Exploration of benzofuran as scaffold for the design of potent and selective *Pf*GSK-3 inhibitors

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Abstract

Control and prevention of malaria in endemic regions rely heavily on vector management (insecticide-treated bed nets and indoor residual spraying) and the limited number of effective antimalarial drugs. Unfortunately, the malaria parasite is highly adaptable and continuously develop resistance to available antimalarial drugs and insecticides. *Plasmodium falciparum*, the malaria species responsible for the majority of malaria-related deaths, in particular is resistant to all antimalarial drugs including the first-line artemisinin-based combination therapies. This necessitates the continual generation of new antimalarial drugs with novel modes of action.

Plasmodial protein kinases have been explored as targets for next-generation antimalarial drugs due to their involvement in various critical processes throughout the life cycle of the malaria parasite. Significant progress has been made thus far with regard to drug development for this target group. However, drug discovery programs for some essential plasmodial kinases have been limited. One such kinase is the *Plasmodium falciparum* glycogen synthase kinase-3 (*Pf*GSK-3). *Pf*GSK-3 was identified as the parasitic orthologue of human glycogen synthase kinase (*Hs*GSK-3) and although its exact biological functions is still unknown, it has been demonstrated to be essential for the survival of erythrocytic *P. falciparum* parasites. Despite the high degree of sequence and structural similarity between *Pf*GSK-3 and the two *Hs*GSK-3 orthologues (*Hs*GSK-3α and *Hs*GSK-3β), studies have shown that it is indeed possible to achieve *Pf*GSK-3 selective inhibition. To date, *Pf*GSK-3 selective inhibitors have been limited to the 4-phenylthieno[2,3-*b*]pyridine class.

The present study explored benzofurans as an alternative chalcone-based scaffold for the design of potent and selective *Pf*GSK-3 inhibitors. A series of benzofuran derivatives (**5a-v**) with varied structural substituents on both ring A and B were synthesised and characterised through nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), high-performance liquid chromatography (HPLC) and melting point (mp) analyses. The benzofuran derivatives were then evaluated as potential inhibitors of recombinantly expressed and purified *Pf*GSK-3 and *Hs*GSK-3β. Of the seven compounds that demonstrated inhibitory activity, five compounds (**5k**, **5m**, **5p**, **5r**, **5s**) preferentially inhibited *Pf*GSK-3 with IC_{50} values ranging from 0.00049 – 1.692 μM. Compounds **5p** and **5m** were the most potent *Pf*GSK-3 inhibitors of the series (IC_{50}: 0.00049 μM and 0.00168 μM, respectively), while compound **5r** was the most selective inhibitor with a 559-fold selectivity towards *Pf*GSK-3. The structure-activity relationships indicated that C6-OCH_3 substitution on ring A promotes

selectivity towards *Pf*GSK-3, while the effect of ring B substitution on activity, in decreasing order was: C4'-CN > C4'-F > C3'-OCH_3 > C3',4'-diCl.

In conclusion, the current study evaluated benzofurans to gain further insight into the structure-activity relationships around this scaffold for the design of potent and selective *Pf*GSK-3 inhibitors. The benzofuran derivatives proved to be highly potent and selective *Pf*GSK-3 inhibitors. As these compounds have potential in the treatment of multidrug-resistant malaria, they are promising hits for future medicinal chemistry efforts.

Keywords

malaria, *Plasmodium falciparum*, protein kinases, glycogen synthase kinase-3, benzofurans

Preface

This thesis is submitted in **article format**, comprising of two peer-reviewed articles, in accordance with the General Academic Rules (A.13.7.33) of the North-West University.

The review article titled **"An update on development of small-molecule plasmodial kinase inhibitors"** was published in *Molecules*, an open-access journal with an impact factor of 3.267 (2019).

The article titled **"Exploration of benzofuran-based compounds as potent and selective *Plasmodium falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) inhibitors"** was published in the *Bioorganic Chemistry* journal with an impact factor of 4.831 (2019).

Author contributions

Paper I presented in Chapter 3: Chantalle Moolman was involved in the review of the literature and manuscript writing. Lesetja Legoabe, Rencia van der Sluis and Richard Beteck were involved in revision and supervision.

Paper II presented in Chapter 4: Chantalle Moolman conducted the experimental work including, compound synthesis, compound characterisation (HPLC and melting point analysis), recombinant protein expression and purification, and the biological assays. Chantalle Moolman was also involved in data analysis and manuscript writing. Lesetja Legoabe was involved in the study design, data analysis (chemistry), revision and supervision. Rencia van der Sluis was involved in data analysis (molecular biology), revision and supervision. Richard Beteck was involved in revision and supervision.

Permission from co-authors

As a co-author, I hereby approve and give consent that the above-mentioned articles may be submitted by Chantalle Moolman as part of her PhD degree in Pharmaceutical Chemistry at the North-West University. I declare that my role in the study, as indicated above, is a representation of my actual contribution.

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List of Abbreviations

Symbols

α	alpha
β	beta
δ	chemical shift
J	coupling constant
°C	degrees Celsius
δ	delta
ε	epsilon
γ	gamma
>	greater-than
≥	greater-than or equal to
<	less-than
%	percent
±	plus-minus

Units of measurement

bp	base pairs
g	gram
Hz	hertz
kcal/mol	kilocalorie per mole
kDa	kilodaltons
MHz	megahertz
µg/ml	micrograms per millilitre
µl	microlitre
µM	micromolar
mg	milligram
mg/kg	milligrams per kilogram
mg/ml	milligrams per millilitre
ml	millilitre
mM	millimolar
mmol	millimoles
ng	nanograms
nm	nanometers
nM	nanomolar
ppm	parts per million
rpm	revolutions per minute

Abbreviations

^{1}H NMR	proton nuclear magnetic resonance
3D-QSAR	three-dimensional structure-activity relationship
^{13}C NMR	carbon nuclear magnetic resonance
A-loop	activation loop
Abl1	tyrosine-protein kinase Abl 1
ACT	artemisinin-based combination therapy

ADME(T)	absorption, distribution, metabolism, excretion (and toxicity)
AGC	protein kinase A, protein kinase G and protein kinase C families
$AlCl_3$	aluminium chloride
AP-1	activator protein-1
APC	adenomatous polyposis coli
APCI	atmospheric pressure chemical ionisation
APE-motif	alanine (A)-proline (P)-glutamic acid (E)-motif
APP	amyloid precursor protein
ATP	adenosine triphosphate
AXK-motif	alanine (A)-any amino acid (X)-lysine (K)-motif
BBr_3	boron tribromide
BKI	bumped kinase inhibitor
Br	bromine (bromo)
BRAF	serine/threonine kinase
BSA	bovine serum albumin
C-spine	catalytic spine
Ca^{2+}	calcium
CAMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CC_{50}	half maximal cytotoxic concentration
$CDCl_3$	deuterated chloroform
CDK	cyclin-dependent kinase
CDPK	calcium-dependent protein kinase
cGMP	cyclic guanosine monophosphate
CIRK	insulin receptor kinase
Cl	chlorine (chloro)
CLK	cyclin-dependent kinase (CDK)-like kinase
CK1	casein kinase 1
CMGC	Cyclin-dependent kinase, Mitogen-activated protein kinase, Glycogen synthase kinase and Cyclin-dependent kinase (CDK)-like kinase families
CN	cyanide (cyano)
COVID-19	Coronavirus of 2019
*Cp*CDPK1	*Cryptosporidium parvum* calcium-dependent protein kinase 1
CREB protein	cAMP response element-binding protein
$CuBr_2$	copper(II) bromide
d	doublet
DAD	diode array detector
DCM	dichloromethane
dd	doublet of doublets
ddd	doublet of doublet of doublets
DFG-motif	aspartate (D)-phenylalanine (P)-glycine (G)-motif
diCl	two chlorine atoms (dichloro)
$diOCH_3$	two methoxy groups (dimethoxy)
DMSO	dimethyl sulfoxide
DMSO-*d6*	deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol

EC_{50}	half maximal effective concentration
ED_{90}	90% effective dose
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
EGFR	epidermal growth factor receptor
ePK	eukaryotic protein kinase
Erk	extracellular signal-regulated kinase
F	fluorine (fluoro)
FDA	Food and Drug Administration
FIKK	phenylalanine (F)-isoleucine (I)-lysine (K)-lysine (K) kinase
GS-1	glycogen synthase-1
GSK-3	glycogen synthase kinase-3
GSK-3α	glycogen synthase kinase-3 alpha isoform
GSK-3β	glycogen synthase kinase-3 beta isoform
HB3	drug-sensitive strain of *Plasmodium falciparum*
HCl	hydrochloric acid
HepG2	human hepatoma cell line
hERG	human ether-a-go-go related gene potassium channel
His-tag	polyhistidine-tag
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
*Hs*CDK2	human cyclin-dependent kinase 2
*Hs*GSK-3	human glycogen synthase kinase-3
*Hs*GSK-3α	human glycogen synthase kinase-3 alpha isoform
*Hs*GSK-3β	human glycogen synthase kinase-3 beta isoform
HuH7	human hepatoma cell line
IC_{50}	half maximal inhibitory concentration
IP_3	inositol (1,4,5)-triphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
IPTi	intermittent preventive treatment for infants
IPTp	intermittent preventive treatment during pregnancy
J-domain	junction domain
K_2CO_3	potassium carbonate
K_d	binding affinity
K_D	equilibrium dissociation constant
$K_{D(app)}$	apparent dissociation constant
K_i	inhibitory constant
LB	lysogeny broth
LCK	human lymphocyte-specific protein tyrosine kinase
LEW buffer	Lysis-Equilibration-Wash buffer
LLE	lipophilic ligand efficiency
LogD	distribution coefficient
m	multiplet
m/z	mass-to-charge-ratio

MAP2K (MAPKK)	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MAP3K7 (TAK1)	mitogen-activated protein kinase kinase kinase 7
MAPK	mitogen-activated protein kinase
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MH^+	protonated molecule
MLM	mouse liver microsomes
MMV	Medicines for Malaria Venture
mp	melting point
mRNA	messenger ribonucleic acid
ND	not determined
NFAT	nuclear factor of activated T-cells
NIH	National Institutes of Health
NMR	nuclear magnetic resonance
NSG	humanised immunodeficient mice
OD_{600}	optical density measured at a wavelength of 600 nm
OH	hydroxy
P	*Plasmodium*
Pb	*Plasmodium berghei*
*Pb*CDPK	*Plasmodium berghei* calcium-dependent protein kinase
*Pb*CLK	*Plasmodium berghei* cyclin-dependent kinase (CDK)-like kinase
*Pb*crk	*Plasmodium berghei* cyclin-dependent kinase (CDK)-related kinase
*Pb*map	*Plasmodium berghei* mitogen-activated protein kinase
Pc	*Plasmodium cynomolgi*
PCR	polymerase chain reaction
PDB ID	Protein Data Bank Identification
PEG-400	polyethylene glycol 400
Pf	*Plasmodium falciparum*
*Pf*3D7	*Plasmodium falciparum* 3D7 (drug-sensitive) strain
*Pf*AMA1	*Plasmodium falciparum* apical membrane antigen 1
*Pf*ark	*Plasmodium falciparum* Aurora-related kinase
*Pf*CDPK	*Plasmodium falciparum* calcium-dependent protein kinase
*Pf*CLK	*Plasmodium falciparum* cyclin-dependent kinase (CDK)-like kinase
*Pf*CK1	*Plasmodium falciparum* casein kinase 1
*Pf*crk	*Plasmodium falciparum* cyclin-dependent kinase (CDK)-related kinase
*Pf*CRT	*Plasmodium falciparum* chloroquine-resistance membrane transporter
*Pf*D6	*Plasmodium falciparum* D6 (drug-sensitive) strain
*Pf*Dd2	*Plasmodium falciparum* Dd2 (drug-resistant) strain
*Pf*FCB1	*Plasmodium falciparum* FCB1 (drug-resistant) strain
*Pf*FIKK	*Plasmodium falciparum* phenylalanine (F)-isoleucine (I)-lysine (K)-lysine (K) kinase
*Pf*GSK-3	*Plasmodium falciparum* glycogen synthase kinase-3
*Pf*HSP90	*Plasmodium falciparum* chaperone protein
*Pf*K1	*Plasmodium falciparum* K1 (drug-resistant) strain
*Pf*map	*Plasmodium falciparum* mitogen-activated protein kinase
*Pf*mrk	*Plasmodium falciparum* MO15-related kinase
*Pf*nek	*Plasmodium falciparum* Never in Mitosis gene A (NIMA)-related kinase

