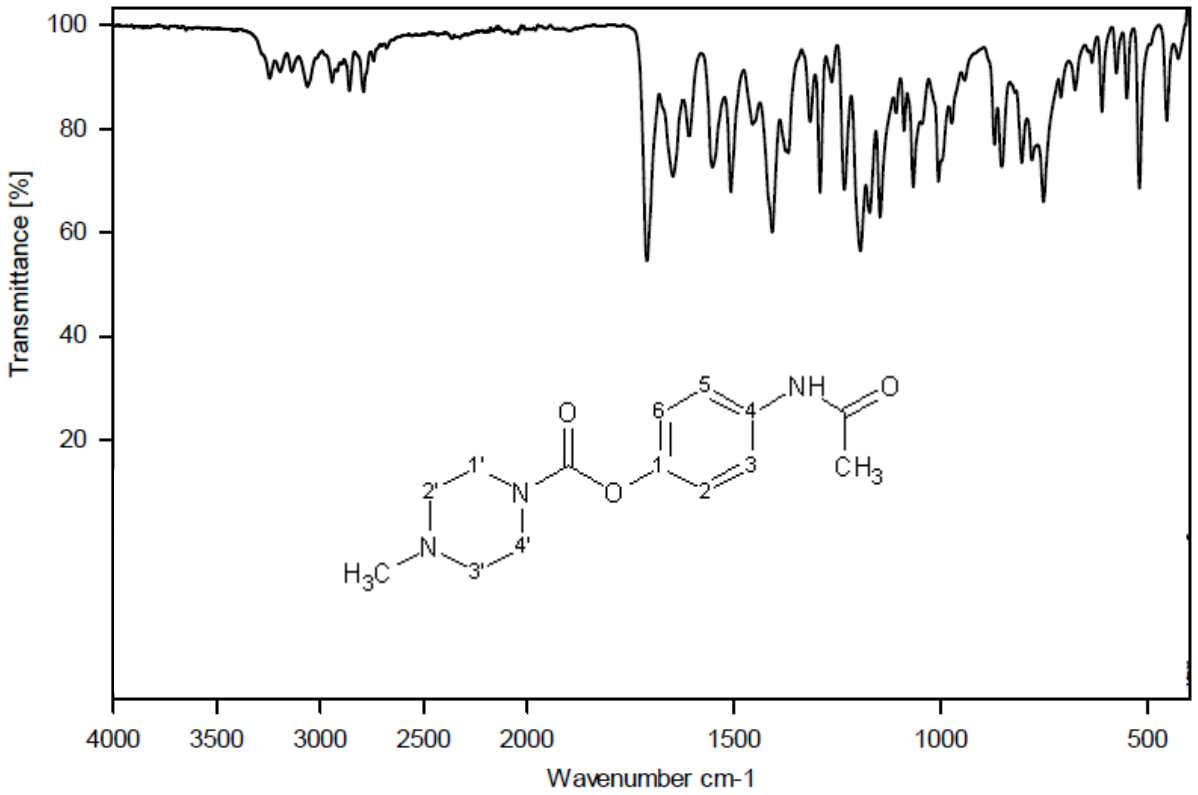
4-(acetylamino)phenyldimethylcarbamate (**10b**)



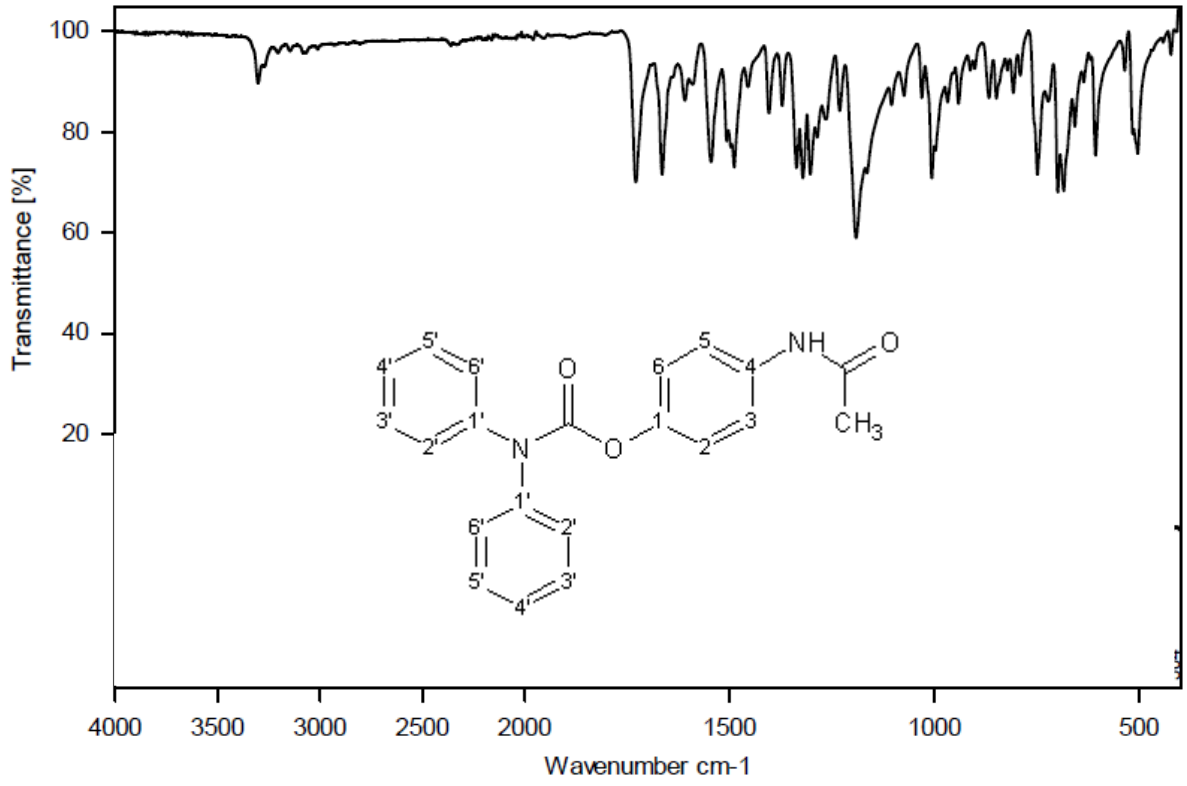
4-(acetylamino)phenyl morpholine-4-carboxylate (**10c**)



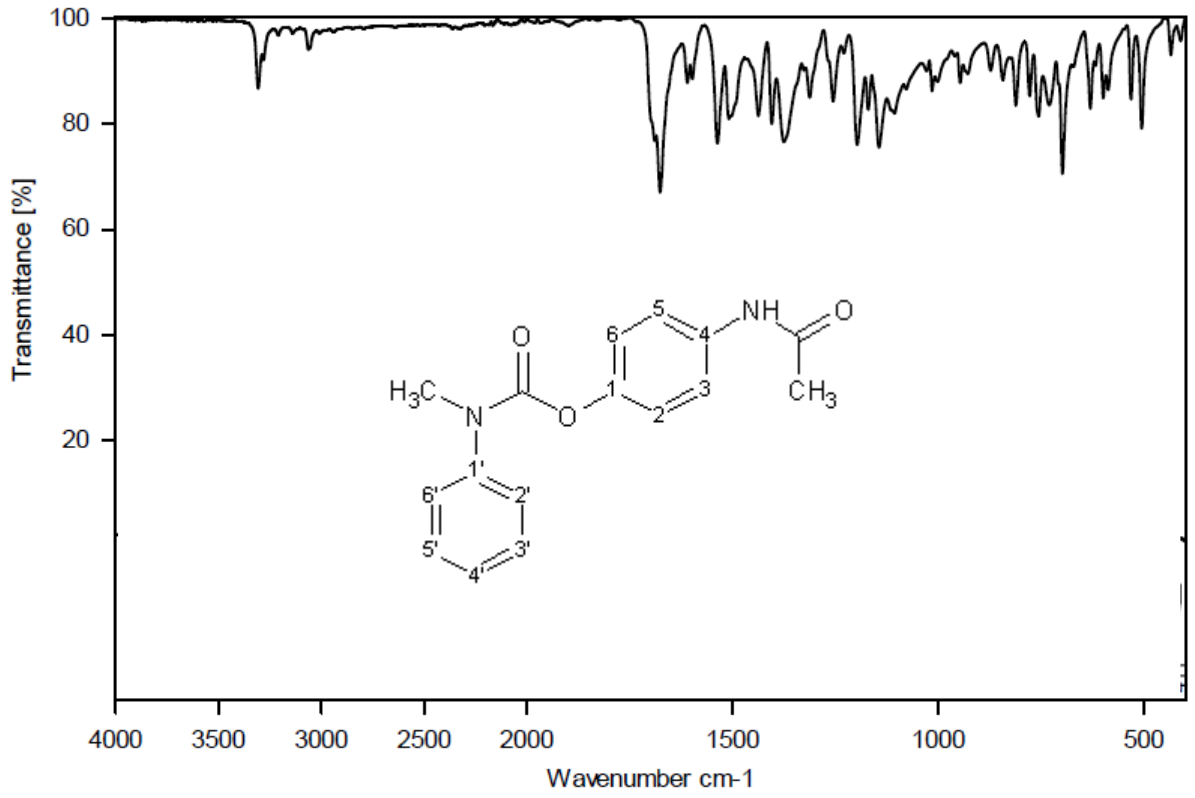
4-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**10d**)



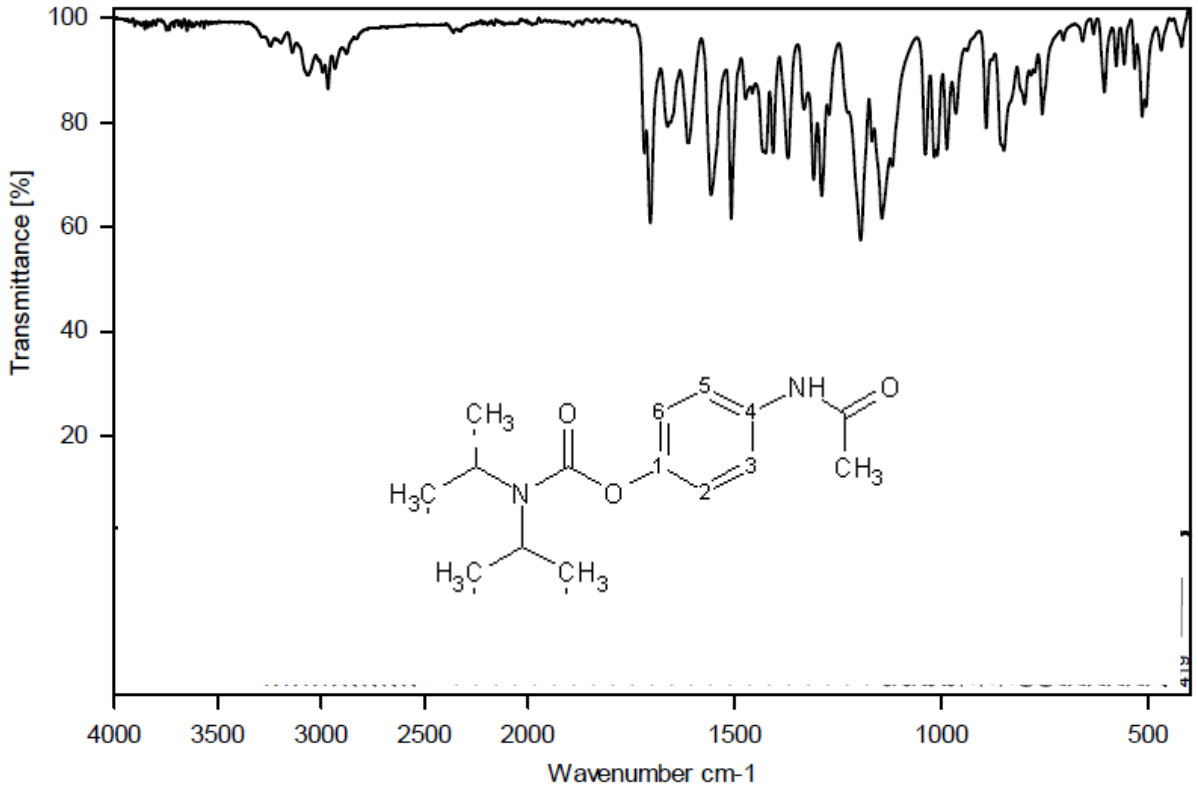
4-(acetylamino)phenyldiphenylcarbamate (**10e**)