*Pf*NF54	*Plasmodium falciparum* NF54 (drug-sensitive) strain
*Pf*NF54-Luc	transgenic *Plasmodium falciparum* NF54 strain expressing the luciferase gene
*Pf*PI3K	*Plasmodium falciparum* phosphoinositide 3-kinase
*Pf*PI4K	*Plasmodium falciparum* phosphatidylinositol 4-kinase
*Pf*PK	*Plasmodium falciparum* protein kinase
*Pf*PKA	*Plasmodium falciparum* protein kinase A
*Pf*PKAc	*Plasmodium falciparum* protein kinase A catalytic subunit
*Pf*PKAr	*Plasmodium falciparum* protein kinase A regulatory subunit
*Pf*PKB	*Plasmodium falciparum* protein kinase B
*Pf*PKG	*Plasmodium falciparum* protein kinase G
*Pf*SR1	*Plasmodium falciparum* serine/arginine protein 1
*Pf*TKL3	*Plasmodium falciparum* tyrosine kinase-like kinase 3
*Pf*UBC13	*Plasmodium falciparum* E2 ubiquitin-conjugating enzyme 13
*Pf*W2	*Plasmodium falciparum* W2 (drug-resistant) strain
pI_{50}	negative log of the IC_{50} value in molar
PI3K	phosphoinositide 3-kinase
PI4K	phosphatidylinositol 4-kinase
PIK	phosphoinositide lipid kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKG	protein kinase G
PKG-Iα	human protein kinase G-1 alpha (cytosolic orthologue)
PKG-Iβ	human protein kinase G-1 beta (cytosolic orthologue)
PKG-II	human protein kinase G-2 (membrane-associated orthologue)
PRP4 (PRPF4B)	pre-mRNA processing factor 4B
Pv	*Plasmodium vivax*
*Pv*CLK	*Plasmodium vivax* cyclin-dependent kinase (CDK)-like kinase
*Pv*FIKK	*Plasmodium vivax* phenylalanine (F)-isoleucine (I)-lysine (K)-lysine (K) kinase
*Pv*PI4K	*Plasmodium vivax* phosphatidylinositol 4-kinase
Py	*Plasmodium yoelii*
q	quartet
R-spine	regulatory spine
RGC	receptor guanylate cyclase
s	singlet
SAR	structure-activity relationship
SASBMB-FASBMB	South African Society of Biochemistry and Molecular Biology – Federation of African Societies of Biochemistry and Molecular Biology
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	serine
SI	selectivity index
Sky1p	yeast serine/arginine-rich protein
SR	serine/arginine-rich
Src	Proto-oncogene tyrosine-protein kinase Src
SRPK	serine/arginine protein kinase
STE	sterile yeast kinase

t	triplet
TCP	target candidate profile
TFP	trifluoperazine
*Tg*CDPK1	*Toxoplasma gondii* calcium-dependent protein kinase 1
Thr	threonine
TK	tyrosine kinase
TKL	tyrosine kinase-like
TLC	thin layer chromatography
TMS	tetramethylsilane
TPP	target product profile
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
Tyr	tyrosine
UBC13	E2 ubiquitin-conjugating enzyme 13
WHO	World Health Organization
x g	times gravity
Y/HRD-motif	tyrosine (Y)/histidine (H)-arginine (R)-aspartate (D)-motif

Chapter 1: Introduction

1.1. Background and motivation

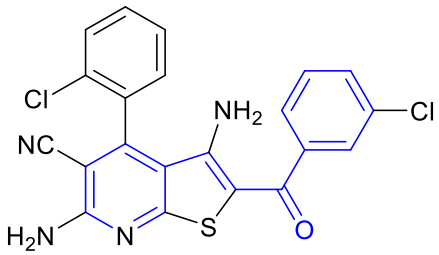
Malaria is a mosquito-borne infectious disease caused by protozoan parasites of the genus *Plasmodium* (Tuteja, 2007:4670). Despite significant progress towards worldwide elimination of malaria, this disease continues to have devastating effects, especially in developing countries. In 2018, an estimated 405 000 malaria-related deaths were reported, of which 94% occurred in sub-Saharan Africa (WHO, 2019a:10). Children under the age of five are particularly vulnerable to infection and sadly accounted for 67% of all malaria-related deaths in 2018 (WHO, 2019a:10).

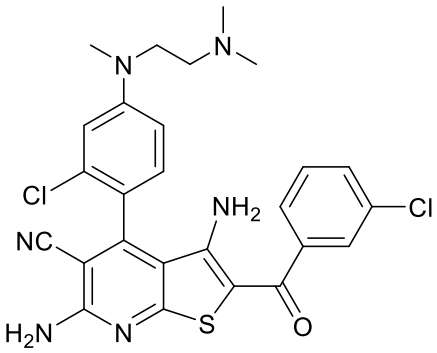
One of the major threats to malaria control and elimination is the parasite's ability to continually develop resistance to available antimalarial drugs. *P. falciparum*, the *Plasmodium* species responsible for the majority of malaria-related deaths, is resistant to all classes of antimalarial drugs, including the current first-line artemisinin-based combination therapy (ACT) (Rout & Mahapatra, 2019:748). Until recently, artemisinin resistance was predominantly confined to Southeast Asia (Menard *et al.*, 2016:2453). However, artemisinin resistance has now also emerged in Rwanda (Uwimana *et al.*, 2020:1606), which can significantly compromise malaria control in sub-Saharan Africa. Therefore, recurring antimalarial drug resistance necessitates the continuous development of new antimalarial drugs with novel modes of action.

The fully sequenced *P. falciparum* genome (Gardner *et al.*, 2002:498) has resulted in the identification of numerous potential targets for next-generation antimalarial drug development (Kumar *et al.*, 2018:10). Plasmodial kinases in particular, are promising targets for next-generation antimalarial drugs due to their involvement in critical cellular processes throughout the life cycle of the parasite (Doerig, 2004:157; Doerig & Meijer, 2007:285; Doerig *et al.*, 2010:605). The *P. falciparum* kinome consists of kinases that cluster within known eukaryotic kinase families, kinases that display homology to multiple eukaryotic kinase families, and kinases that have no mammalian orthologues (Doerig *et al.*, 2008:570). It has been established that 36 of these kinases are likely essential for survival of the erythrocytic stages of *P. falciparum* (Solyakov *et al.*, 2011:3). One such essential kinase is the *P. falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) which clusters within the eukaryotic glycogen synthase kinase (GSK-3) family. *Pf*GSK-3 shares a high degree of sequence similarity with both human GSK-3 isoforms, *Hs*GSK-3α (62.4%) and *Hs*GSK-3β (58.9%) (Droucheau *et al.*, 2004:188). Despite the

highly conserved nature of *Pf*GSK-3, certain key structural differences have been identified (Droucheau *et al.*, 2004:188; Kruggel & Lemcke, 2009a:890; Osolodkin *et al.*, 2011:733), which can be exploited in designing selective *Pf*GSK-3 inhibitors.

To date, drug discovery programs have been limited for this target and all *Pf*GSK-3 selective inhibitors belong to the 4-phenylthieno[2,3-*b*]pyridine class. Fugel *et al*. (2013:271) designed the first series of *Pf*GSK-3 selective inhibitors with the thieno[2,3-*b*]pyridine motif as parent scaffold. One of the most potent compounds of this series (**1**, Figure 1.1) selectively inhibited *Pf*GSK-3 at a submicromolar concentration and demonstrated *in vitro* antiplasmodial activity when tested against the erythrocytic stages of *P. falciparum*. Masch *et al*. (2019:8) further optimised compound **1** by attaching various substituents to the *para*-position of the 4-phenyl ring. Compound **2** (Figure 1.1) was the most promising of the series as it retained *Pf*GSK-3 selectivity, whilst demonstrating improved aqueous solubility and *in vitro* antiplasmodial activity compared to compound **1**.





	1	**2**
***Pf*GSK-3 IC_{50}**	0.48 µM	0.72 µM
***Hs*GSK-3β IC_{50}**	3.3 µM	40.2 µM
***Pf*NF54-Luc EC_{50}**	5.5 µM	1.2 µM

Figure 1.1: Structures and biological data of *Pf*GSK-3 selective inhibitors belonging to the 4-phenylthieno[2,3-*b*]pyridine class. The structural core of these compounds is based on a well-known chalcone scaffold (**1**, highlighted in blue). IC_{50}: half maximal inhibitory concentration; *Pf*NF54-Luc: transgenic *Plasmodium falciparum* NF54 strain expressing the luciferase gene; EC_{50}: half maximal effective concentration.

Molecular docking studies of compound **1** with a *Pf*GSK-3 homology model (Fugel *et al.*, 2013:269; Masch *et al.*, 2019:4) suggested that the nitrogen of the pyridine moiety, the C3-amino group and the

C5-cyano group are not involved in the *Pf*GSK-3 active site interactions, and are therefore not likely to be essential for activity (Figure 1.2).

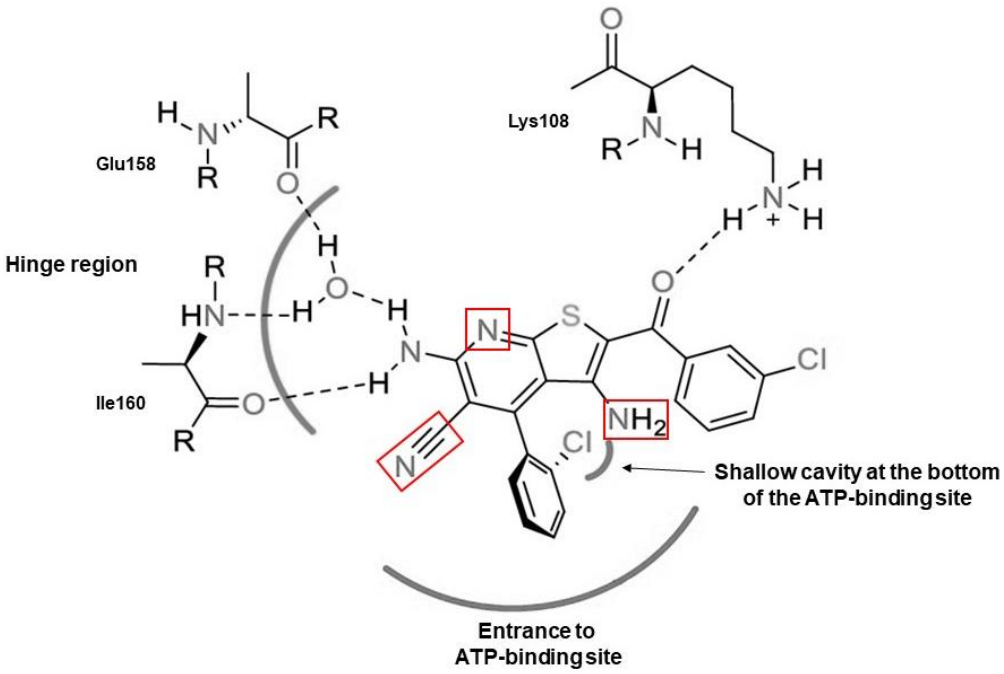


Figure 1.2: Interaction of the 4-phenylthieno[2,3-*b*]pyridine inhibitor with the ATP-binding site of *Pf*GSK-3, as predicted by molecular docking studies. The nitrogen of the pyridine moiety, the C3-amino group and the C5-cyano group (highlighted in red) do not interact with the active site. Figure reproduced from (Masch *et al.*, 2019:4) which is freely available for public use (http://creativecommons.org/licenses/by/4.0/).

Based on these results the present study will use the thieno[2,3-*b*]pyridine core to design new *Pf*GSK-3 inhibitors. This structural core is based on a well-known chalcone scaffold (highlighted in blue, **1**, Figure 1.1), which suggests the relevance of using chalcones in the design of new *Pf*GSK-3 inhibitors. Herein, we explore an alternative benzofuran-based chalcone scaffold (**3**, Figure 1.3), which is somewhat structurally similar to the thieno[2,3-*b*]pyridine motif. A series of benzofuran-based compounds (**5a-v**) with a variety of polar and lipophilic substituents on both ring A (R_1, R_2, R_3) and B (R_1', R_2') were synthesised and evaluated as potential inhibitors of recombinantly expressed and purified *Pf*GSK-3 and *Hs*GSK-3β.

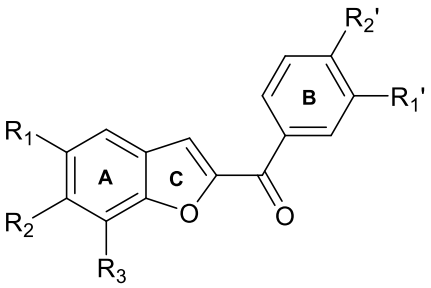


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Figure 1.3: Structure of the benzofuran-based chalcones explored for the design of potent and selective *Pf*GSK-3 inhibitors during the present study. The benzofuran scaffold was substituted with a variety of polar and lipophilic groups on both ring A (R_1, R_2, R_3) and B (R_1', R_2').

1.2. Aim and objectives

This study aimed to gain further insight into the structure-activity relationship of the benzofuran-based chalcone scaffold for the design of potent and selective *Pf*GSK-3 inhibitors.

The specific objectives of this study included:

1. The design and synthesis of a series of benzofuran derivatives.
2. Characterisation of the synthesised benzofurans by means of nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), high-performance liquid chromatography (HPLC) and melting point (mp) analysis.
3. Optimising expression and purification conditions for active recombinant *Pf*GSK-3 and *Hs*GSK-3β.
4. Optimising the ADP-Glo™ Kinase Assay conditions for both enzymes.
5. Evaluating the synthesised compounds as inhibitors of recombinant *Pf*GSK-3 and *Hs*GSK-3β.
6. Evaluating the structure-activity relationships of the benzofuran derivatives essential for *Pf*GSK-3 selectivity.

1.3. Thesis outline

This thesis is presented in five chapters which includes two peer-reviewed articles.

Chapter 1 provides a short introduction and motivation for this study, as well as the aim and specific objectives.

In **Chapter 2** the relevant literature is reviewed. The various aspects of malaria are discussed, including malaria statistics, the parasite life cycle, current antimalarial chemotherapy, and ways to address the problem of drug-resistant malaria. Other potential drug targets for malaria are discussed, with the main focus on the protein kinase group. Protein kinase classification, structure, types of inhibitors, and the human kinome vs the plasmodial kinome, are discussed. Lastly, human glycogen synthase kinase-3 (*Hs*GSK-3) and its plasmodial orthologue (*Pf*GSK-3) are discussed.

Chapter 3 consists of a peer-reviewed article (Paper I), **"An update on development of small-molecule plasmodial kinase inhibitors"**, published in *Molecules*. It gives an overview of plasmodial kinases that cluster within established eukaryotic protein kinase groups as well as orphan kinases that have no mammalian orthologues. It summarises the different types of small-molecule inhibitors developed for each group and highlights those kinases that are yet to be targeted by drug discovery programs.

Chapter 4 consists of a peer-reviewed article (Paper II), **"Exploration of benzofuran-based compounds as potent and selective *Plasmodium falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) inhibitors"**, published in *Bioorganic Chemistry*. Herein, the experimental findings of this study are presented, including methods for expressing and purifying active recombinant *Hs*GSK-3β and *Pf*GSK-3, and the structure-activity relationships of benzofuran derivatives as selective inhibitors of *Pf*GSK-3.

Chapter 5 includes a summary of the findings of this study, contributions made to the field and future recommendations.

The **Annexures** at the end of the thesis include the instructions to authors for *Molecules* (**A**) and *Bioorganic Chemistry* (**B**), the supplementary materials of Paper II (**C**), the poster presented at the SASBMB-FASBMB 2018 Conference (**D**), and permission to reproduce figures in Chapter 2 and Paper I (**E**).

1.4. Ethics

Ethical approval for the use of genetically modified cell lines was obtained from the Health Research Ethics Committee (HREC) of the North-West University (NWU-00368-16-A1).

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Chapter 2: Literature review

2.1. Malaria and the challenges of multidrug-resistance

2.1.1. Introduction

In the last decade, substantial progress has been made in the fight against malaria. However, in recent years the progress towards reducing the number of new malaria cases has slowed. Between 2014 and 2018 the global malaria incidence rate remained at ±57 cases per 1000 people at risk (WHO, 2019a:7). In 2018, an estimated 228 million malaria cases and 405 000 malaria-related deaths were reported globally (WHO, 2019a:4). Developing countries, including countries in sub-Saharan Africa, are more severely affected by malaria due to poverty, limited access to adequate health care and preventive measures, and recurring resistance to insecticides and antimalarial drugs (Dhiman, 2019:5). Sub-Saharan Africa accounted for 93% of all malaria cases and 94% of deaths reported in 2018 (WHO, 2019a:4). Pregnant women and children under the age of five are particularly vulnerable to infection and developing severe malaria, as pregnancy reduces immunity and young children have not yet acquired partial immunity against the disease (WHO, 2019a:14). The World Health Organization (WHO) reported an estimated 11 million pregnant women in sub-Saharan Africa were infected with malaria in 2018, and as a result 872 000 infants were born with a low birth weight (WHO, 2019a:18). Only about 31% of pregnant women received the three or more recommended doses of preventive therapy (WHO, 2019a:22). Another major concern is that nearly 40% of children in sub-Saharan Africa presenting with fever did not receive any medical attention (WHO, 2019a:22). As a result, children under the age of five accounted for 67% of all malaria-related deaths (WHO, 2019a:10).

The ongoing coronavirus (COVID-19) pandemic could also have a significant impact on the progress made towards malaria control and eradication (WHO, 2020:1). Patients presenting with malaria symptoms or requiring treatment for malaria are less likely to visit health care facilities out of fear of contracting the virus (Nghochuzie *et al.*, 2020:3). Lockdown periods have significantly disrupted the supply of antimalarial drugs, rapid diagnostic tests and other malaria interventions (Nghochuzie *et al.*, 2020:1). Antimalarial drugs (e.g. artemisinin, chloroquine and hydroxychloroquine) have been repurposed for COVID-19 treatment, despite a lack of scientific evidence and approval (Nghochuzie *et al.*, 2020:2). This has resulted in a shortage of these drugs and, in the long-run, an increased risk of drug resistance due to improper use of antimalarial monotherapies (Nghochuzie *et al.*, 2020:2).

To effectively control and eliminate malaria in the future, an integrated strategy is needed which includes addressing the inadequacies of the health care systems, effectively monitoring insecticide and drug

resistance in malaria-endemic regions, maintaining malaria control activities during pandemics (e.g. COVID-19), and continually developing new antimalarial drugs and vaccines.

2.1.2. Overview of malaria

Malaria is caused by protozoan parasites of the genus *Plasmodium* that uses the female *Anopheles* mosquito as vector for transmission (Tuteja, 2007:4670). In humans, malaria is caused by five *Plasmodium* species namely, *P. falciparum*, *P. vivax*, *P. ovale, P. malariae* and *P. knowlesi* (WHO, 2015a:2). Among these *Plasmodium* species, *P. falciparum* and *P. vivax* infections are the most common. *P. falciparum* is the most prevalent in Africa and responsible for almost all malaria-related deaths (WHO, 2015a:3), while *P. vivax* has a wider geographical distribution as it can survive and develop in harsher conditions than the other species (WHO, 2015a:3). *P. vivax* can also remain dormant in the liver of the human host and can reactivate when conditions are favourable, causing a relapse of symptoms (Tuteja, 2007:4671). Infection with *P. vivax* is relatively low in African countries due to the absence of the Duffy gene in many African populations (WHO, 2015a:3). Expression of this gene produces a protein that is necessary for the *P. vivax* parasite to invade the erythrocytes of the human host. The least common species are *P. ovale,* which is restricted to West Africa, *P. malariae*, which can be found worldwide but in low frequencies (Tuteja, 2007:4671), and *P. knowlesi*, which is predominantly found in Malaysia (Zaw & Lin, 2019:681).

2.1.3. Life Cycle

The life cycle of the parasite (Figure 2.1) can be divided into the asexual stage (pre-erythrocytic and erythrocytic phases), which takes place within the human host, and the sexual stage, which takes place within the mosquito vector (Tuteja, 2007:2671).

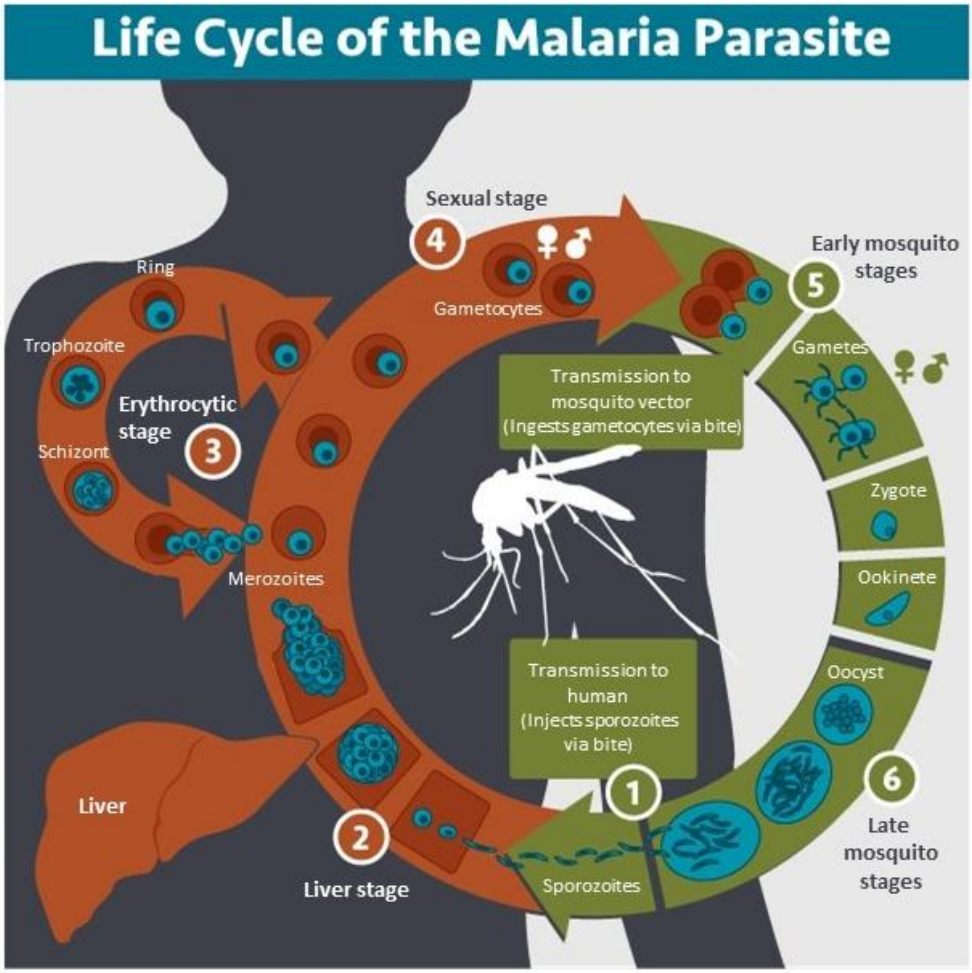


Figure 2.1: The life cycle of the malaria parasite. Image is freely available for public use (https://creativecommons.org/licenses/by/4.0/legalcode) from the National Institutes of Health (NIH) website (https://www.niaid.nih.gov/diseases-conditions/malaria-parasite).

2.1.3.1. Pre-erythrocytic or liver stage

This stage begins when an infected female *Anopheles* mosquito injects sporozoites into the human host during a blood meal. The sporozoites slowly migrate from the site of injection to the liver and penetrate the hepatocytes (Prudencio *et al.*, 2006:850; Tuteja, 2007:4671). The sporozoites can either immediately trigger asexual replication or remain dormant (*P. vivax* and *P. ovale* hypnozoites) for long periods within the hepatocytes (Tuteja, 2007:4671). During the asexual replication cycle each sporozoite develops into a schizont that contains tens of thousands of merozoites (Prudencio *et al.*, 2006:853). The merozoites are then released into the bloodstream with the help of hepatocyte-derived merosomes (Prudencio *et al.*, 2006:854). The merosomes act as shuttles that carry the merozoites into the bloodstream while providing protection against the host's immune response (Prudencio *et al.*, 2006:855; Tuteja, 2007:4671). The pre-erythrocytic stage is known as the "silent" stage as no symptoms are experienced by the host (Prudencio *et al.*, 2006:849).

2.1.3.2. Erythrocytic stage

After entering the blood circulation, merozoites invade the erythrocytes through a complex process. This process is facilitated by molecular interactions between ligands on the merozoite surface and receptors on the membrane of the erythrocytes (Cowman *et al.*, 2012:963). *P. vivax* merozoites generally only invade erythrocytes that display the Duffy-binding protein on its surface (Cowman *et al.*, 2012:961), while *P. falciparum* merozoites recognize several different receptor families on the erythrocyte surface and alternate their invasion pathways (Cowman *et al.*, 2012:964). The invasion process happens rapidly in order for the parasite to evade the host's immune response.