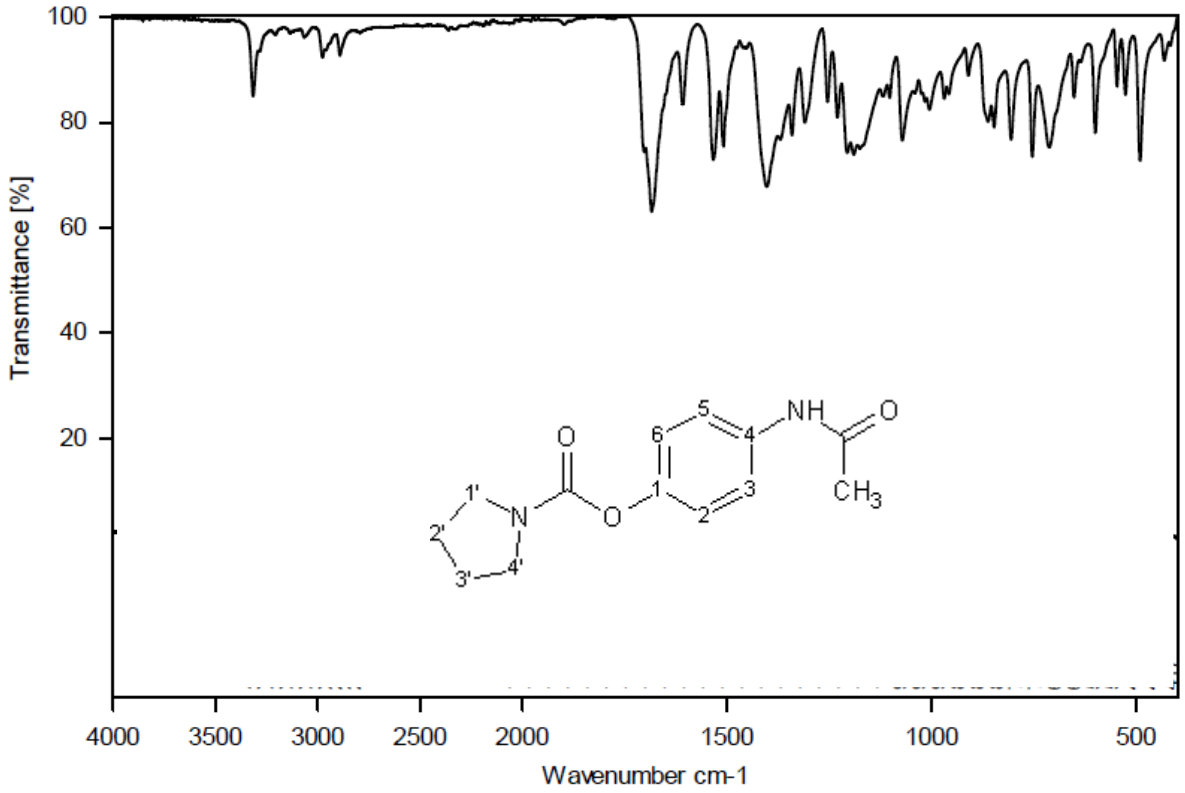
4-(acetylamino)phenyl methyl(phenyl)carbamate (**10f**)



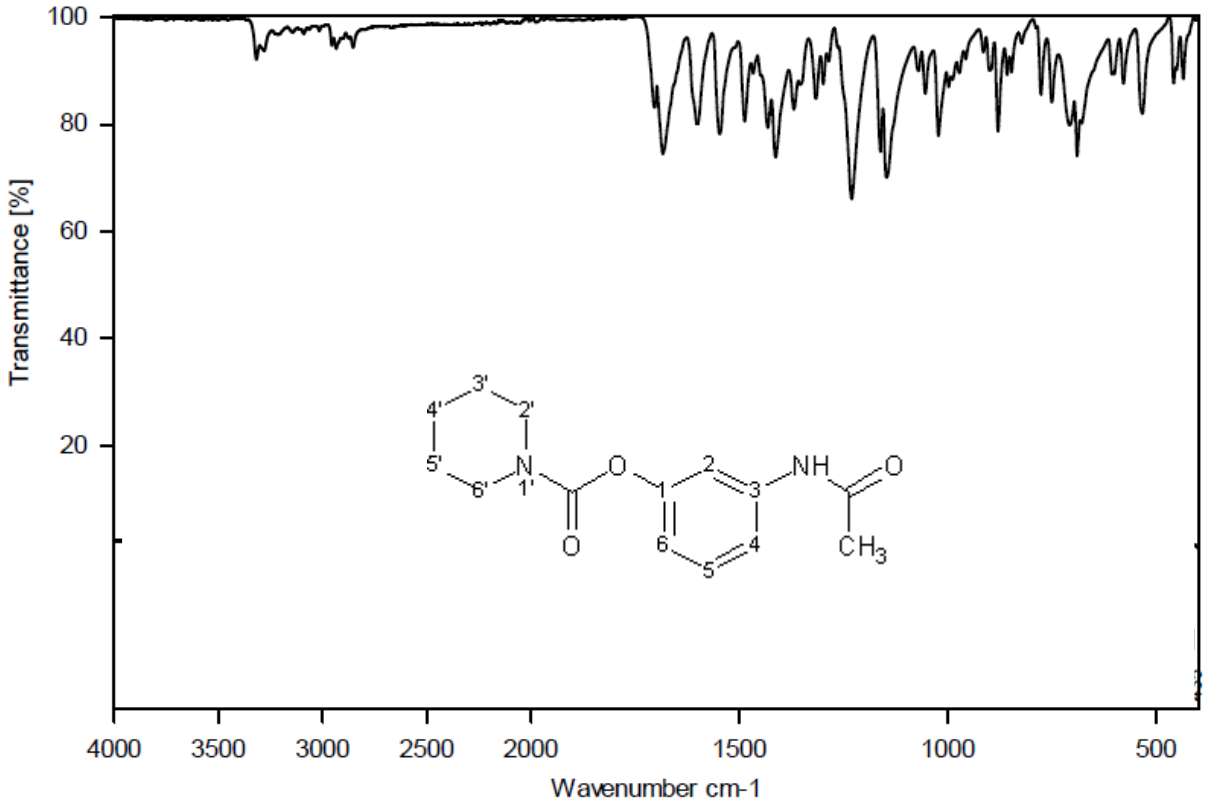
4-(acetylamino)phenyl dipropan-2-ylcarbamate (**10g**)



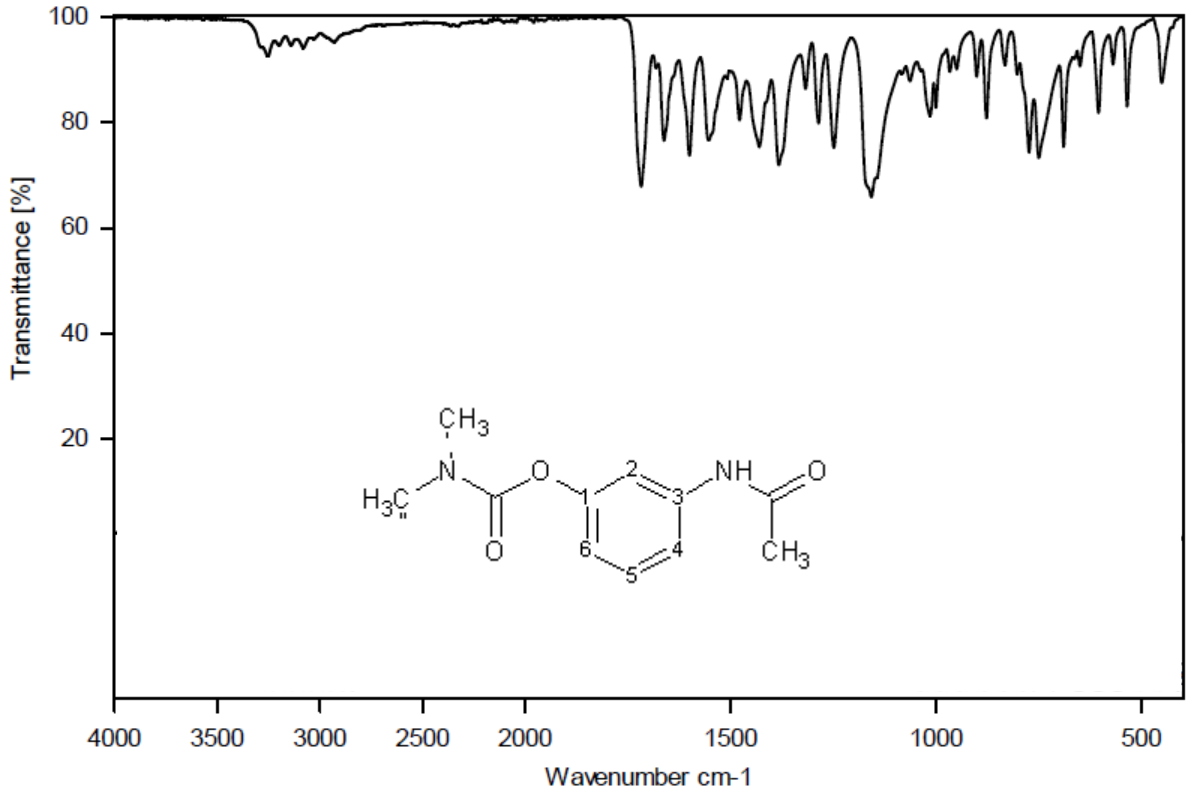
4-(acetylamino)phenyl pyrrolidine-1-carboxylate (**10h**)



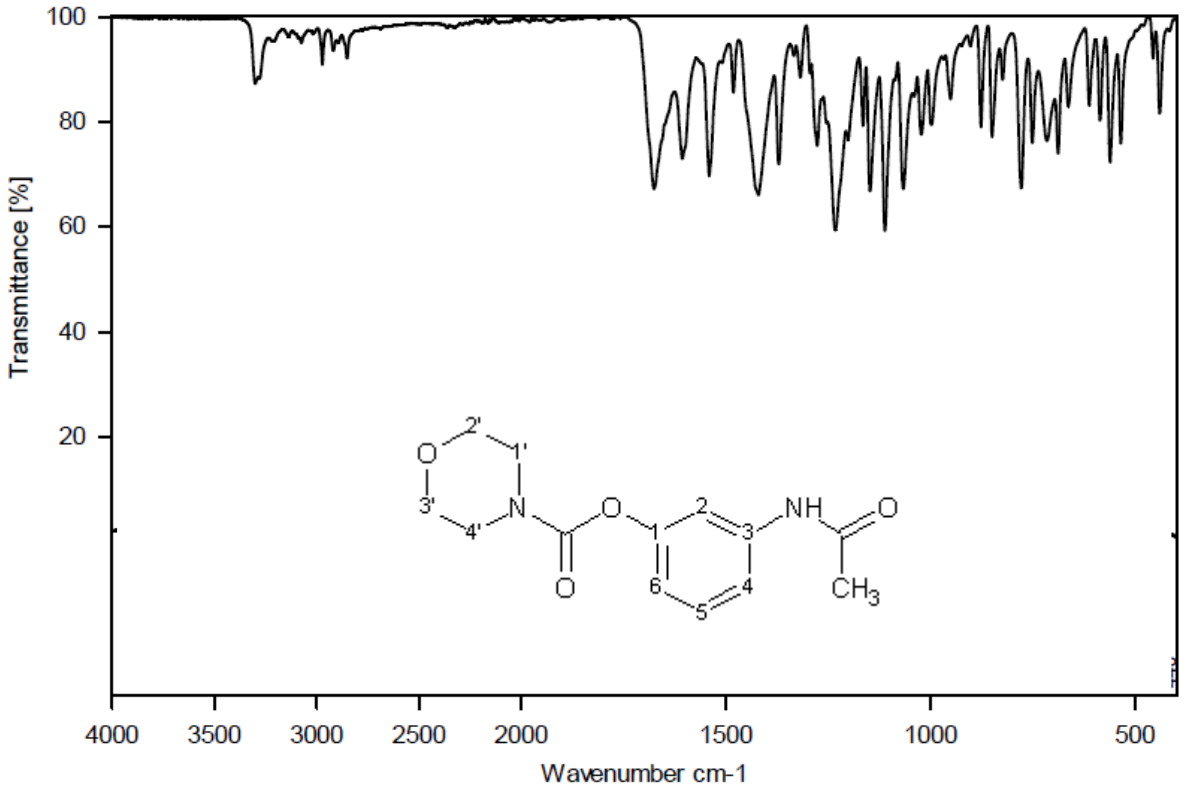
3-(acetylamino)phenyl piperidine-1-carboxylate (**11a**)



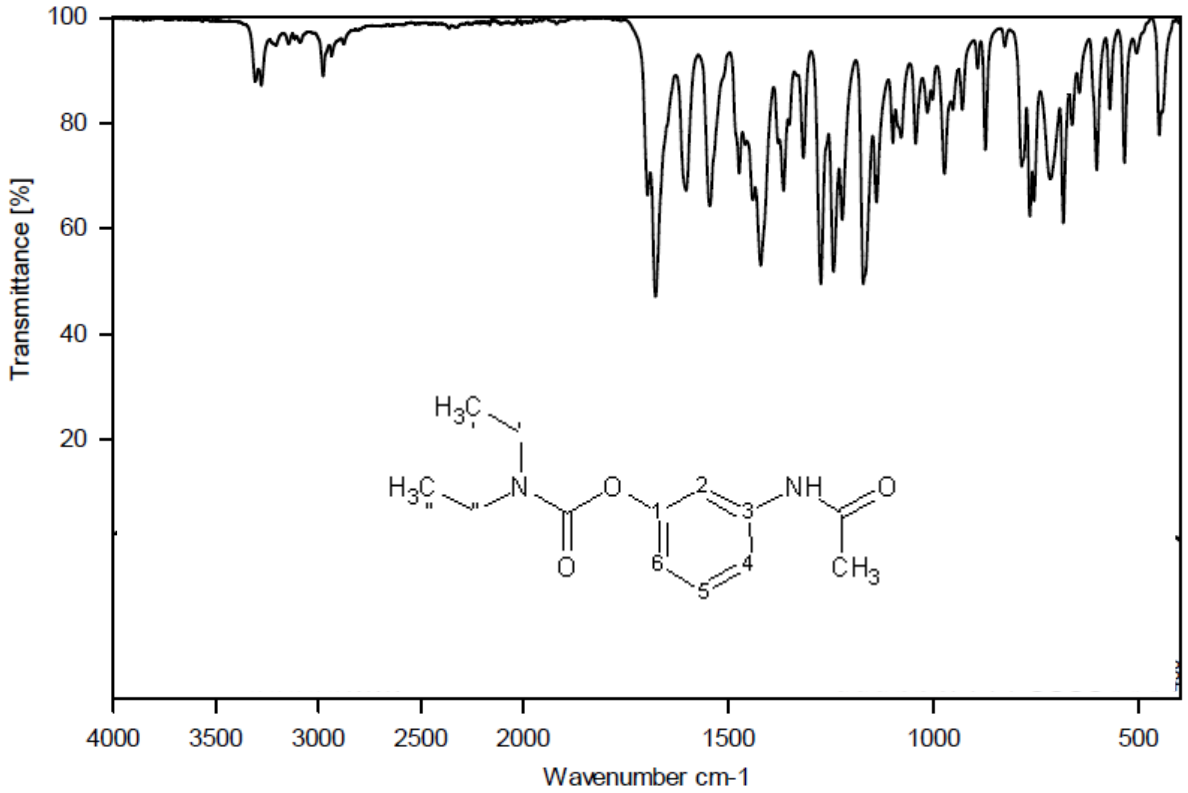
3-(acetylamino)phenyldimethylcarbamate (**11b**)



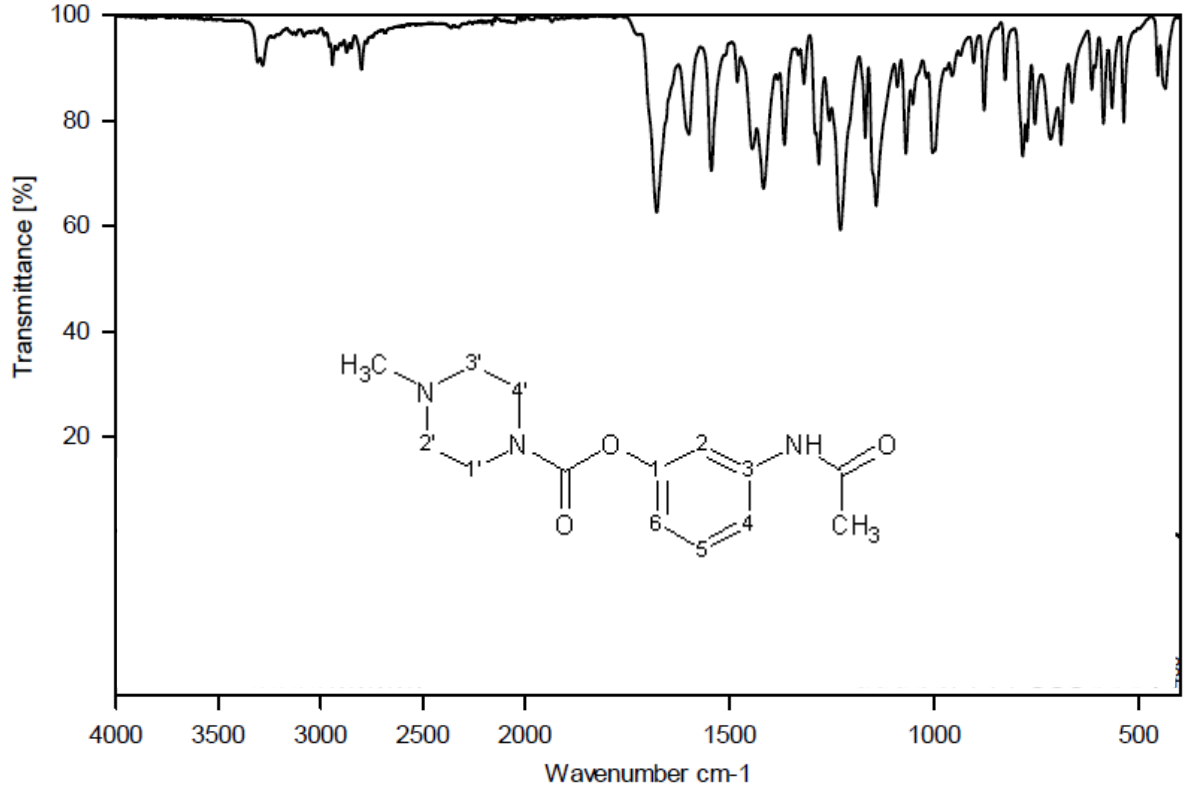
3-(acetylamino)phenyl morpholine-4-carboxylate (**11c**)



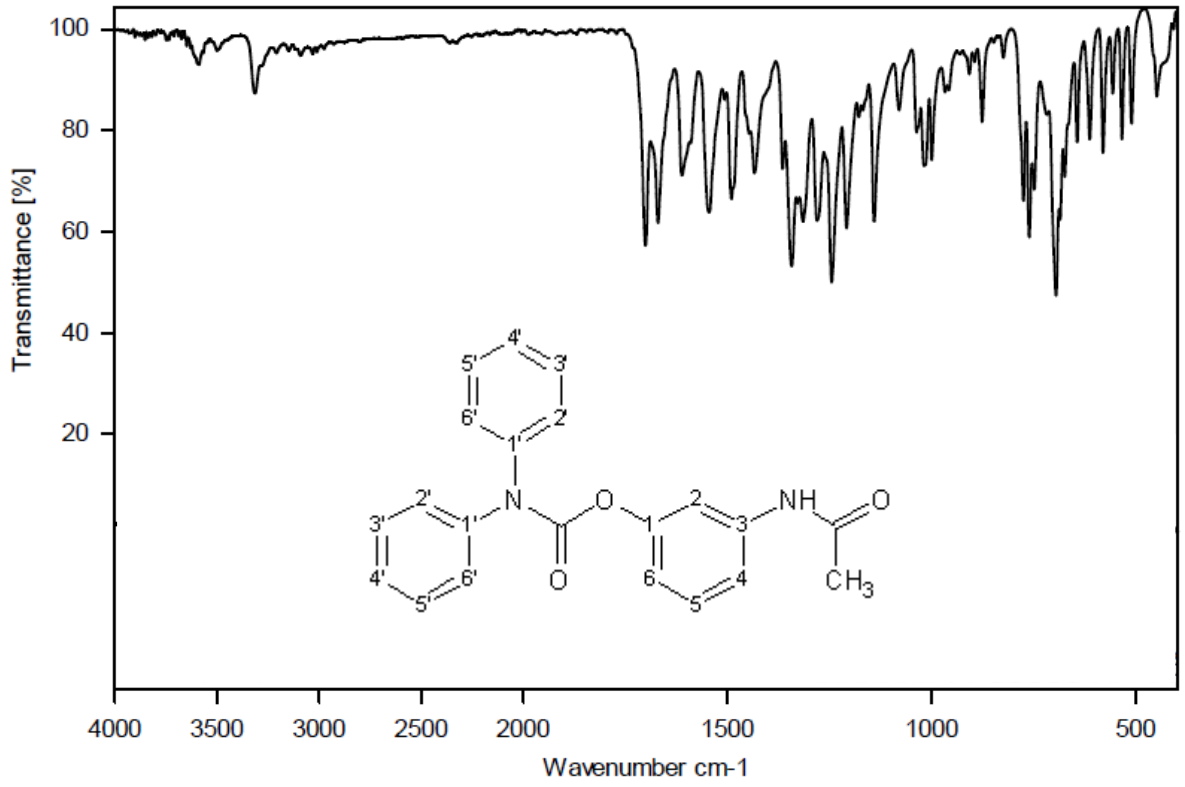
3-(acetylamino)phenyldiethylcarbamate (**11d**)



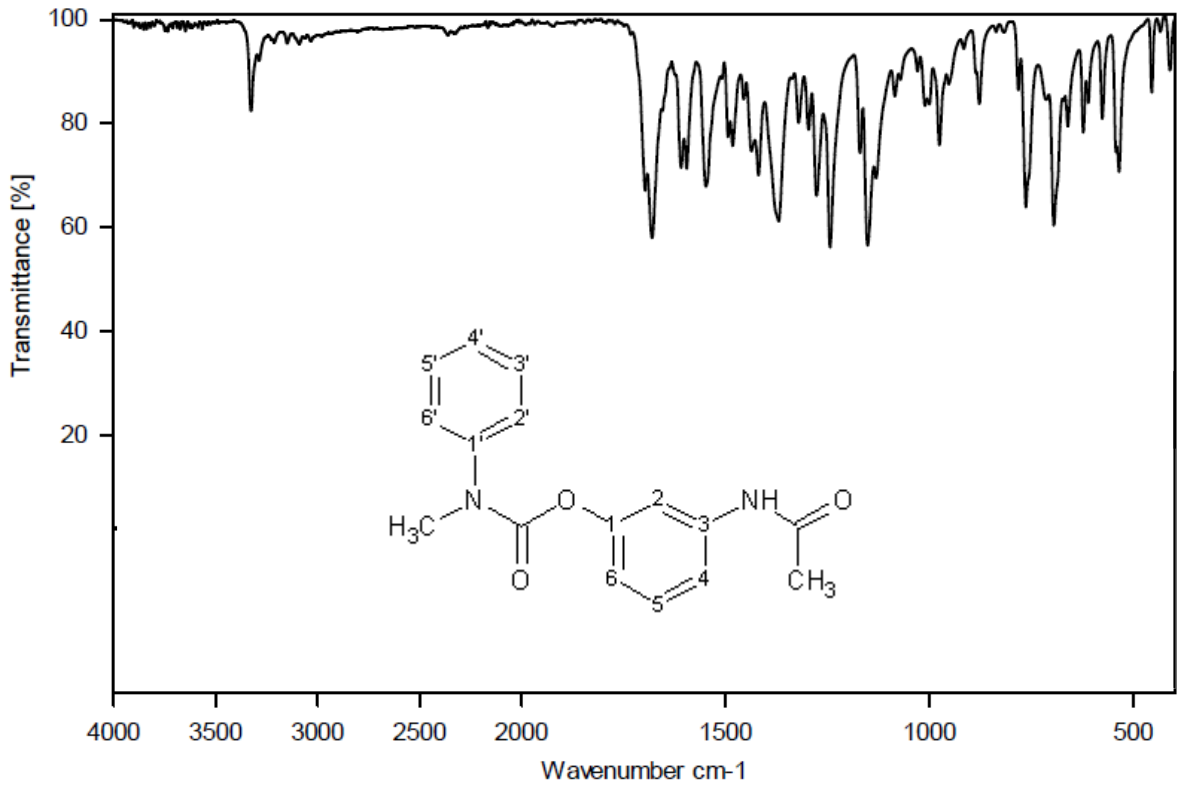
3-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**11e**)