Within the erythrocyte, merozoites undergo asexual replication to form immature ring stage trophozoites (Tuteja, 2007:4672). Trophozoites mature into schizonts which contain merozoites for further invasion of unaffected erythrocytes (Tuteja, 2007:4672). The erythrocyte ruptures when the merozoites are released and this stimulates the production of inflammatory cytokines which are responsible for the clinical symptoms of malaria (Tuteja, 2007:4673). This asexual replication cycle occurs repeatedly, every 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, and every 72 hours for *P. malariae* (Tuteja, 2007:4673). After a variable number of asexual replication cycles, some merozoites differentiate to produce male and female gametocytes, in a process known as gametocytogenesis (Josling & Llinas, 2015:573). The formation of gametocytes is essential for transmission to the mosquito vector (Tuteja, 2007:4673).

2.1.3.3. Sexual stage

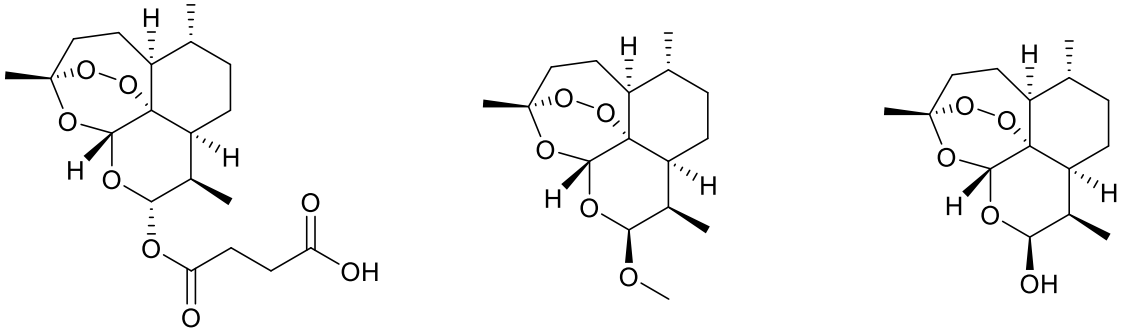
A female *Anopheles* mosquito may ingest the gametocytes during a blood meal from an infected individual. Within the gut of the mosquito, the erythrocytes rupture and release the gametocytes (Tuteja, 2007:4673). The gametocytes develop into sexually mature gametes; triggered by the change in temperature and pH, as well as the presence of xanthurenic acid within the mosquito (Josling & Llinas, 2015:573). Male and female gametes fuse to form zygotes which further develop into ookinetes (Josling & Llinas, 2015:574; Tuteja, 2007:4673). The ookinetes burrow through the midgut wall and form oocysts (Josling & Llinas, 2015:574). Further division of each oocyst produces thousands of sporozoites which are released into the body cavity of the mosquito when the oocysts rupture (Josling & Llinas, 2015:575; Tuteja, 2007:4673). The sporozoites can then migrate to the salivary glands, ready to infect another human host (Josling & Llinas, 2015:575; Tuteja, 2007:4673).

2.1.4. Malaria chemotherapy

Currently, malaria control and prevention relies heavily on the limited number of available antimalarial drugs. The existing antimalarial drug arsenal is highly stage-specific, with the majority of these drugs targeting the symptomatic erythrocytic stages and only a few which are active against the liver and sexual stage parasites (Raphemot *et al.*, 2016:2013; Rout & Mahapatra, 2019:739; Yahiya *et al.*, 2019:1). Antimalarial drugs can be grouped into several different chemical classes (Okombo & Chibale, 2018:438), as discussed below.

2.1.4.1. Artemisinins

Artemisinin derivatives (Figure 2.2), e.g. artesunate (**3**), artemether (**4**) and dihydroatemisinin (**5**), are potent antimalarial drugs; active against the erythrocytic and sexual stages of all *Plasmodium* species affecting humans (Achieng *et al.*, 2017:2117). The endoperoxide bridge is said to be responsible for the antiplasmodial activity of artemisinins, however, the specific mechanism(s) of action are still unclear (Tse *et al.*, 2019:3). Due to their short half-life, artemisinins are used in combination with partner drugs from different chemical classes. This combination is known as artemisinin-based combination therapy (ACT) (WHO, 2015b:34). The rationale for ACT is that artemisinin rapidly reduces parasitemia, while the longer half-life partner drug clears the remaining parasites and prevents the development of resistance to artemisinin (WHO, 2015b:34). ACTs are currently recommended by WHO as first-line treatment for uncomplicated *P. falciparum* malaria as well as chloroquine-resistant *P. vivax* malaria in adults and children (WHO, 2015b:34; WHO, 2019b:23). Severe malaria is treated with administration of parenteral artesunate for a minimum of 24 hours, followed by a full ACT course when the patient can tolerate oral medication (WHO, 2015b:72).

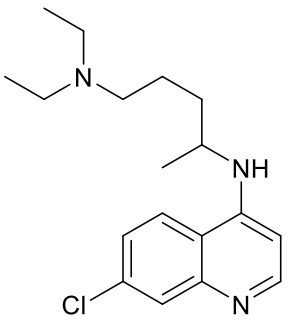


3 **4** **5**

Figure 2.2: Chemical structures of artemisinin derivatives, artesunate (**3**), artemether (**4**) and dihydroartemisinin (**5**).

2.1.4.2. Quinolines

The quinoline class (Figure 2.3) includes 4-aminoquinolines, 8-aminoquinolines and aryl-amino alcohols. 4-Aminoquinolines, e.g. chloroquine (**6**), piperaquine (**7**), amodiaquine (**8**) and pyronaridine (**9**), are active against the erythrocytic stage and are said to interfere with detoxification of haem; a toxic by-product of haemoglobin digestion (Achieng *et al.*, 2017:2117). Chloroquine is used to treat uncomplicated *P. vivax*, *P. ovale* and *P. knowlesi* infections, in areas where parasites are still susceptible to this drug (WHO, 2015b:65). The 8-aminoquinolines, e.g. primaquine (**10**) and tafenoquine (**11**), are the only drugs that target the hypnozoites of *P. vivax* and *P. ovale* as well as the gametocyte stage of *P. falciparum* (Rout & Mahapatra, 2019:744). Therefore, these drugs are primarily used to prevent *P. vivax* and *P. ovale* relapses and to reduce *P. falciparum* transmission (Cowell & Winzeler, 2019a:318). However, treatment with 8-aminoquinolines may result in acute haemolytic anaemia in patients with a glucose-6-phosphate dehydrogenase deficiency (Rout & Mahapatra, 2019:744). The mechanism of action of the 8-aminoquinolines is still unknown (Cowell & Winzeler, 2019a:318; Rout & Mahapatra, 2019:744).



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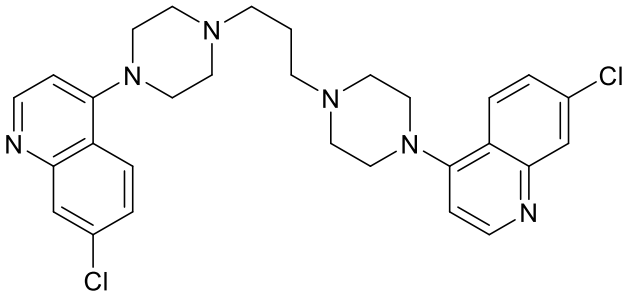

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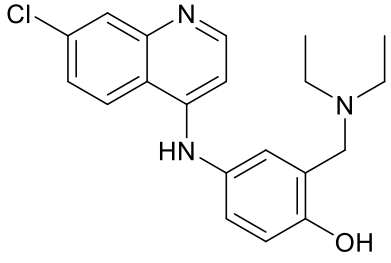

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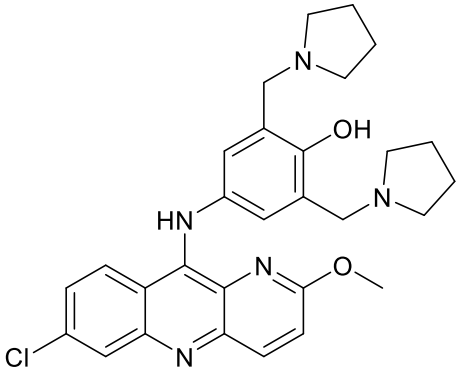

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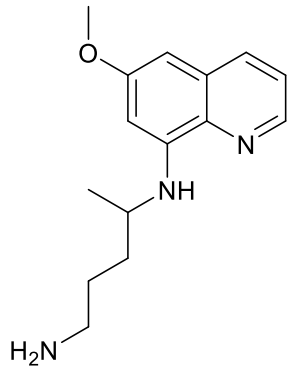
Figure 2.3 *(continued)*



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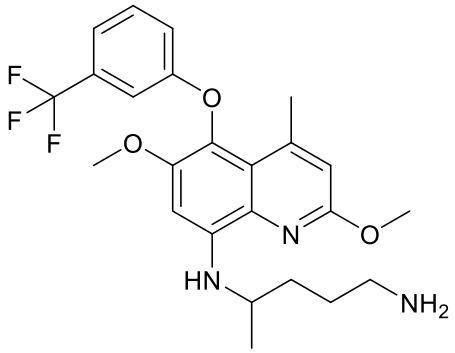

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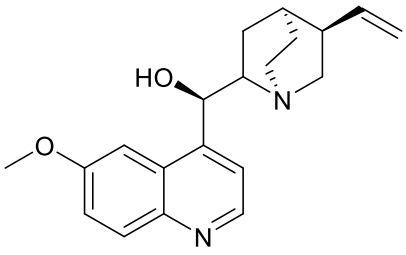
Figure 2.3: Chemical structures of 4-aminoquinolines; chloroquine (**6**), piperaquine (**7**), amodiaquine (**8**) and pyronaridine (**9**); and 8-aminoquinolines, primaquine (**10**) and tafenoquine (**11**).

Members of the aryl-amino alcohol class (Figure 2.4), e.g. quinine (**12**), mefloquine (**13**) and lumefantrine (**14**), are active against erythrocytic stage parasites (Rout & Mahapatra, 2019:742). Their mechanism of action is still uncertain, although it is suggested that they too interfere with haem detoxification (Schlitzer, 2008:151). Despite serious side effects caused by quinine, it is often used in combination with antibiotics (doxycycline, clindamycin and tetracycline) as treatment for uncomplicated and severe malaria when first-line treatment fails or is unavailable (Schlitzer, 2008:151; WHO, 2015b:78).



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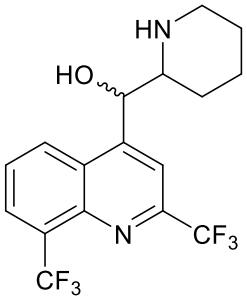

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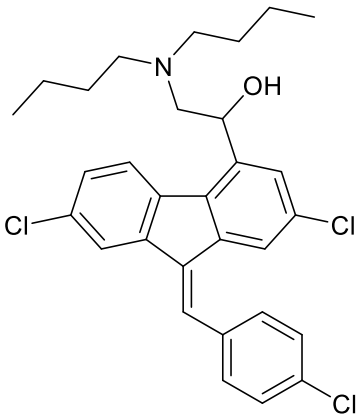

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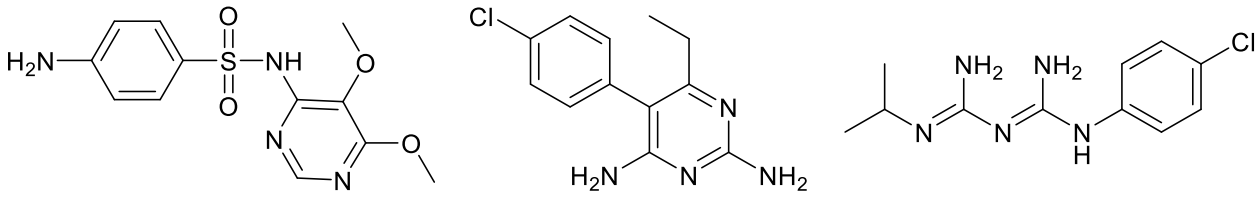
Figure 2.4: Chemical structures of the aryl-amino alcohols, quinine (**12**), mefloquine (**13**) and lumefantrine (**14**).