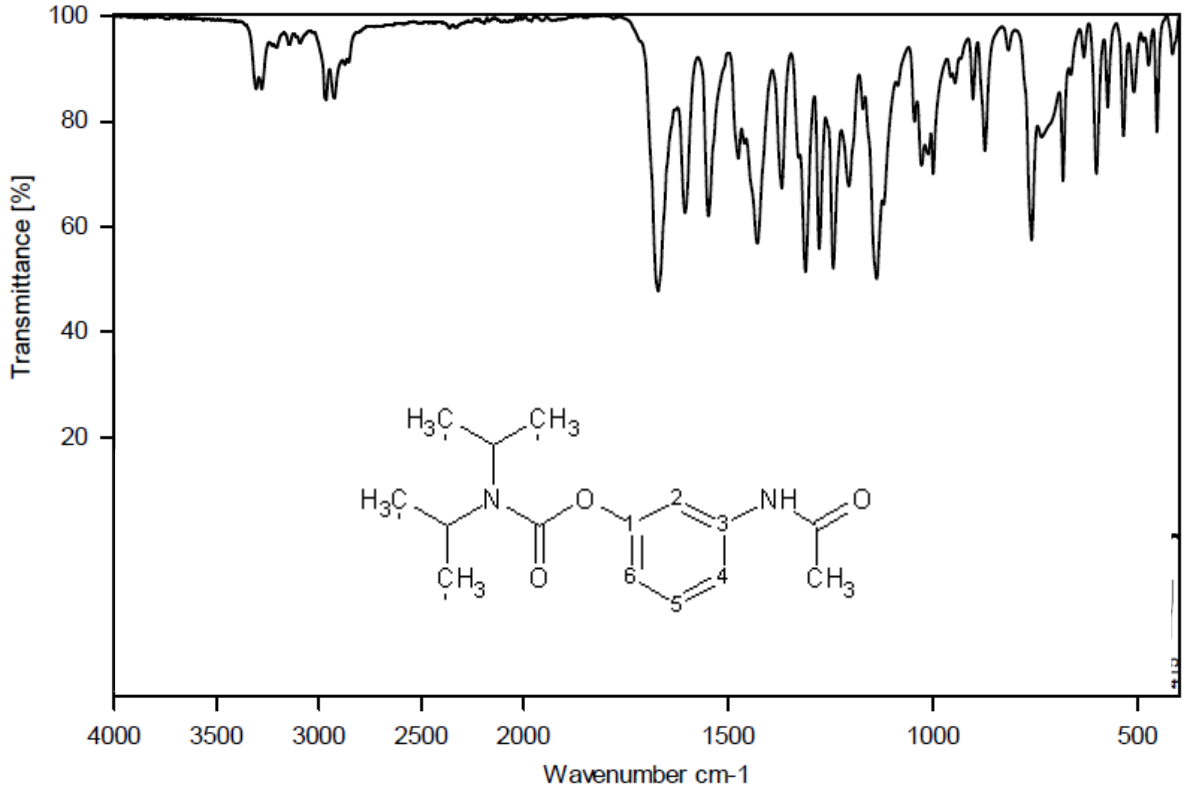
3-(acetylamino)phenyldiphenylcarbamate (**11f**)



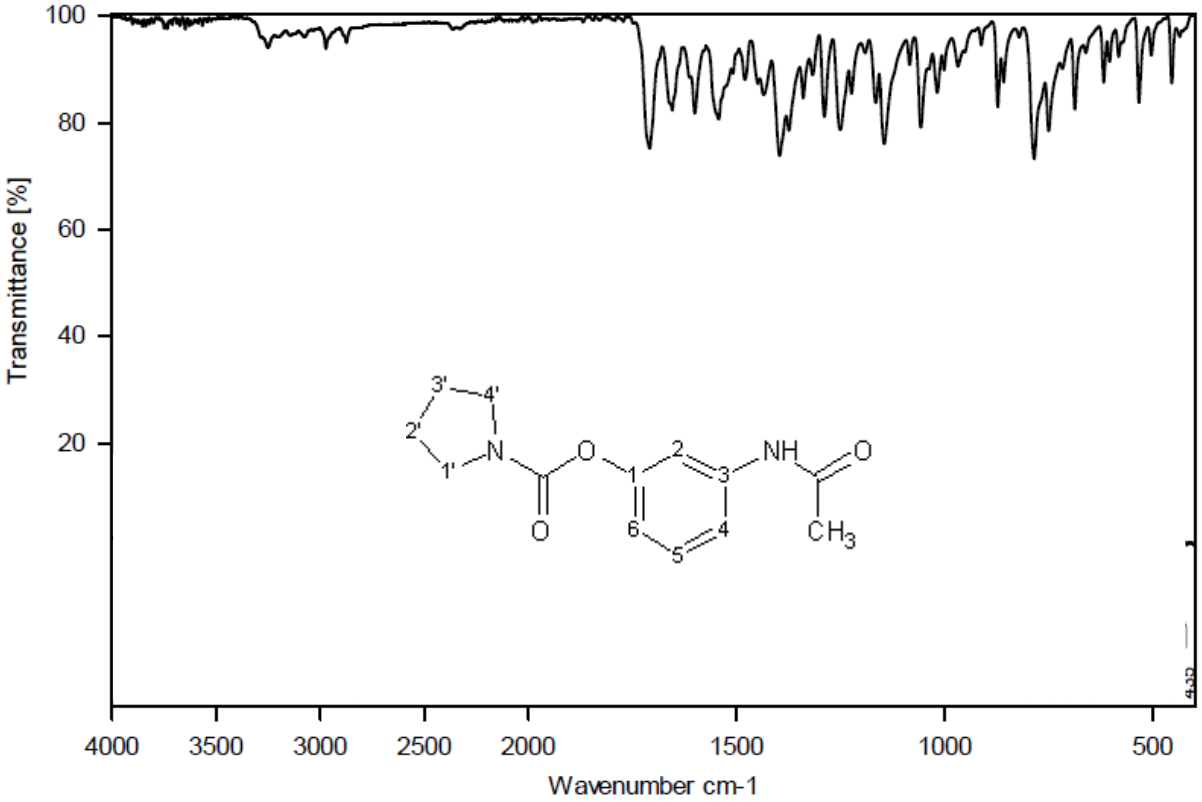
3-(acetylamino)phenyl methyl(phenyl)carbamate (**11g**)



3-(acetylamino)phenyl dipropan-2-ylcarbamate (**11h**)

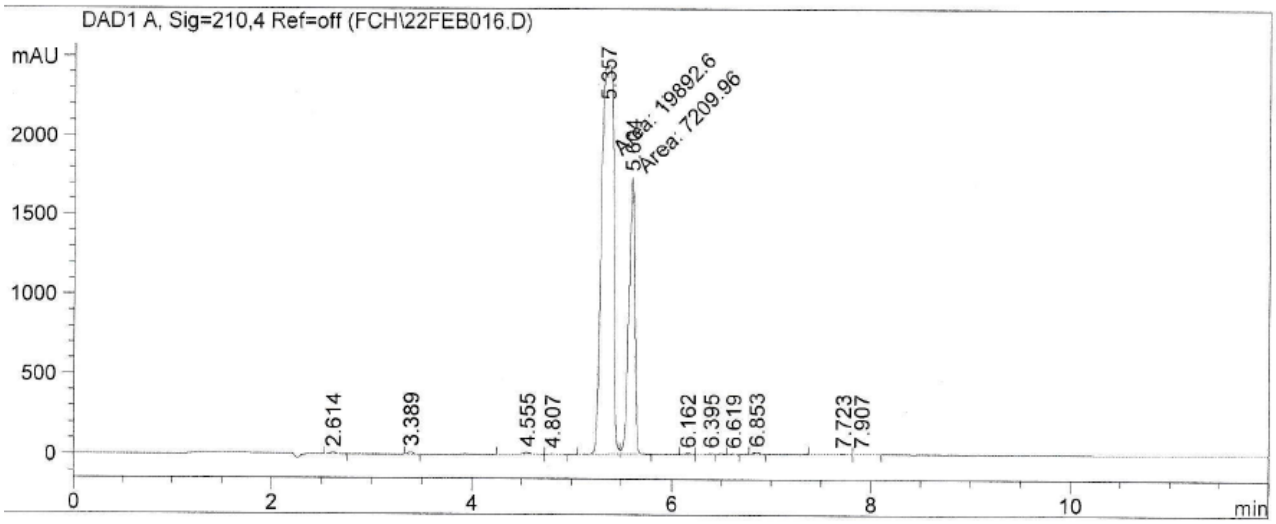


3-(acetylamino)phenyl pyrrolidine-1-carboxylate (**11i**)

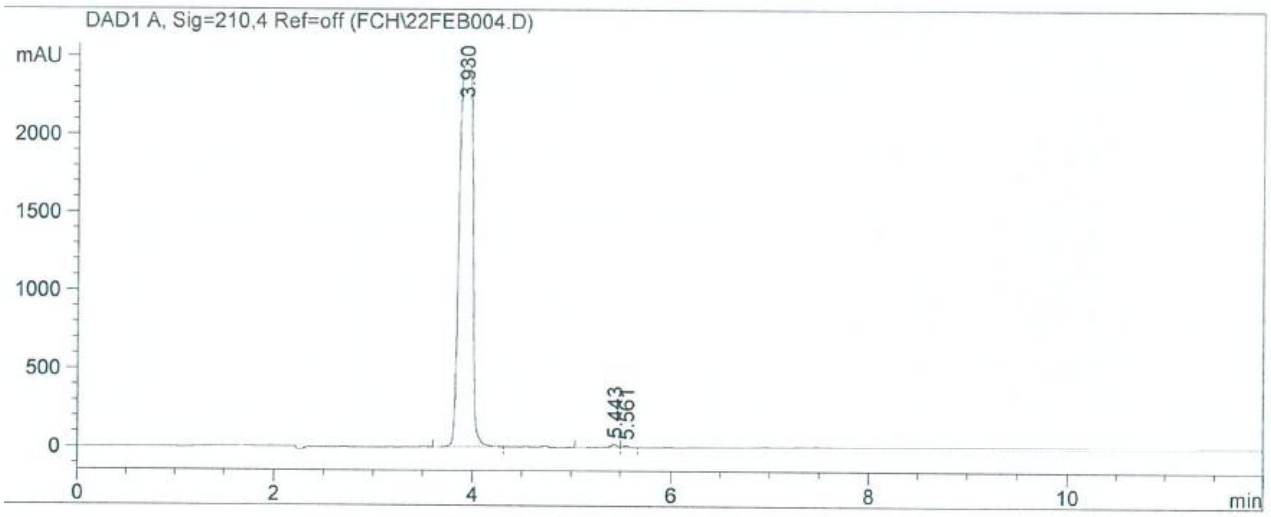


HPLC DATA

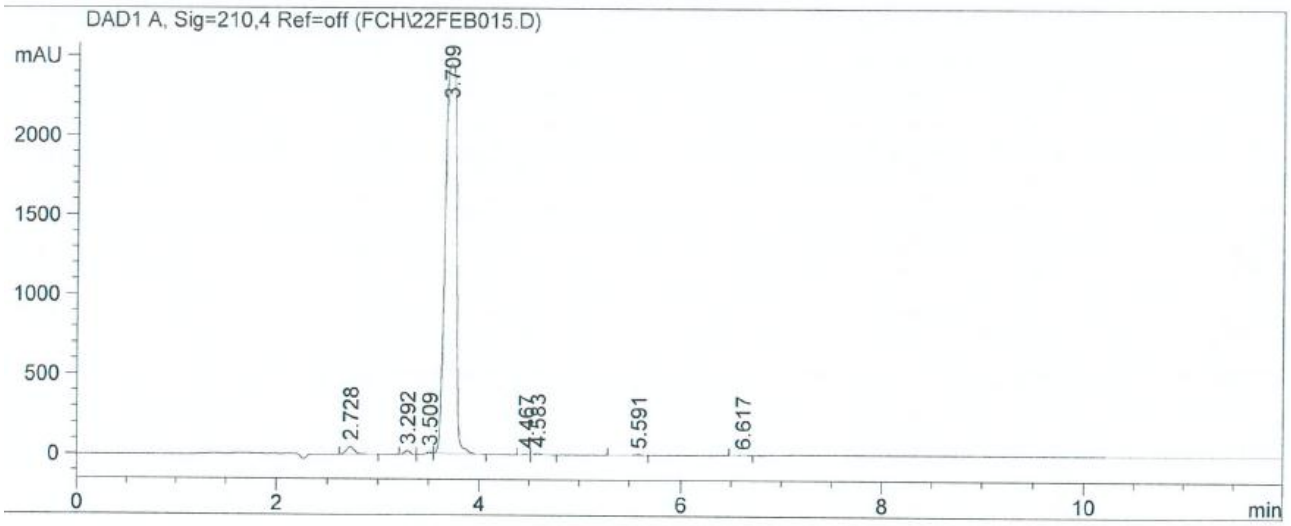
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl piperidine-1-carboxylate (**8a**)



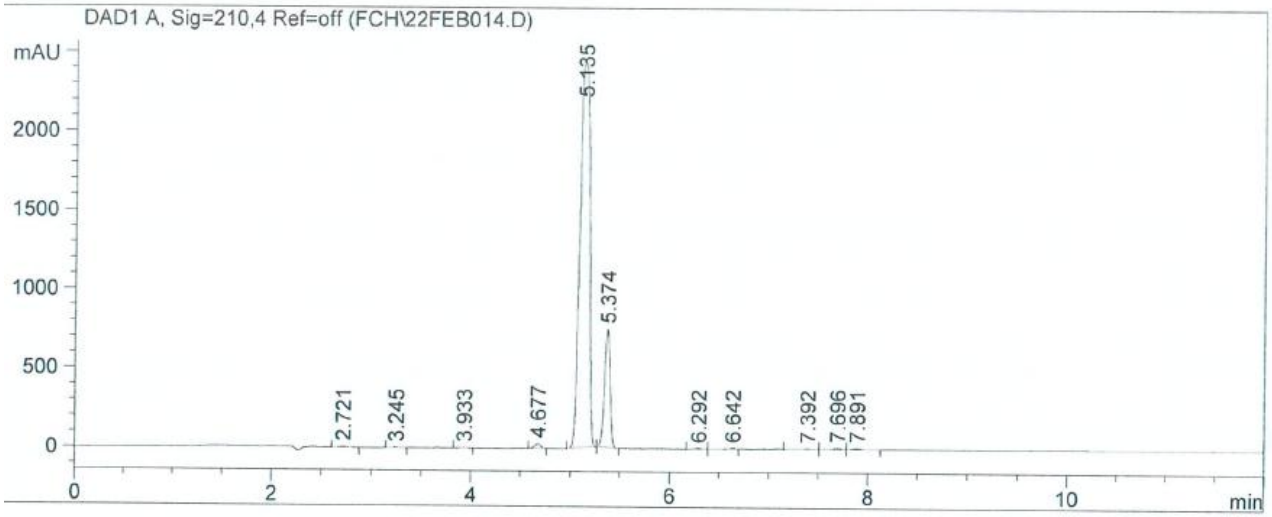
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl dimethylcarbamate (**8b**)



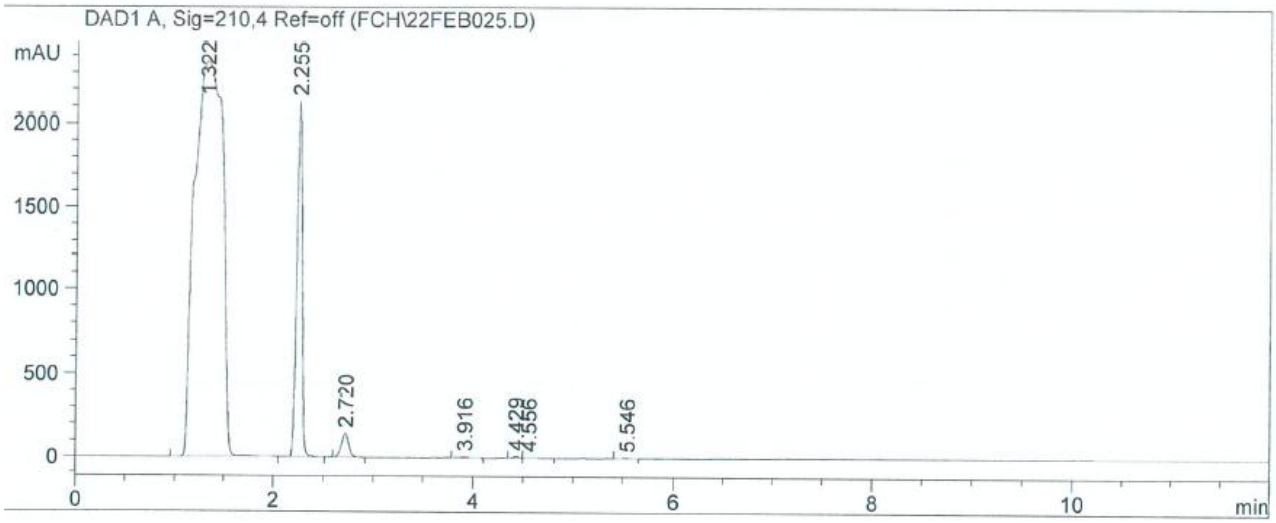
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**8c**)



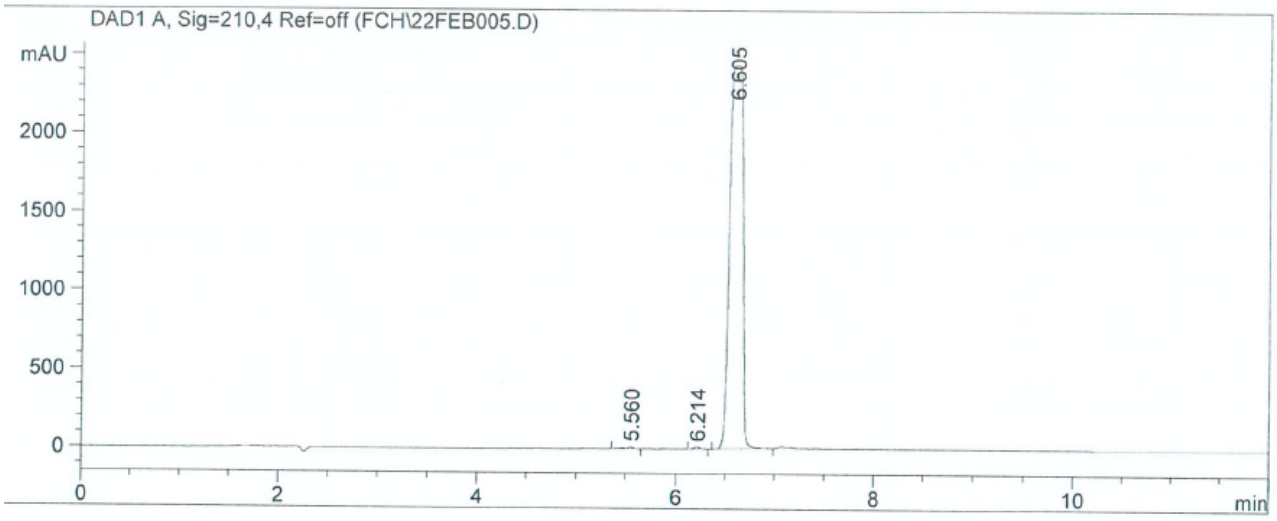
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diethylcarbamate (**8d**)



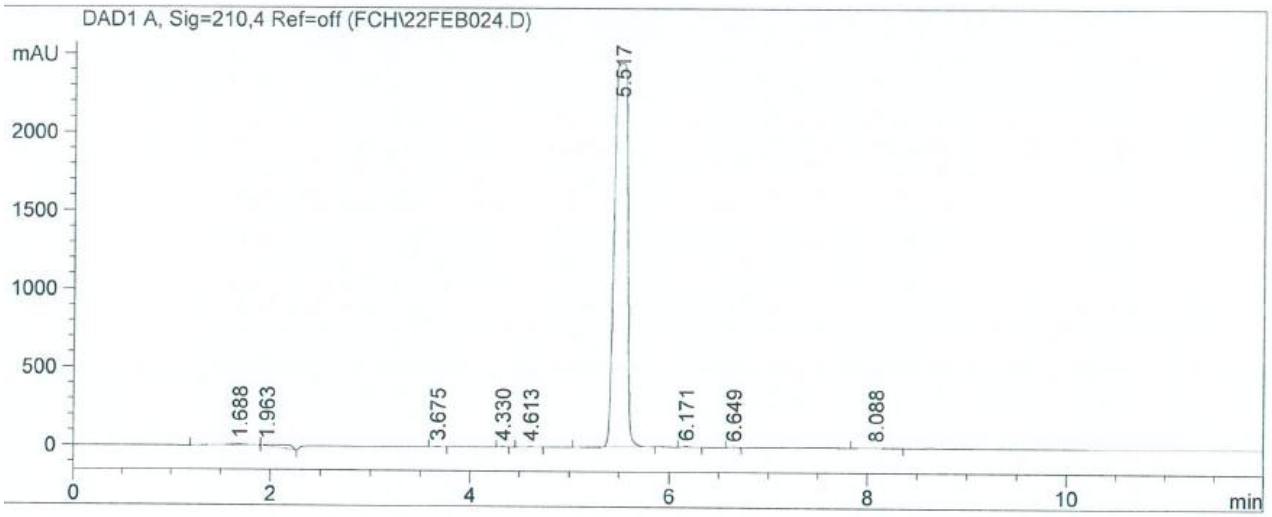
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl 4-methylpiperazine-1-carboxylate (**8e**)



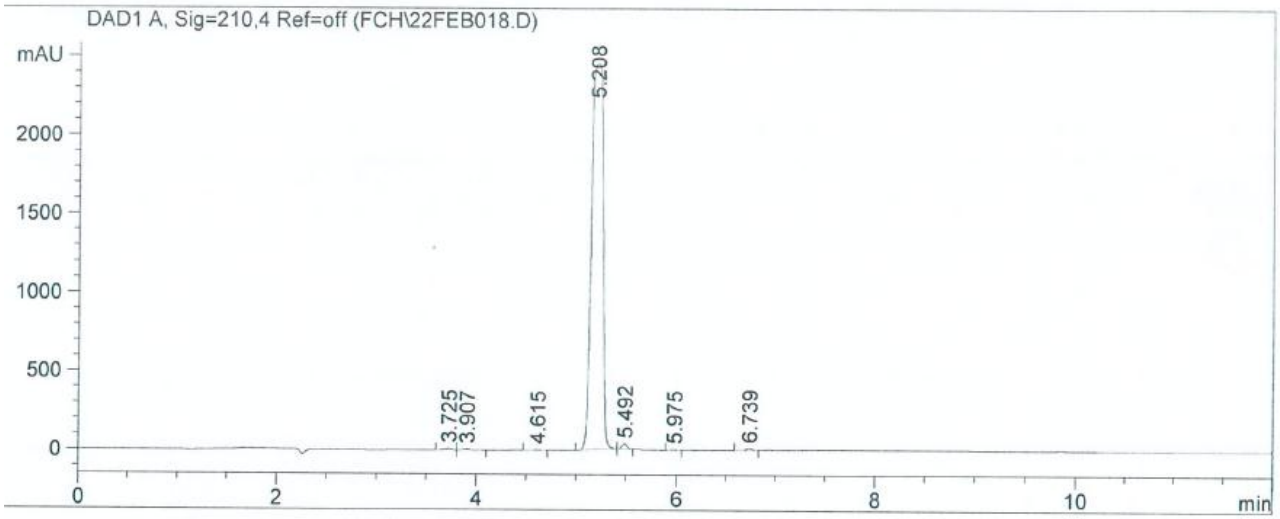
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl diphenylcarbamate (**8f**)



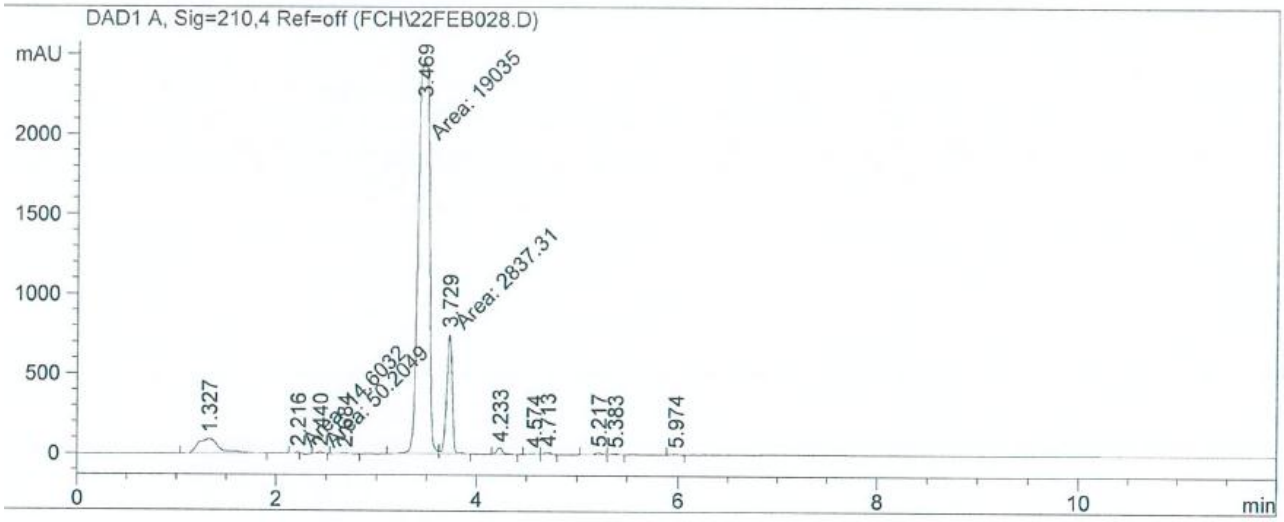
2-oxo-1,2,3,4-tetrahydroquinolin-7-yl methyl(phenyl)carbamate (**8g**)



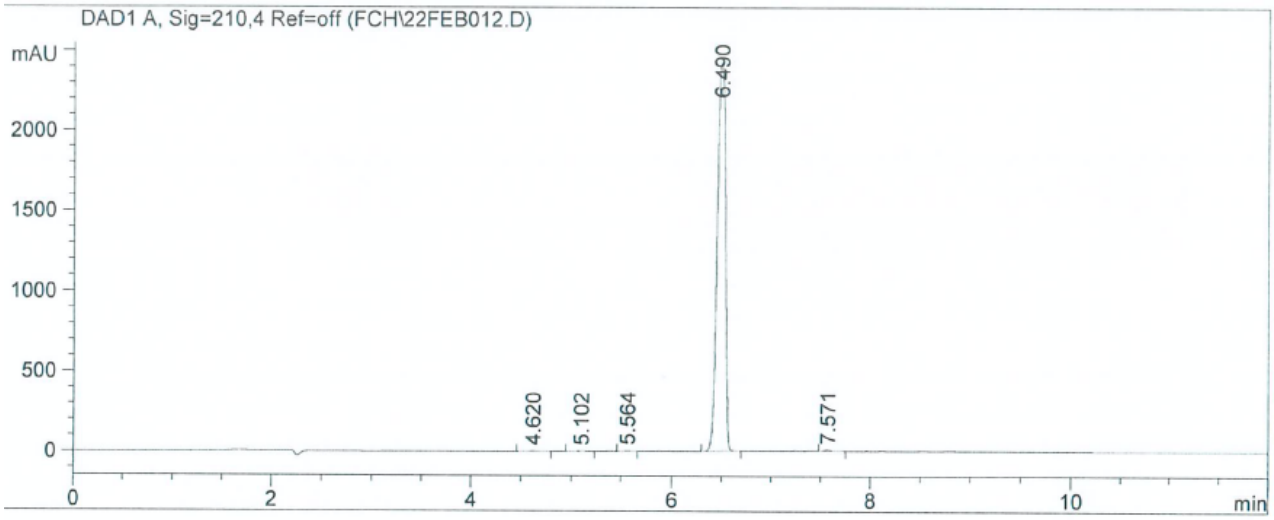
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl piperidine-1-carboxylate (**9a**)