2.1.4.3. Antifolates

The antifolate class (Figure 2.5) consists of type I, e.g. sulfadoxine (**15**), and type II antifolates, e.g. pyrimethamine (**16**) and proguanil (**17**). Antifolates inhibit key enzymes in the folate biosynthetic pathway,

namely dihydropteroate synthase (type I) and dihydrofolate reductase (type II) (Rout & Mahapatra, 2019:744). Sulfadoxine and pyrimethamine are administered concurrently to provide intermittent preventive treatment during pregnancy (IPTp) and for infants (IPTi) in malaria-endemic areas (WHO, 2019a:14). In areas with high seasonal malaria transmission rates, sulfadoxine-pyrimethamine is combined with amodiaquine to provide seasonal malaria chemoprevention in young children (WHO, 2019a:14).

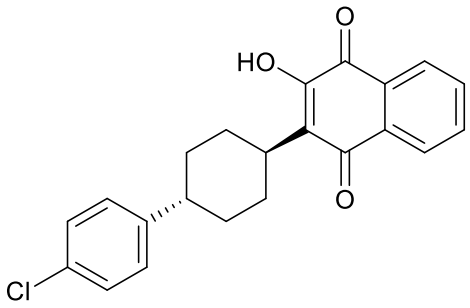


15 **16** **17**

Figure 2.5: Chemical structures of the antifolates, sulfadoxine (**15**), pyrimethamine (**16**) and proguanil (**17**).

2.1.4.4. Hydroxynaphthoquinones

From the hydroxynaphthoquinone class, atovaquone (**18**, Figure 2.6) is the only antimalarial drug in clinical use. This drug inhibits the parasitic mitochondrial electron transport chain at complex *bc1* (Tse *et al.*, 2019:4). The use of atovaquone in combination with proguanil is mainly indicated for malaria prophylaxis (WHO, 2015b:100).



18

Figure 2.6: Chemical structure of atovaquone.

2.1.4.5. Antibiotics

Antibiotics, such as doxycycline (**19**, Figure 2.7), are slow-acting antimalarial drugs active against the erythrocytic stage parasites (Gaillard *et al.*, 2015:3). Antibiotics are mainly indicated for prophylaxis and treatment of uncomplicated or severe malaria (in combination with artesunate or quinine) (WHO,

2015b:246; WHO, 2019b:24). It is suggested that doxycycline inhibits protein synthesis in the apicoplast of the malaria parasite (Gaillard *et al.*, 2015:2).

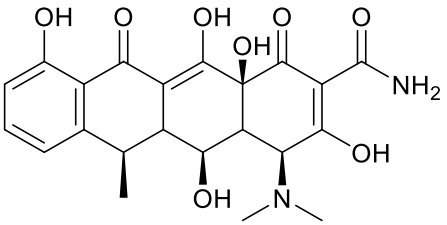


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Figure 2.7: Chemical structure of doxycycline.

2.1.5. Drug-resistant malaria and strategies to address this problem

One of the major challenges in the fight against malaria is the recurring emergence of *Plasmodium* resistance against available antimalarial drugs. Antimalarial drug resistance is defined as the ability of a *Plasmodium* strain to survive and/or multiply despite proper administration and absorption of an antimalarial drug at the recommended dose (WHO, 2015b:113).

Drug resistance is mainly seen in *P. falciparum*, while *P. vivax* is slower to develop resistance (Noisang *et al.*, 2019:1). *P. falciparum* resistance to chloroquine was first reported in the late 1950s along the Thai-Cambodian border, and have since spread to most malaria-endemic regions of the world (Payne, 1987:241). Resistant *P. falciparum* strains now exist for all classes of antimalarial drugs including artemisinin, with some strains demonstrating resistance to multiple drugs (multidrug-resistant malaria) (Cowell & Winzeler, 2019a:314; Rout & Mahapatra, 2019:748). Artemisinin resistance is prevalent in Southeast Asia and has also recently emerged in Rwanda (Uwimana *et al.*, 2020:1606). *P. vivax* drug resistance was unknown until the late 1980s when the first chloroquine-resistant cases were reported by travellers returning from Papua New Guinea (Rieckmann *et al.*, 1989:1184). Since then chloroquine-resistant *P. vivax* strains have emerged in many South American and Asian malarial endemic regions (Price *et al.*, 2014:989).

Various strategies have been proposed to address the issue of antimalarial drug resistance. The first approach is to optimise existing antimalarial drug therapy by rotational use of existing drug combinations, developing new drug combinations, or changing dosing regimens (Grimberg & Mehlotra, 2011:686). A second approach is to develop/optimise drugs that act as resistance reversers (chemosensitisers) for existing antimalarial drugs (Mishra *et al.*, 2017:1314). Chloroquine-resistance for example, is caused by an increased efflux of chloroquine from the digestive vacuole due to mutation of the *P. falciparum* chloroquine-resistance membrane transporter (*Pf*CRT) (Deane *et al.*, 2014:576). This decreases the chloroquine concentration at its

intended site of action (digestive vacuole), but it does not alter the vulnerability of chloroquine's target (Deane *et al.*, 2014:576). Several drugs have demonstrated potential to inhibit this efflux mechanism in chloroquine-resistant parasites, however, these drugs are yet to be deployed in a clinical setting (Deane *et al.*, 2014:580; Mishra *et al.*, 2017:1314; Obaldia *et al.*, 2018:5; Ye *et al.*, 2013:3). A third approach is to develop analogues of existing antimalarial drugs to improve its antiplasmodial activity and pharmacokinetic properties (Mishra *et al.*, 2017:1305). However, this strategy could result in cross-resistance which occurs between antimalarial drugs from the same chemical class or with similar mechanisms of action (Rout & Mahapatra, 2019:748). A fourth approach repurposes existing drugs developed for other diseases, for antimalarial therapy (Grimberg & Mehlotra, 2011:688; Mishra *et al.*, 2017:1311). This approach greatly reduces the time and cost of drug development, as the drug has already been clinically approved, and its safety and pharmacokinetic profiles have been established. For example, atovaquone is used to prevent and treat *Pneumocystis carinii* pneumonia, but when combined with proguanil it is highly effective for prophylaxis and treatment of uncomplicated malaria (Baggish & Hill, 2002:1163). Similarly, antiretroviral drugs (e.g. lopinavir) have also demonstrated antimalarial activity (Achan *et al.*, 2012:2116; Nsanzabana & Rosenthal, 2011:5076; Parikh *et al.*, 2005:2984).

The above mentioned strategies are mostly short-term options in order to minimise or delay the development of antimalarial drug resistance. The optimal approach to combat drug resistance would be to explore novel plasmodial targets and chemical scaffolds for next-generation antimalarial drug and vaccine development (Mishra *et al.*, 2017:1312). Novel chemical scaffolds can be identified by means of virtual, phenotypic- and target-based high-throughput screening strategies (Grimberg & Mehlotra, 2011:692). Rational drug design relies on a target-based approach where compound libraries are screened against a specific plasmodial target that is known to be essential for parasite survival (Hovlid & Winzeler, 2016:698). However, more studies opt for a phenotypic-based approach where prior knowledge on the plasmodial target is not required (Hovlid & Winzeler, 2016:698). Phenotypic-based screening identifies chemical scaffolds that have the desired *in vitro* antimalarial activity against the whole cell, and also readily eliminates compounds that do not have the required permeability (Cowell & Winzeler, 2019b:2). Virtual screening is a computational approach that can select compounds with desirable pharmacokinetic properties and screen them against the three-dimensional structure of a specific plasmodial target (Neves *et al.*, 2018:2). This is a cost-effective approach that speeds up the drug discovery process and limits the number of compounds that have to be evaluated *in vitro* (Neves *et al.*, 2018:2).

Overall, recurring antimalarial drug resistance have shown us that continued research on new antimalarial drug development is essential towards effective control and eventual eradication of malaria.

2.1.6. Potential drug targets for malaria

The fully sequenced genomes of several *Plasmodium* species, including *P. falciparum* and *P. vivax* (accessible from the *Plasmodium* genomic database, PlasmoDB) (Aurrecoechea *et al.*, 2009:D539), have resulted in the identification of numerous potential drug targets. These targets include various transport pathways as well as basic metabolic and parasite-specific processes (Figure 2.8), and was discussed in detail in a recent review (Kumar *et al.*, 2018:10).

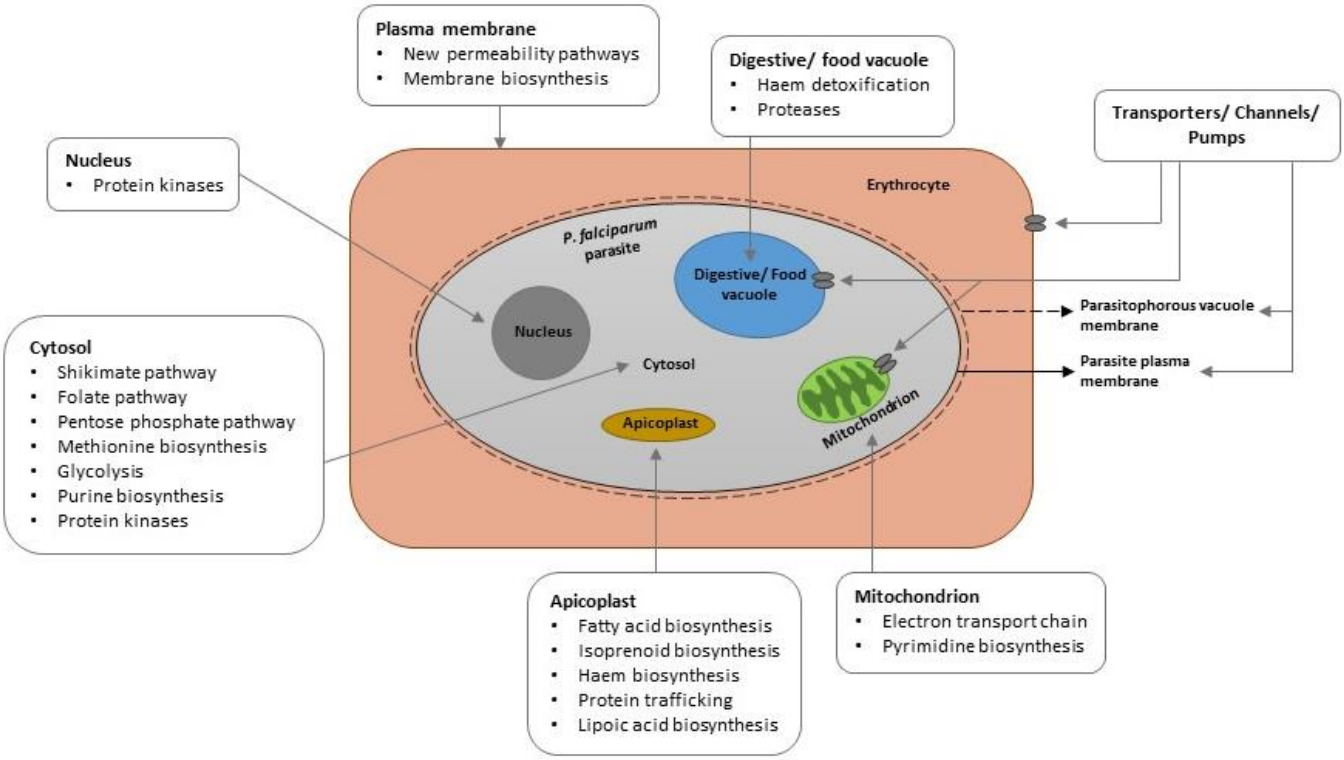


Figure 2.8: Potential targets for antimalarial drug development. Figure created with information from (Kumar *et al.*, 2018; Meier *et al.*, 2018; Tibon *et al.*, 2020).

Protein transporters, channels and pumps provide the required nutrients, eliminates waste/ excess nutrients, and mediate osmoregulation for intrahepatocytic and intraerythrocytic parasites (Meier *et al.*, 2018:3; Vaughan & Kappe, 2017:8). The parasite also significantly alters the permeability of hepatocytes and erythrocytes by inducing new permeability pathways, integrating plasmodial proteins into the host cell membrane, and upregulating existing host pathways (Meier *et al.*, 2018:3; Posfai *et al.*, 2018:4). These transporters are of interest as potential drug targets and antimalarial drug delivery routes. Targeting the transporters located at the host cell membrane could be beneficial as it prevents direct contact between the drug and the parasite, which limits the parasite's options when it comes to developing drug resistance (Meier *et al.*, 2018:17). However, this strategy would require a better understanding of the proteins involved, how it would be tolerated by the host, as well as the susceptibility and adaptability of the parasites (Meier *et al.*,

2018:4). Targeting the parasitic organelle membrane transporters is a more challenging task as these drugs need to cross several membranes to reach its intended target (Meier *et al.*, 2018:9). The parasite can also interfere with the drug action by preventing drug uptake, chemically modifying the drug, or by transporting the drug away from its intended site of action (Meier *et al.*, 2018:9).