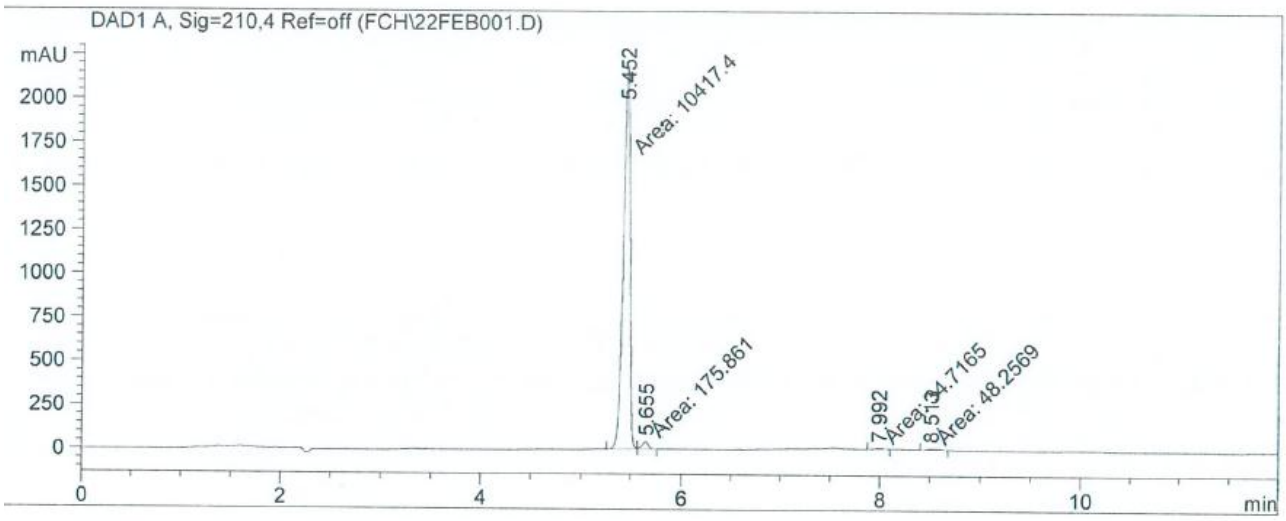
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl morpholine-4-carboxylate (**9b**)



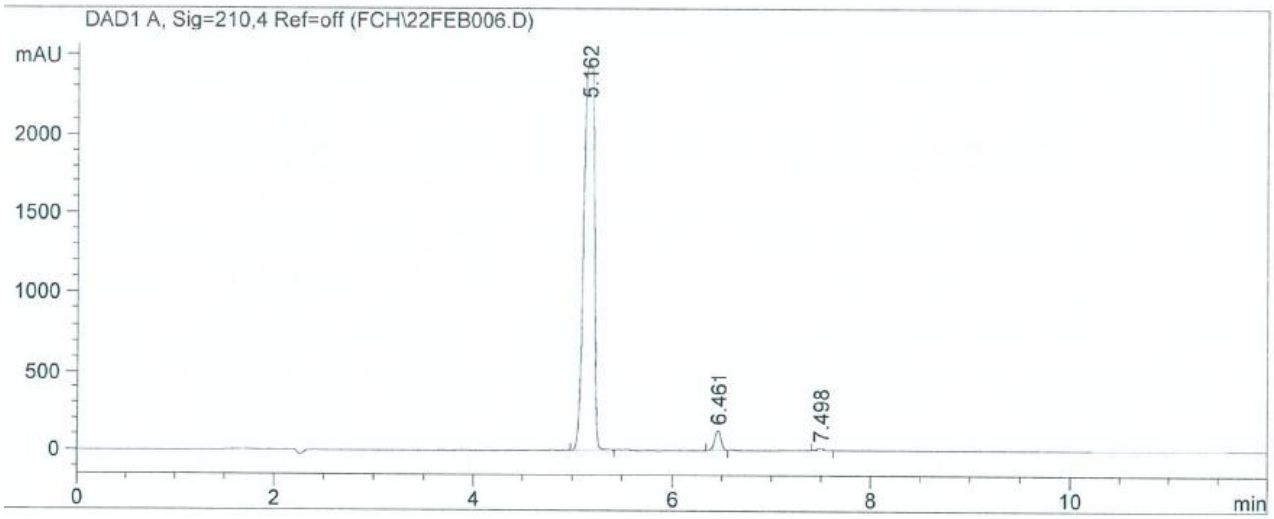
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl diphenylcarbamate (**9c**)



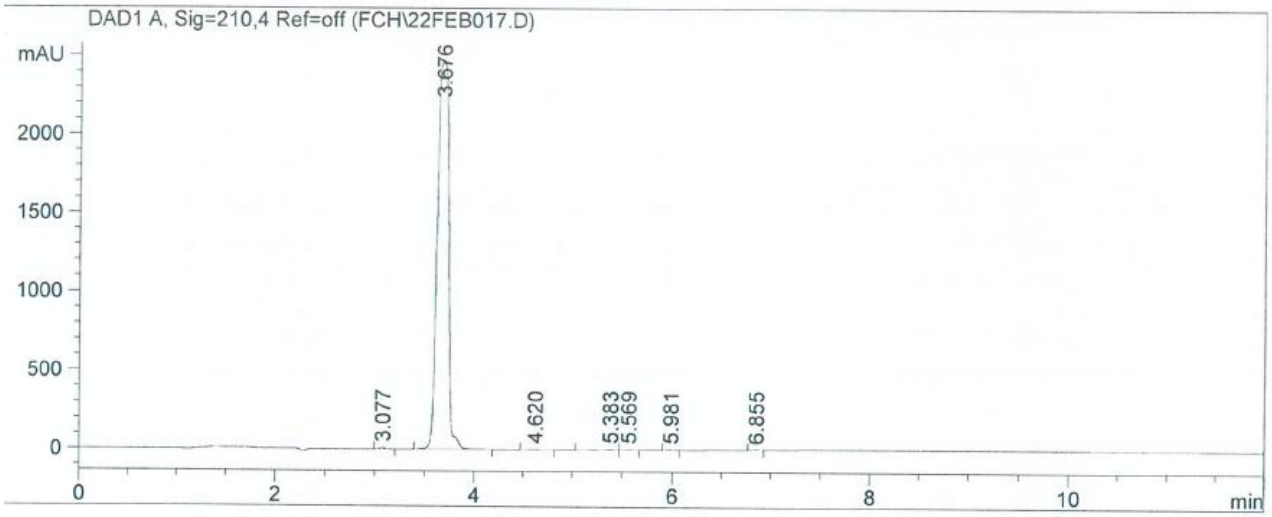
2-oxo-1,2,3,4-tetrahydroquinolin-6-yl methyl(phenyl)carbamate (**9d**)



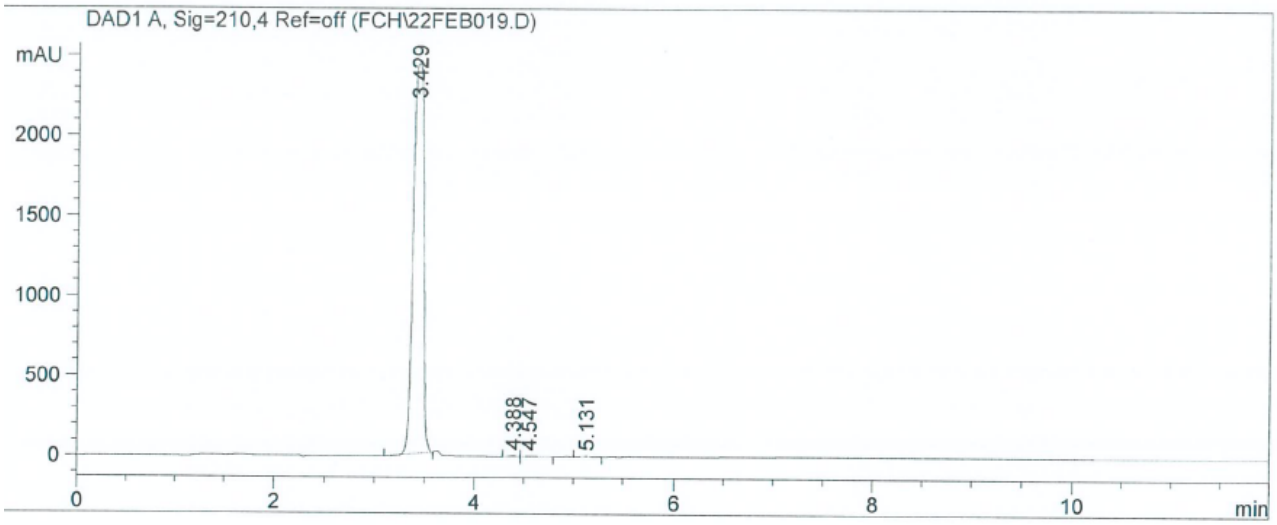
4-(acetylamino)phenyl piperidine-1-carboxylate (**10a**)



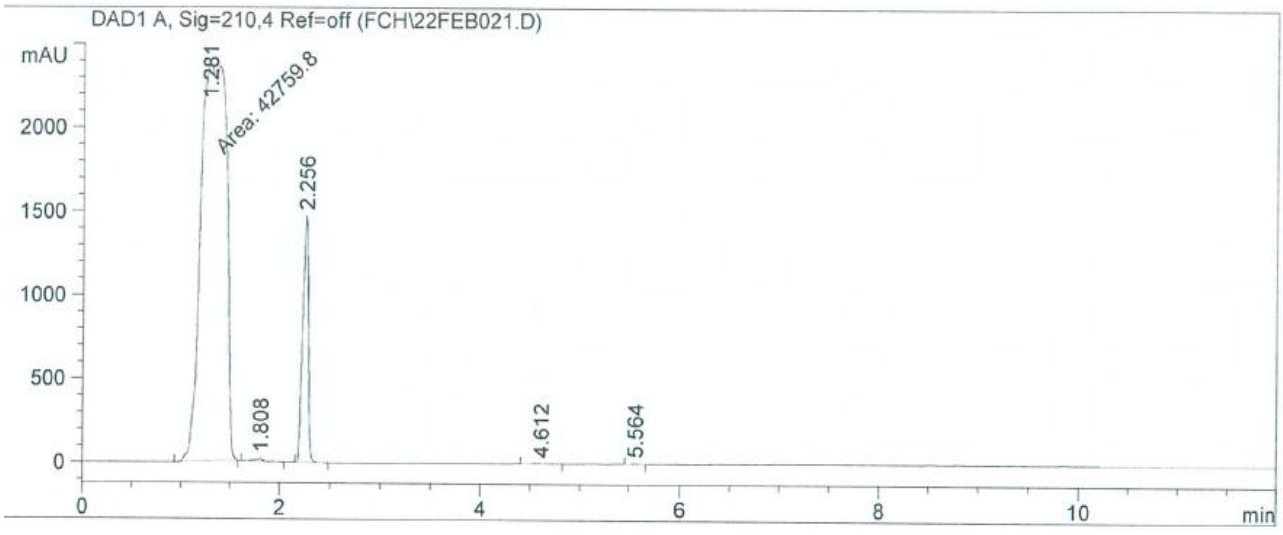
4-(acetylamino)phenyldimethylcarbamate (**10b**)