Parasite-specific processes (e.g. invasion and egress) and metabolic pathways (e.g. shikimate pathway) that are absent from the human host are considered ideal targets for antimalarial drug development (Kumar *et al.*, 2018:12). Targeting these processes/pathways limits the risk of unwanted side-effects caused by off-target activity. However, plasmodial pathways that are conserved in the human host should not be discarded when it comes to drug development. The proteins involved in these pathways often demonstrate significant structural and/or functional differences that can be exploited for parasite-specific drug development (Kumar *et al.*, 2018:16). The major challenge with these novel targets is to identify those that are essential to parasite survival. As drug development is a costly and time consuming process, it is critical to validate a potential target prior to inhibitor development (Batista *et al.*, 2020:189; Smith, 2003:341). Although significant progress has been made in the last decade, the exact biological functions, interactions and compensatory mechanisms are still unclear for numerous plasmodial targets (Cabrera *et al.*, 2018:8069; Sexton *et al.*, 2019:1269).

Proteases and kinases are two of the major enzyme groups that are conserved in the human host, but are also involved in numerous essential pathways throughout the parasitic life cycle. Plasmodial proteases play a key role in erythrocytic invasion and egress, as well as haemoglobin degradation in the parasitic food vacuole (Deu, 2017:2607; Kumar *et al.*, 2018:11). However, only a third of the approximately 170 predicted proteases have been studied to date, and very few have been validated as drug targets (Deu, 2017:2606). Genetic validation studies also demonstrated a high degree of protease redundancy, especially those that function within the haemoglobin degradation pathway (Deu, 2017:2606). Plasmodial kinases are involved in various signalling pathways that regulate essential cellular processes (Cabrera *et al.*, 2018:8061). Although numerous plasmodial kinases still need to be characterised, a number of kinases have been validated and successfully targeted by medicinal chemistry campaigns (Cabrera *et al.*, 2018:8063). However, inhibitor development for some essential plasmodial kinases have been limited or completely absent (Cabrera *et al.*, 2018:8068). Thus for the purposes of this study the plasmodial kinases, specifically the protein kinases, were chosen as the target group.

2.2. Protein kinases

2.2.1. Introduction

Kinases are one of the largest and most functionally diverse group of enzymes. Through phosphorylation, kinases can direct the activity, cellular location and function of various substrates (proteins, lipids, nucleotides, etc.), which are actively involved in critical cellular and extracellular processes (Fabbro *et al.*, 2015:2676; Manning *et al.*, 2002:1912; Martin *et al.*, 2010:1). Protein kinases are the largest group of kinases found in eukaryotes and phosphorylate either tyrosine residues (tyrosine-specific protein kinases), serine-threonine residues (serine-threonine-specific protein kinases), or both tyrosine and serine-threonine residues (dual-specificity protein kinases) (Fabbro *et al.*, 2015:2676).

The human kinome contains 518 protein kinases, 478 of which are classical/typical protein kinases that share the eukaryotic protein kinase catalytic domain, and 40 of which are atypical protein kinases that exhibit kinase activity but their catalytic domains differ from typical protein kinases (Manning *et al.*, 2002:1913). Protein kinases were first classified into different groups, families and subfamilies, primarily based on the sequence similarity of their catalytic domains (Hanks & Hunter, 1995:595). Since then this classification has been extended by several researchers, to also take into consideration the overall sequence and structural similarity outside of the catalytic domain, their biological functions and modes of regulation (Manning *et al.*, 2002:1914; Martin *et al.*, 2010:8; Miranda-Saavedra & Barton, 2007:910). Based on these classifications, typical protein kinases have been divided into nine main groups (Roskoski, 2015:4):

1. AGC group, which includes the cAMP-dependent protein kinase or protein kinase A (PKA), the cGMP-dependent protein kinase or protein kinase G (PKG), and the protein kinase C (PKC) families.
2. CMGC group, which includes the cyclin-dependent kinase (CDK), the mitogen-activated protein kinase (MAPK), the glycogen synthase kinase (GSK), and CDK-like (CLK) kinase families.
3. CAMK group, consisting of calcium/calmodulin-dependent kinases.
4. CK1 group, consisting of casein kinase 1 isoforms (CK1α, β, γ1, γ2, γ3, δ and ε).
5. Tyrosine kinase (TK) group, which includes receptor and non-receptor tyrosine kinase families.
6. Tyrosine kinase-like (TKL) group, a diverse group of mostly serine-threonine kinases with sequence similarity to the TK group.
7. RGC group, consisting of receptor guanylate cyclases.
8. STE group, which consists of three kinase families (MAP4K, MAP3K and MAP2K) that are related to sterile yeast proteins (STE20, STE11 and STE7, respectively).
9. Other, a diverse group of kinases that could not be assigned to one of the eight other kinase groups.

In addition to typical and atypical protein kinases, the eukaryotic kinome also contains pseudokinases (Manning *et al.*, 2002:1916). Pseudokinases lack one or more of the catalytic domain motifs that are essential

for catalysis, and are therefore either inactive or only weakly active (Byrne *et al.*, 2017:245). Despite catalytic inactivity, some pseudokinases can still bind and hydrolyse ATP (Byrne *et al.*, 2017:245). The non-catalytic functions of pseudokinases are still poorly understood, but they appear to regulate the activity of functional kinases and act as scaffolds during the assembly of multiprotein complexes (Boudeau *et al.*, 2006:448).

2.2.2. Protein kinase structure

The protein kinase domain is extremely well conserved within the eukaryotic protein kinase family (Hanks & Hunter, 1995:576; Huse & Kuriyan, 2002:275). It comprises a small N-terminal lobe and a larger C-terminal lobe. The N-terminal lobe is connected to the C-terminal lobe through a flexible hinge region. The N-terminal lobe consists mainly of β-sheets (β1-β5) and a single α-helix (αC-helix), and is responsible for binding and orientating the magnesium-ATP complex. The C-terminal lobe, on the other hand, consists mainly of α-helices and is responsible for binding the peptide substrate and initiating the phosphate transfer to the substrate. Both lobes contain key secondary structural elements (Figure 2.9) that are important for catalysis and regulation of kinase activity. (Huse & Kuriyan, 2002:275; Roskoski, 2015:6)

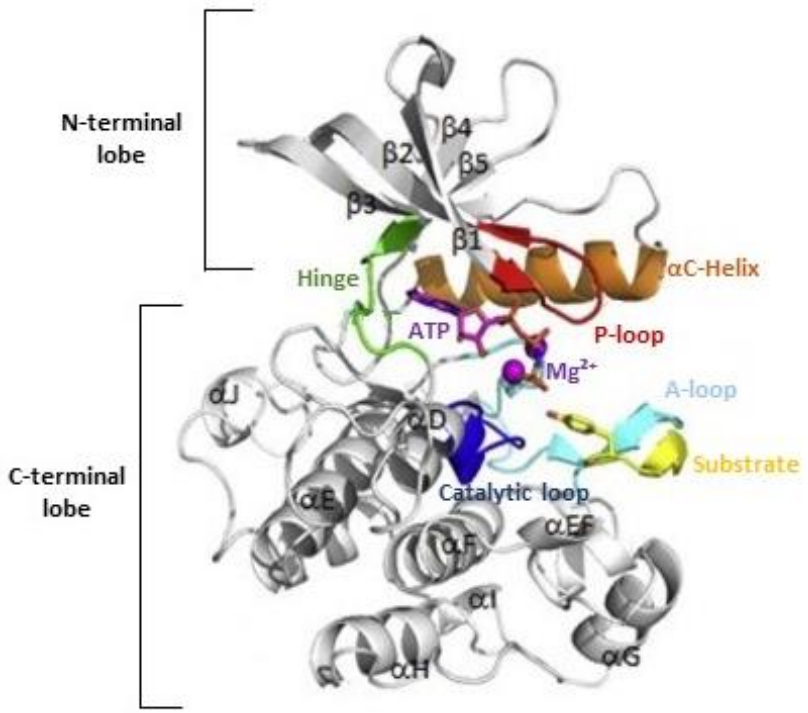


Figure 2.9: Protein kinase structure indicating the different secondary elements. Figure reproduced from (Fabbro *et al.*, 2015:2682) with permission from John Wiley and Sons.

The ATP-binding site is located in a cleft between the two lobes. The adenosyl moiety of ATP binds to the hydrophobic hinge region through hydrogen bonding, while the ribose moiety binds within the sugar/ribose pocket (Bayliss *et al.*, 2012:6). The P-loop, a flexible glycine-rich loop, folds over the ATP molecule and positions the gamma-phosphate for catalysis (Fabbro *et al.*, 2015:2681). The orientation of the αC-helix is crucial for catalysis and kinase activity. During activation of a protein kinase, the αC-helix adopts the 'C-helix-in' conformation, which allows a conserved glutamate residue of the αC-helix to interact with the lysine residue of the AXK-motif (Alanine-Any amino acid-Lysine, located on the β3-strand of the N-lobe) (Fabbro *et al.*, 2015:2683). This interaction forms a lysine-glutamate salt bridge, which stabilizes the interaction with the phosphate groups of ATP and correctly positions the ATP molecule for catalysis (Fabbro *et al.*, 2015:2683; Roskoski, 2015:6). An inactive kinase state is caused by rotation of the αC-helix to the 'C-helix-out' conformation, which prevents salt bridge formation (Fabbro *et al.*, 2015:2681).

The activation segment, located in the C-lobe, is also involved in regulating protein kinase activity. The activation segment includes several important structural elements such as the magnesium binding loop, the activation loop (A-loop), and the P+1 loop (Nolen *et al.*, 2004:661). The magnesium binding loop is located on the N-terminal side of the activation segment and contains the conserved DFG-motif (Aspartate-Phenylalanine-Glycine) (Nolen *et al.*, 2004:662). The position of the DFG-motif is of great importance for catalysis. Upon activation of the kinase, the DFG-motif moves from the 'DFG-out' conformation to the 'DFG-in' conformation (Fabbro *et al.*, 2015:2683). In this conformation, the aspartate side chain faces the active site, where it coordinates a magnesium ion to activate the γ-phosphate of ATP for transfer to the substrate (Fabbro *et al.*, 2015:2683). For activation, most protein kinases require phosphorylation of specific residues located on the A-loop of the activation segment (Roskoski, 2015:7). In its unphosphorylated state, the A-loop collapses and blocks the entrance to the active site (Huse & Kuriyan, 2002:276). When phosphorylated, the A-loop undergoes conformational changes to allow ATP and the peptide substrate access to the active site (Huse & Kuriyan, 2002:276). The C-terminal side of the activation segment consists of the P+1 loop and the APE-motif (Alanine-Proline-Glutamic acid), which are both involved in substrate binding. The P+1 loop recognizes and interacts with the residue that is adjacent to the phosphorylated residue of the substrate (Bayliss *et al.*, 2012:7). The glutamic acid residue of the APE-motif, together with the short αEF-helix next to the activation segment, forms the substrate-binding site (Fabbro *et al.*, 2015:2683). The catalytic loop contains another motif that is essential for catalysis, namely the Y/HRD-motif (Tyrosine/ Histidine-Arginine-Aspartate). The aspartate residue of this motif is highly conserved in all eukaryotic protein kinases (Fabbro *et al.*, 2015:2681). It acts as a base catalyst and accepts the hydrogen that is removed from the peptide substrate's hydroxyl group during phosphorylation (Bayliss *et al.*, 2012:7). Unlike most of the other elements, the conformation of the catalytic loop stays the same regardless of the activation state (active or inactive) of the protein kinase (Fabbro *et al.*, 2015:2683).

Stabilization of the protein kinase structure in its active form is achieved by "spine" formation. Spine formation involves the interaction of various hydrophobic residues located in both lobes of the protein kinase. Two spines have been identified in active protein kinases, namely the regulatory spine (R-spine) and the catalytic spine (C-spine) (Kornev *et al.*, 2006:17788; Roskoski, 2015:8). The R-spine is formed when residues from the DFG-motif, the β4-strand of the N-lobe, the αC-helix, and the Y/HRD-motif of the catalytic loop, are properly aligned during activation. The C-spine consists of interaction between a residue from the β2-strand, the alanine residue from the AXK-motif of the β3-strand, and the adenine ring of the ATP molecule (Fabbro *et al.*, 2015:2683). The R-spine is mainly responsible for positioning of the peptide substrate for catalysis, while the C-spine facilitates the binding of ATP (Roskoski, 2015:8).

2.2.3. Protein kinase inhibition

Aberrant protein kinase function has been implicated in the pathogenesis of various diseases, thus making this enzyme family one of the major drug targets of interest (Roskoski, 2020:3). Protein kinase inhibitors were primarily developed for their application in the field of oncology (Fabbro *et al.*, 2015:2679). However, in recent years this application has also expanded to inflammatory, immunological, neurodegenerative and cardiovascular diseases (Ferguson & Gray, 2018:1). To date, the Food and Drug Administration (FDA) has approved 52 small-molecule protein kinase inhibitors of which 46 are used for cancer therapy and only 8 are used for non-oncologic indications (Roskoski, 2020:1).

The majority of small-molecule protein kinase inhibitors exhibit the same mode of action, whereby they target the ATP-binding site. These ATP-competitive inhibitors can further be classified as either type I, I½ or II inhibitors based on their interaction with the target. Type 1 (classical) inhibitors (Figure 2.10.A) target the catalytically active protein kinase conformation (DFG-in, α-C helix-in) (Lamba & Ghosh, 2012:2936). These compounds mimic the binding of ATP by forming hydrogen bonds with the backbone residues of the hinge region (Müller *et al.*, 2015:819). Due to the highly conserved and rigid nature of the ATP-binding site of active protein kinases, type I inhibitors are generally promiscuous (Zuccotto *et al.*, 2010:2684). However, the selectivity of type I inhibitors can be improved by also targeting unique features within the active site such as small gatekeeper residues, unusual amino acids and inserts (Müller *et al.*, 2015:819). FDA-approved type I inhibitors include erlotinib, dasatinib, sunitinib, and gefitinib (Fabbro *et al.*, 2015:2684). Type II inhibitors (Figure 2.10.C) target the inactive protein kinase conformation (DFG-out, variable α-C helix) (Roskoski, 2016:40). The DFG-out conformation creates an additional allosteric site adjacent to the ATP-binding site (Zuccotto *et al.*, 2010:2684). When this pocket is utilized by type II inhibitors, the protein kinase structure is locked in its inactive conformation (Fabbro *et al.*, 2015:2684). As inactive protein kinase conformations are structurally diverse, type II inhibitors are generally more selective than type I inhibitors (Fabbro *et al.*, 2015:2684). Type I½ inhibitors (Figure 2.10.B) target both the ATP-binding site and the hydrophobic pocket

at the back of the ATP-binding site while the kinase is in the inactive conformation (DFG-in, variable α-C helix) (Roskoski, 2016:38; Zuccotto *et al.*, 2010:2685). Access to the hydrophobic back pocket is controlled by the gatekeeper residue (Zuccotto *et al.*, 2010:2685). The vast majority of human protein kinases have large gatekeeper residues with bulky side chains (e.g. methionine), resulting in a small cavity with limited access to inhibitors (Müller *et al.*, 2015:819; Zuccotto *et al.*, 2010:2685). In contrast, smaller gatekeeper residues (e.g. threonine) result in a large cavity that is accessible to small molecule inhibitors (Zuccotto *et al.*, 2010:2685).

Type III-VI inhibitors are allosteric inhibitors that target sites other than the ATP-binding site. Type III inhibitors (Figure 2.10.D) bind to the allosteric site adjacent to the ATP-binding site of inactive kinases (variable DFG, α-C helix-out) and may displace ATP in the process (Roskoski, 2016:44). Type IV inhibitors (Figure 2.10.E) bind to allosteric sites outside of the active site (variable DFG and α-C helix) (Cox *et al.*, 2011:32; Roskoski, 2016:44). Although allosteric inhibitors often demonstrate high selectivity for their target kinases, these inhibitors generally exhibit low potency compared to ATP-competitive inhibitors (Cox *et al.*, 2011:34; Lamba & Ghosh, 2012:2939). Type V (bisubstrate and bivalent) inhibitors (Figure 2.10.F) aim to increase the potency of allosteric inhibitors by chemically linking an active site-directed moiety to an allosteric moiety, thus simultaneously targeting two distinct protein kinase regions (Cox *et al.*, 2011:34; Lamba & Ghosh, 2012:2939). Type VI inhibitors (Figure 2.10.G) such as the FDA-approved drugs, afatinib and ibrutinib, bind covalently to their target protein kinase (Roskoski, 2016:44).

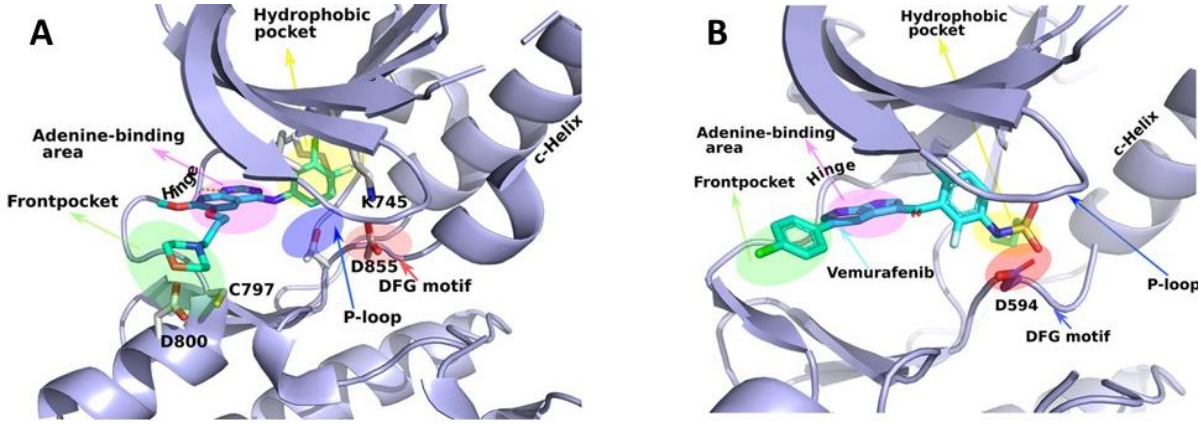


Figure 2.10 *(continued)*

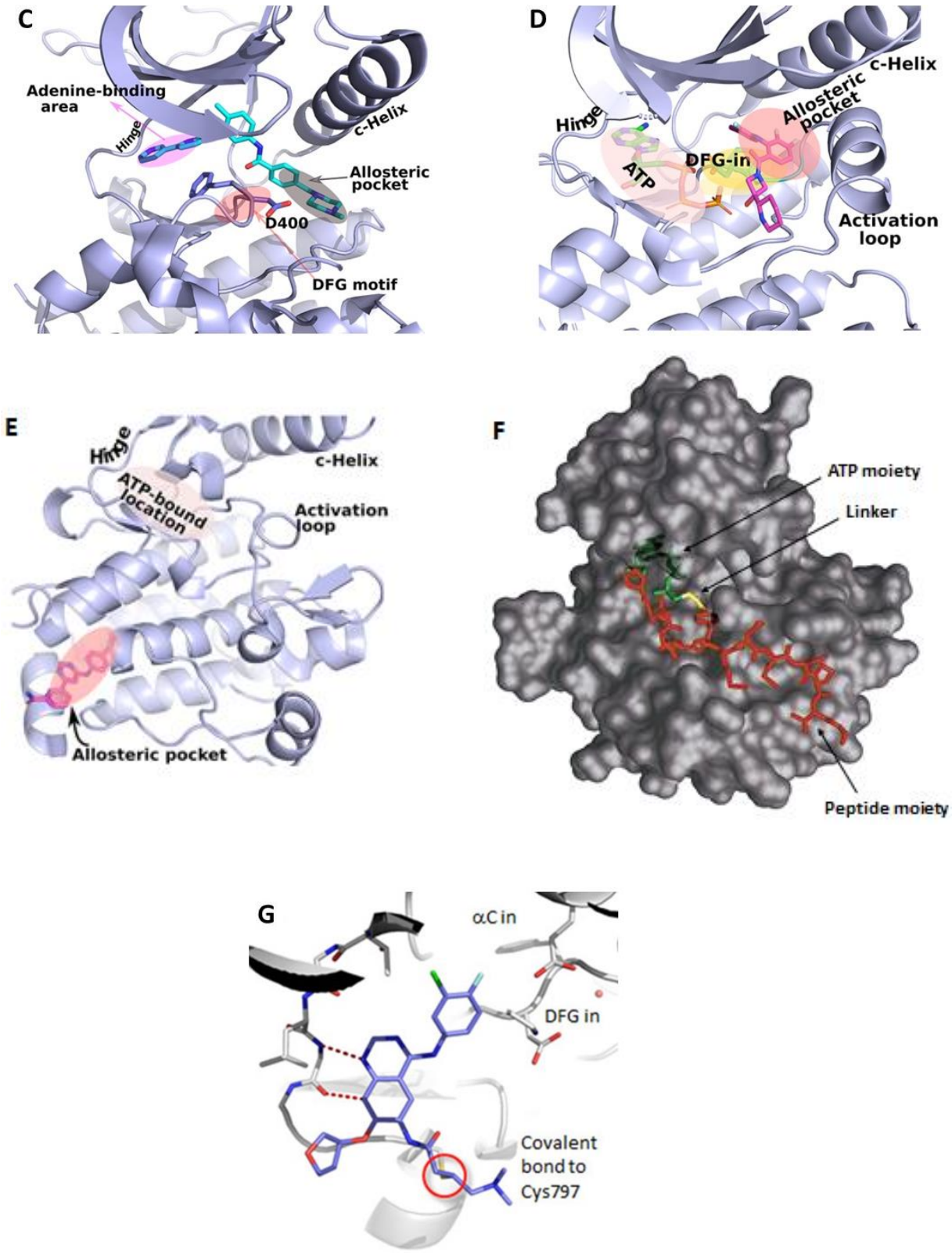


Figure 2.10: Protein kinase inhibitor binding modes. **A**) Type I inhibitor (gefitinib) bound to the ATP-binding site of the epidermal growth factor receptor (EGFR). **B**) Type I½ inhibitor (vemurafenib) bound to the ATP-binding site and the hydrophobic back pocket of BRAF kinase. **C**) Type II inhibitor (imatinib) bound to the ATP-binding site and the allosteric site adjacent to the ATP-binding site of the non-receptor tyrosine kinase, Abl1. **D**) Type III inhibitor (cobimetinib) bound to the allosteric site adjacent to the ATP-binding site of

mitogen-activated protein kinase kinase (MAP2K). **E**) Type IV inhibitor (GNF-2) bound to the allosteric site located in the C-lobe of Abl kinase. **F**) Type V (bisubstrate) inhibitor bound to the insulin receptor kinase (CIRK). The ATP site-directed moiety of the inhibitor is shown in green, the peptide substrate moiety shown in red, and the linker connecting the two moieties shown in yellow. **G**) Type VI inhibitor (afatinib) forms a covalent bond with the cysteine 797 residue of EGFR. Image **A**-**E** reproduced from (Zhao & Bourne, 2020:21) with permission from Springer Nature. Image **F** reproduced from (Parang *et al.*, 2001:40) with permission from Springer Nature. Image **G** was reproduced from (Fabbro *et al.*, 2015:2683) with permission from John Wiley and Sons.

2.2.4. *Plasmodium* protein kinases

The use of protein kinase inhibitors as treatment for other diseases has also stimulated an interest in exploring parasitic protein kinases as targets for treating infectious diseases such as malaria (Cabrera *et al.*, 2018:8061; Canduri *et al.*, 2007:390; Doerig, 2004:157; Doerig *et al.*, 2002:366; Doerig *et al.*, 2005:145). The complex life cycle of the malaria parasite requires efficient cellular and molecular mechanisms to respond to the changing intra- and extra-cellular environments (Doerig *et al.*, 2009:170). Although the roles of many malarial protein kinases are still unknown, studies have demonstrated that protein phosphorylation is essential for each stage of the parasite's life cycle (Doerig *et al.*, 2009:169; Solyakov *et al.*, 2011:2; Treeck *et al.*, 2011:410).