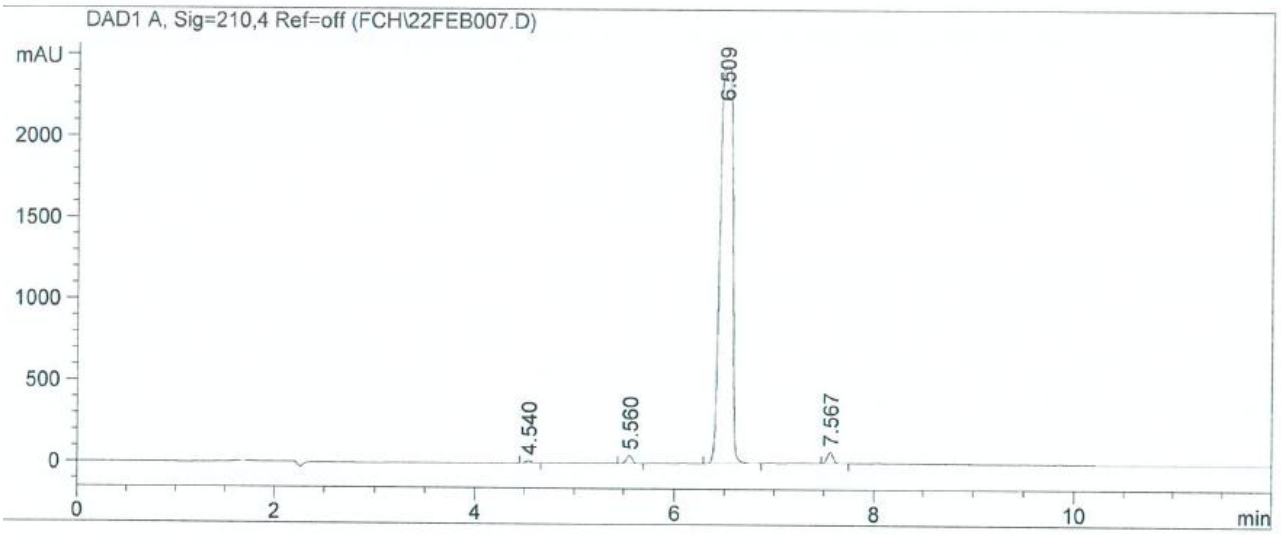
4-(acetylamino)phenyl morpholine-4-carboxylate (**10c**)



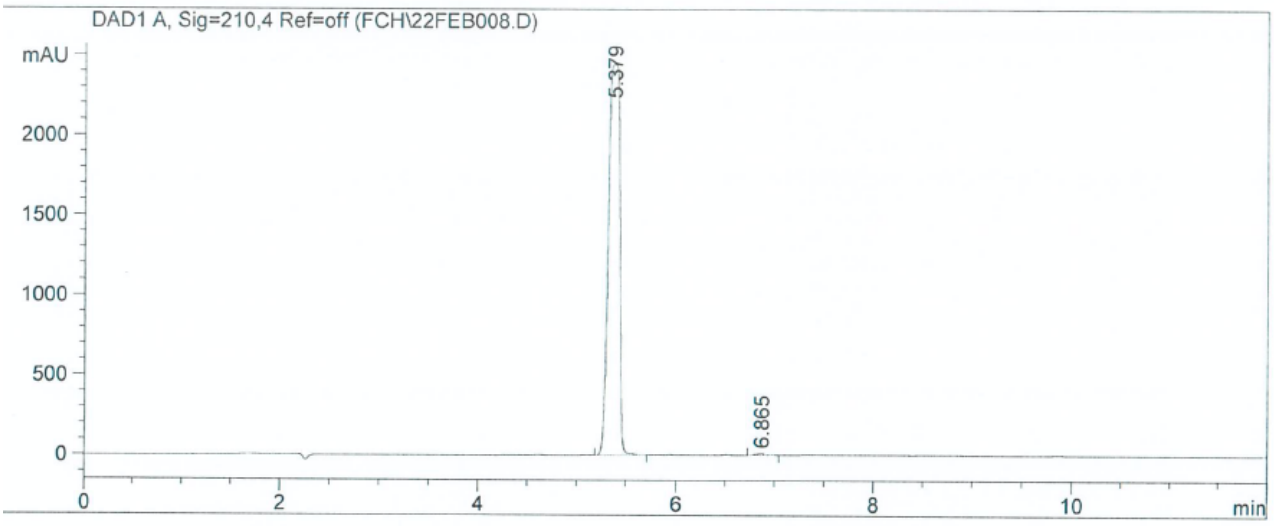
4-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**10d**)



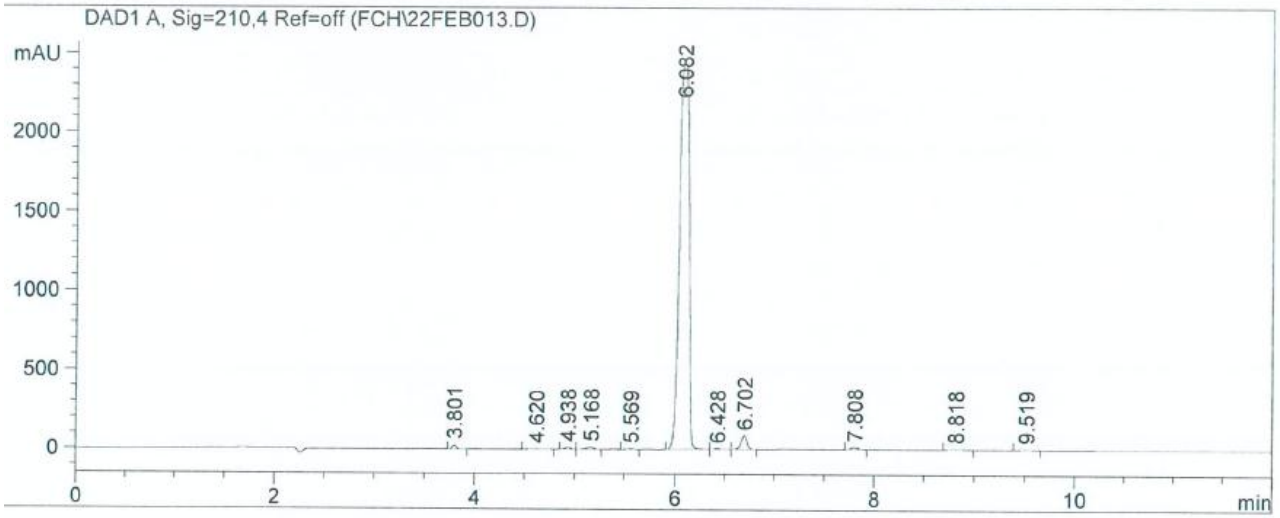
4-(acetylamino)phenyldiphenylcarbamate (**10e**)



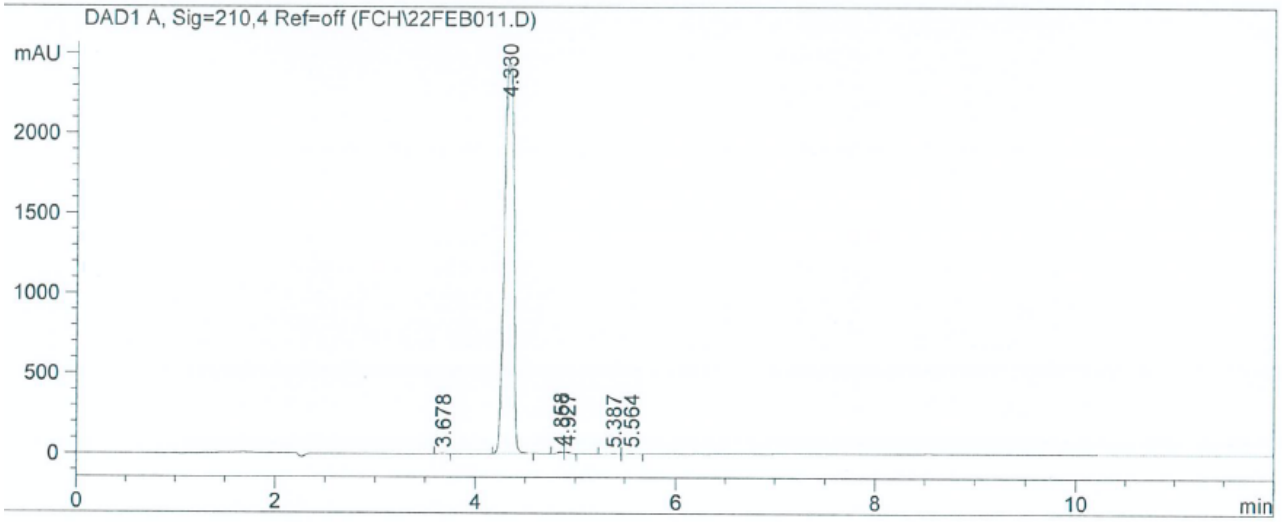
4-(acetylamino)phenyl methyl(phenyl)carbamate (**10f**)



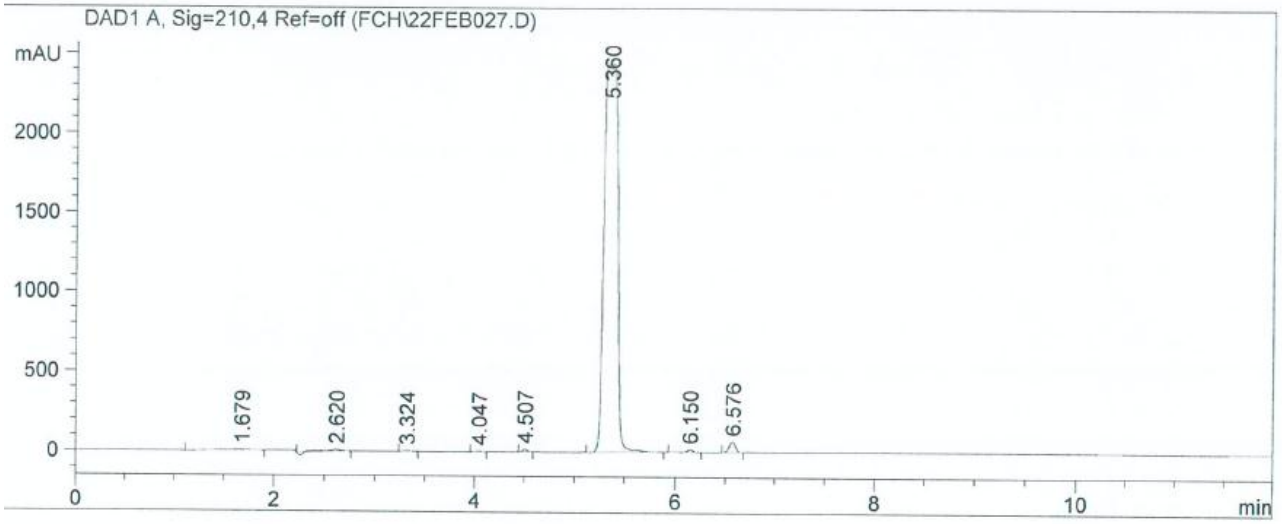
4-(acetylamino)phenyl dipropan-2-ylcarbamate (**10g**)



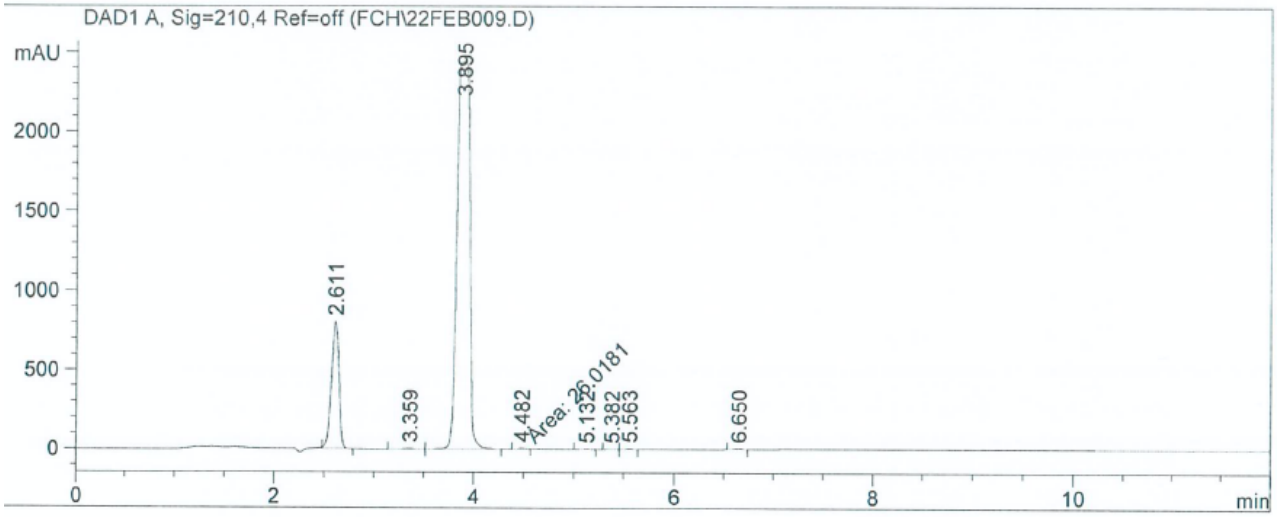
4-(acetylamino)phenyl pyrrolidine-1-carboxylate (**10h**)



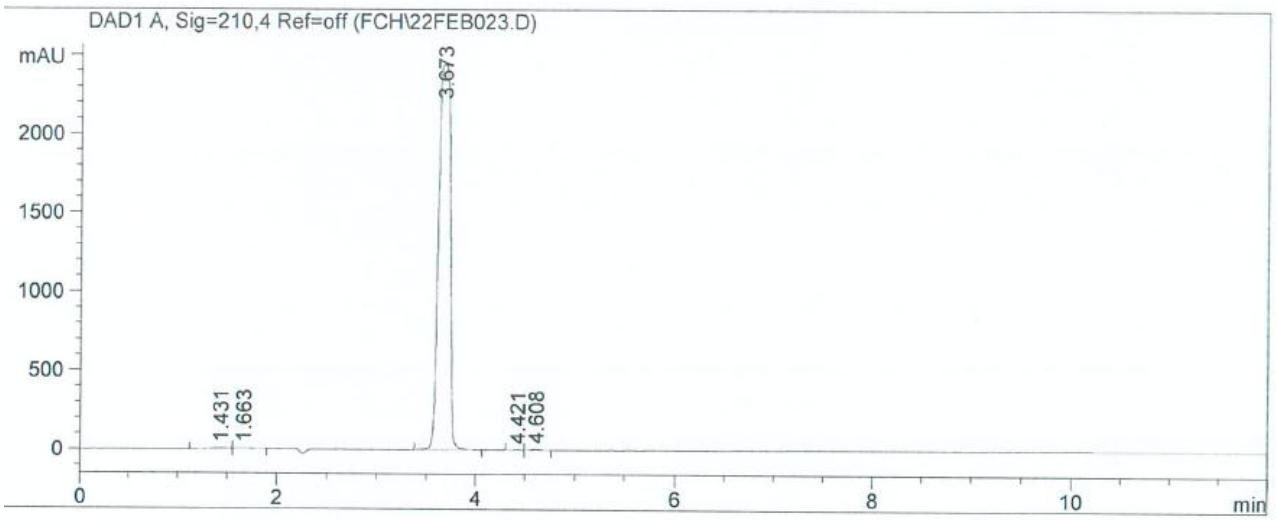
3-(acetylamino)phenyl piperidine-1-carboxylate (**11a**)



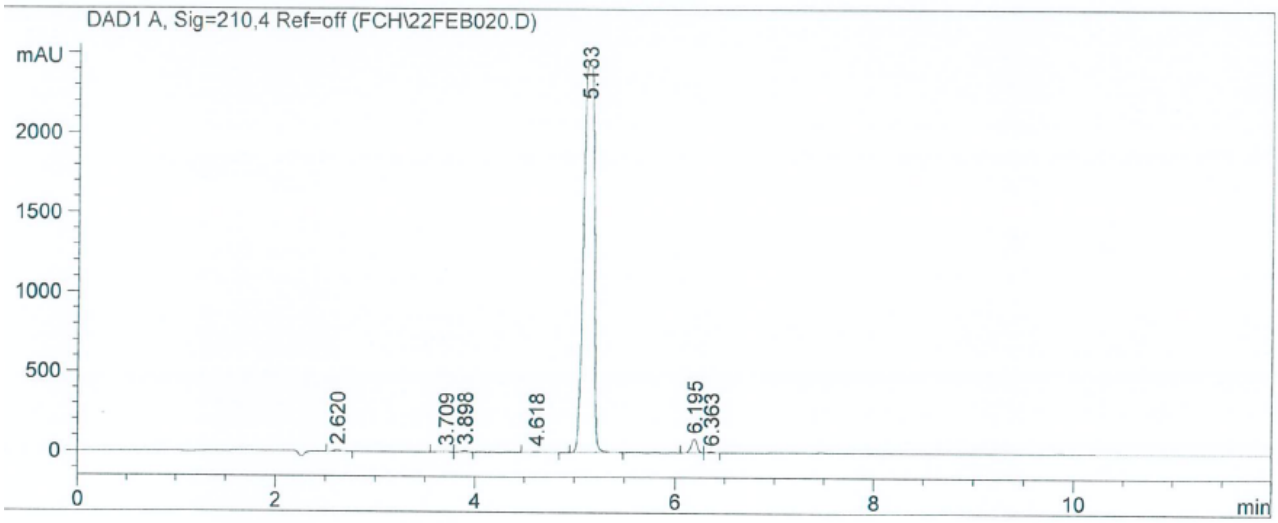
3-(acetylamino)phenyldimethylcarbamate (**11b**)



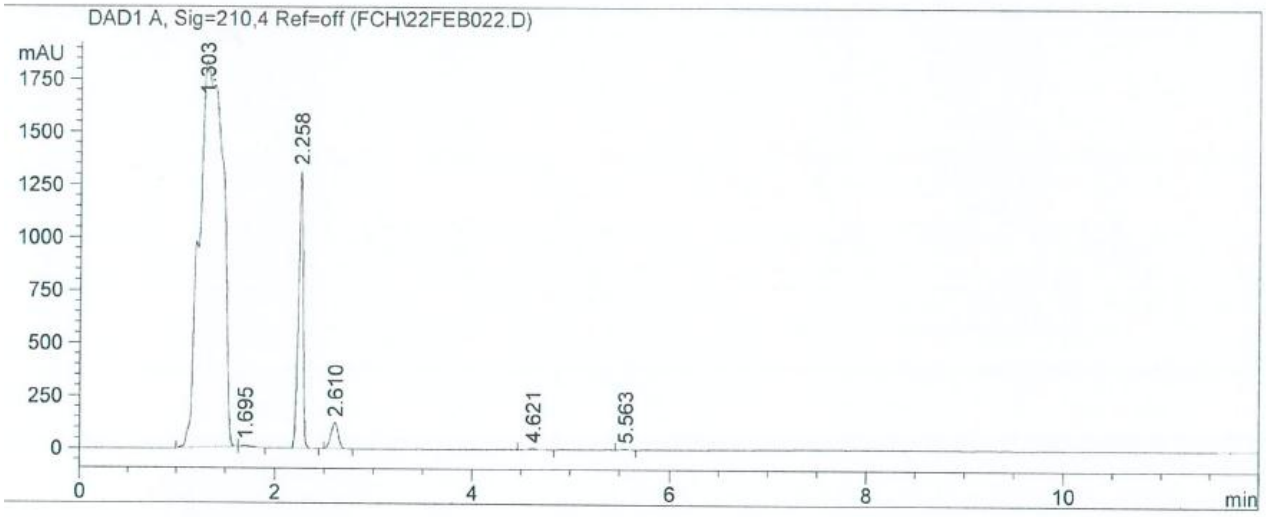
3-(acetylamino)phenyl morpholine-4-carboxylate (**11c**)



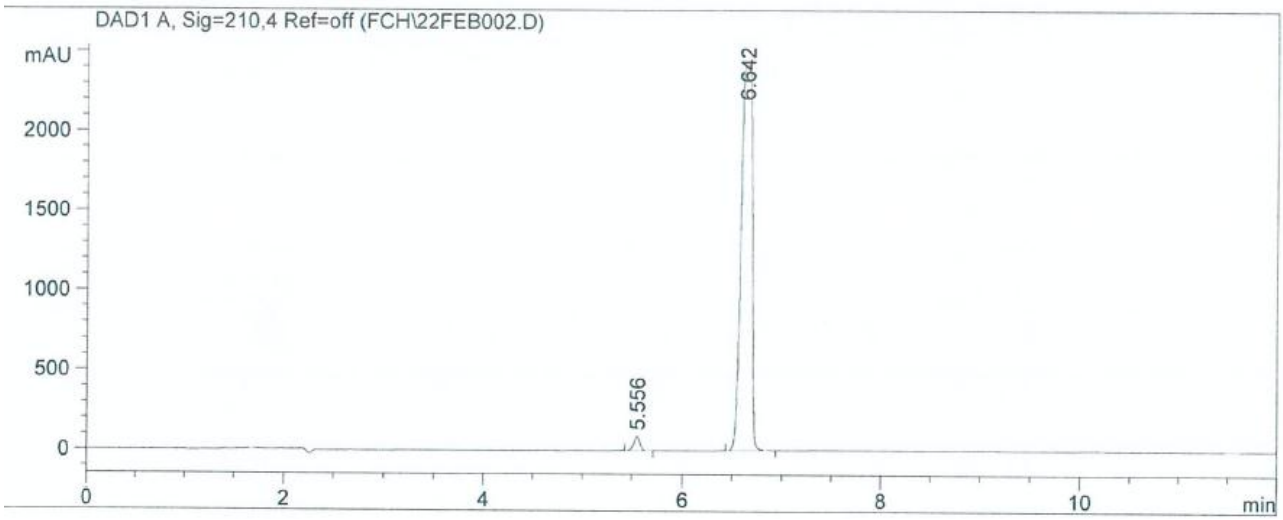
3-(acetylamino)phenyldiethylcarbamate (**11d**)



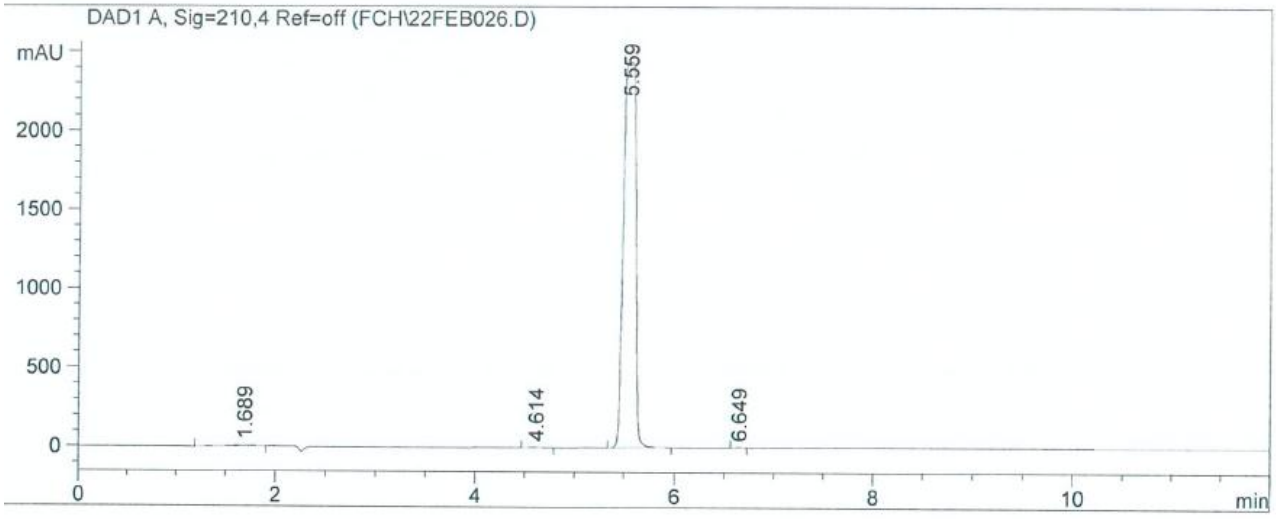
3-(acetylamino)phenyl 4-methylpiperazine-1-carboxylate (**11e**)



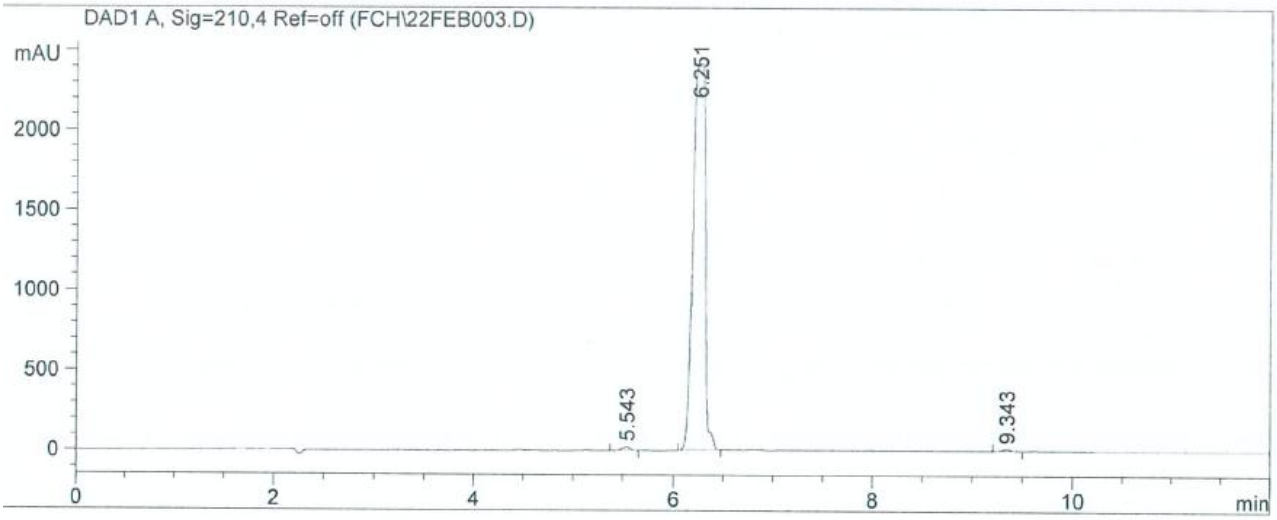
3-(acetylamino)phenyldiphenylcarbamate (**11f**)



3-(acetylamino)phenyl methyl(phenyl)carbamate (**11g**)



3-(acetylamino)phenyl dipropan-2-ylcarbamate (**11h**)



3-(acetylamino)phenyl pyrrolidine-1-carboxylate (**11i**)