The completion of the *P. falciparum* genome project in 2002 (Gardner *et al.*, 2002:498), has provided an opportunity for genome-wide investigation of the parasite's kinome. Two independent studies done on the *P. falciparum* genome have identified 85 (Ward *et al.*, 2004:1) or 99 (Anamika *et al.*, 2005:182) genes that encode for putative protein kinases. The number of genes differs between the two studies due to a difference in the stringency of inclusion criteria. Of these protein kinases, 65 are related to eukaryotic protein kinases and many cluster within established eukaryotic protein kinase families such as AGC, CMGC, CK1, CaMK, and TK (Anamika *et al.*, 2005:182; Doerig *et al.*, 2008:570; Ward *et al.*, 2004:3). The two studies also revealed profound differences between the *P. falciparum* and the mammalian kinome with regards to the kinome composition itself and organization of signalling pathways (Anamika *et al.*, 2005:186; Ward *et al.*, 2004:2). Many of the plasmodial protein kinases that cluster within established families display atypical structural and functional characteristics that are not found in their mammalian counterparts (Doerig, 2004:158). These atypical characteristics include large C-terminal or N-terminal extensions to the catalytic site, large insertions inside the catalytic site and the presence of an atypical regulatory site (Doerig, 2004:158). The *P. falciparum* kinome also contains protein kinases that have no mammalian orthologues (orphan kinases) or display homology to more than one of the established eukaryotic protein kinase groups (composite or hybrid kinases) (Doerig, 2004:159). Some protein kinase families (e.g. tyrosine kinases) and

classic signalling pathways (e.g. MAPK pathway) are notably absent from the *P. falciparum* kinome (Doerig, 2004:159).

These plasmodial kinase groups and the small-molecule inhibitors developed thus far for each group, are discussed in detail in Chapter 3 (Paper I published in *Molecules*). Figure 2.11 gives an overview of the different plasmodial kinase groups and their role during the various stages of the parasite's life cycle.

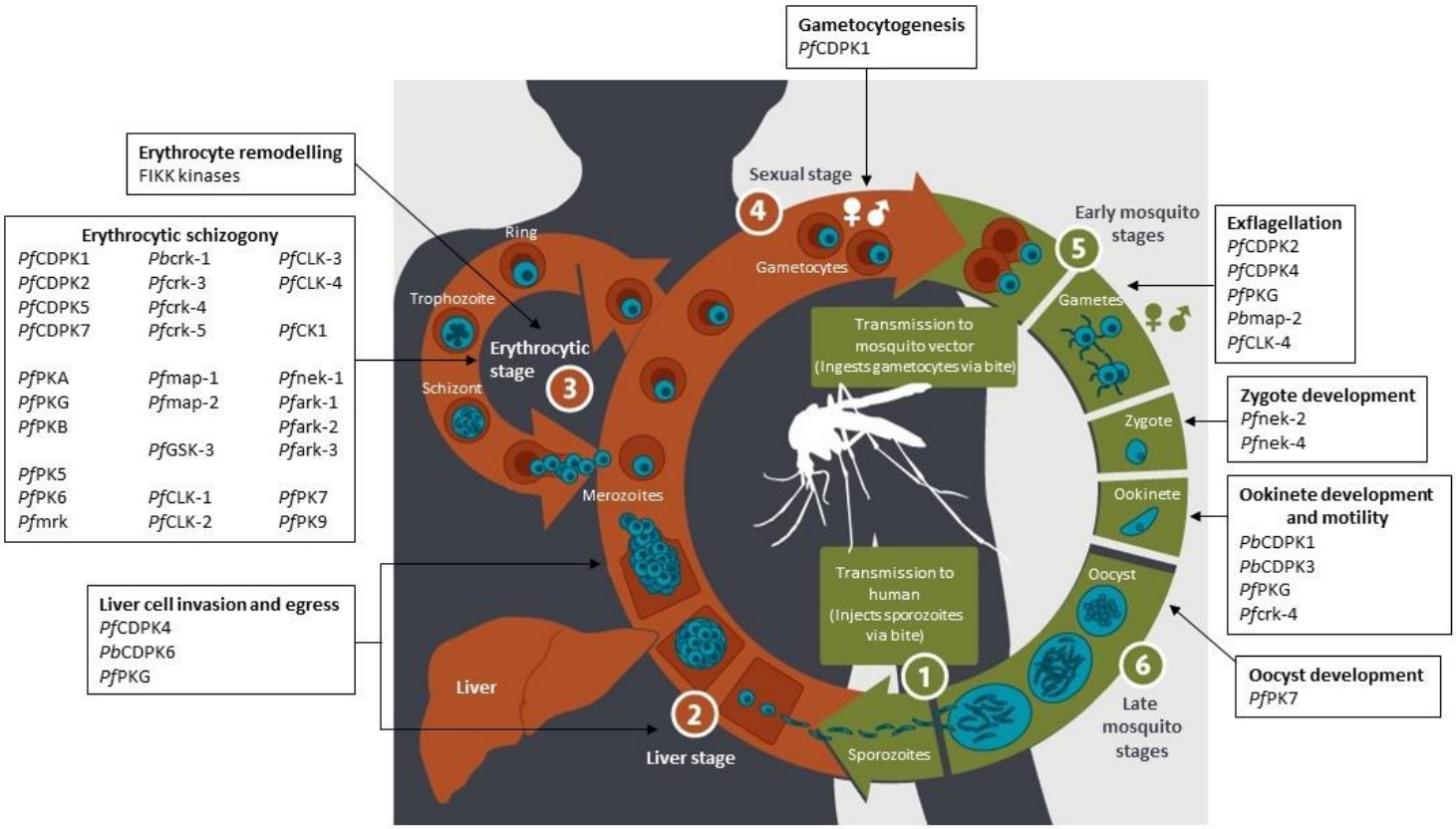


Figure 2.11: Involvement of plasmodial protein kinases during the different stages of the *Plasmodium* parasite's life cycle. Calcium-dependent protein kinases (*Pf*CDPK1 to 7); AGC group (*Pf*PKA, *Pf*PKB and *Pf*PKG); CDK and CDK-related kinases (*Pf*PK5, *Pf*PK6, *Pf*mrk and *Pf*crk-1 to -5); MAPKs (*Pf*map-1 and *Pf*map-2); GSK-3 (*Pf*GSK-3); CLKs (*Pf*CLK-1 to -4); CK1 group (*Pf*CK1); NIMA (Never In Mitosis, gene A)-related kinases (*Pf*nek-1 to -4); Aurora-related kinases (*Pf*ark-1 to -3); Orphan kinases (FIKK kinases, *Pf*PK7 and *Pf*PK9). Image was adapted from the NIH website (https://www.niaid.nih.gov/diseases-conditions/malaria-parasite).

The *P. falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) enzyme was chosen as target for this study. Although *Pf*GSK-3 has been genetically validated as an essential plasmodial kinase, medicinal chemistry programs targeting this enzyme are limited thus far. *Pf*GSK-3 and its human orthologue, *Hs*GSK-3 are discussed below.

2.3. Glycogen synthase kinase-3

2.3.1. Human glycogen synthase kinase-3

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase that belongs to the CMGC family (Saraswati *et al.*, 2018:844). This multifunctional enzyme phosphorylates a diverse range of substrates which in turn regulates several processes including glycogen metabolism, Wnt and insulin signalling, embryonic development, and neuronal function and development (Rayasam *et al.*, 2009:885). In humans, two closely related isoforms of GSK-3 namely *Hs*GSK-3α and *Hs*GSK-3β are expressed (Woodgett, 1990:2433). These two isoforms share 85% overall sequence homology and 98% homology within their catalytic domains (Kaidanovich-Beilin & Woodgett, 2011:1; Woodgett, 1990:2433). Despite the high level of similarity between the two isoforms, their functions are not interchangeable as demonstrated by GSK-3 knock-out studies done on mice (Kaidanovich-Beilin & Woodgett, 2011:11). An embryonic-lethal phenotype was observed when the GSK-3β gene was knocked out (Hoeflich *et al.*, 2000:86), while mice lacking the GSK-3α gene were still viable (MacAulay *et al.*, 2007:329). Both isoforms are expressed in most mammalian tissues with especially high levels expressed in the brain (Kaidanovich-Beilin & Woodgett, 2011:1; Woodgett, 1990:2434).

The GSK-3 enzyme displays some unusual characteristics that are rarely seen in other protein kinases. Firstly, the GSK-3 enzyme is constantly active during resting (unstimulated) cellular conditions, and it is only inactivated in response to external signals from membrane-bound receptors (Kaidanovich-Beilin & Woodgett, 2011:2). Secondly, phosphorylation of GSK-3 substrates generally results in inhibition of their activity rather than activation (Kaidanovich-Beilin & Woodgett, 2011:2). Thirdly, GSK-3 activity is subject to multiple levels of regulation, which is critical due to its involvement in so many cellular processes. GSK-3 regulation is mediated by a combination of phosphorylation, substrate specificity, intracellular localisation and interactions with other proteins (Beurel *et al.*, 2015:115; Kaidanovich-Beilin & Woodgett, 2011:2).

Phosphorylation can either inhibit or activate GSK-3 depending on the amino acid (serine, threonine or tyrosine) being phosphorylated. GSK-3 is inhibited by phosphorylation of the N-terminal serine residue (Ser21 for *Hs*GSK-3α and Ser9 for *Hs*GSK-3β), which plays an important role in the regulation of insulin signalling (Sutherland & Cohen, 1994:37; Sutherland *et al.*, 1993:15). Upon phosphorylation, the N-terminal acts as a pseudosubstrate which binds to the substrate-binding site and blocks access to GSK-3 substrates (Beurel *et al.*, 2015:115; Frame *et al.*, 2001:1321). Inactivation of GSK-3β in the brain occurs primarily through p38 MAPK phosphorylation of the serine (Ser389) and threonine (Thr390) residues in the C-terminal region of the enzyme (Thornton *et al.*, 2008:2). Additionally, extracellular signal-regulated kinase (Erk) can also inactivate *Hs*GSK-3β by phosphorylating the Thr43 residue (Ding *et al.*, 2005:161). Activation of the GSK-3 enzyme occurs through phosphorylation of a tyrosine residue located in the A-loop (Tyr279 for *Hs*GSK-3α and Tyr216 for *Hs*GSK-3β) (Cole *et al.*, 2004:249; Hughes *et al.*, 1993:806). The unphosphorylated Tyr279/216

side-chain impedes access to the substrate-binding site (Harwood, 2001:823; Kaidanovich-Beilin & Woodgett, 2011:2). Phosphorylation of this residue results in a conformational change that allows substrates to bind to GSK-3 (Harwood, 2001:823; Kaidanovich-Beilin & Woodgett, 2011:2).

With regards to substrate specificity, GSK-3 has a unique preference for substrates that are pre-phosphorylated or "primed" at a serine or threonine residue located next to the GSK-3 phosphorylation site (Beurel *et al.*, 2015:117; Fiol *et al.*, 1990:6061; Kaidanovich-Beilin & Woodgett, 2011:6). The primed amino acid residue serves as a signal to GSK-3 to initiate its phosphorylation process. Access of GSK-3 to its substrates is controlled by mechanisms that regulate the intracellular localisation of GSK-3 (Beurel *et al.*, 2015:118; Kaidanovich-Beilin & Woodgett, 2011:4). For example, *Hs*GSK-3β is found primarily in the cytoplasm, however, smaller pools of *Hs*GSK-3β are also located in the nucleus and mitochondria (Bijur & Jope, 2003:2415). The nuclear and mitochondrial *Hs*GSK-3β pools demonstrate less Ser9 phosphorylation and are therefore 5- to 10-fold more active than the cytosolic *Hs*GSK-3β (Bijur & Jope, 2003:2418). In response to apoptotic signalling, *Hs*GSK-3β accumulates within the nucleus which allows interaction with its nuclear substrates (Bijur & Jope, 2001:37441).

The Wnt signalling pathway is a classic example of how the function of GSK-3 is regulated by its interaction with other proteins (Beurel *et al.*, 2015:118; Kaidanovich-Beilin & Woodgett, 2011:4). When the Wnt pathway is inactive, GSK-3 forms part of a "destruction" complex with three other proteins, namely Axin, adenomatous polyposis coli (APC) and casein kinase 1α (CK1α). When the substrate (β-catenin) binds to this complex, it is simultaneously in contact with both the priming kinase (CK1α) and GSK-3, allowing phosphorylation and subsequent degradation of the substrate. Activation of the Wnt pathway results in dissolution of the destruction complex and GSK-3 is no longer able to phosphorylate β-catenin.

2.3.2. GSK-3 as a therapeutic target

Abnormal GSK-3 activity has been implicated in a variety of disorders (Figure 2.12) such as diabetes (Henriksen & Dokken, 2006:1439), cancer (Luo, 2009:198; Manoukian & Woodgett, 2002:223), Alzheimer's disease (Hooper *et al.*, 2008:1436; Maqbool *et al.*, 2016:77), neurodegeneration (Giese, 2009:519), cardiovascular diseases (Hardt & Sadoshima, 2002:1060; Lal *et al.*, 2015:145), and psychiatric disorders (Jope & Roh, 2006:15). Therefore, GSK-3 has been explored as a potential target for the treatment of these diseases (Takahashi-Yanaga, 2013:192).

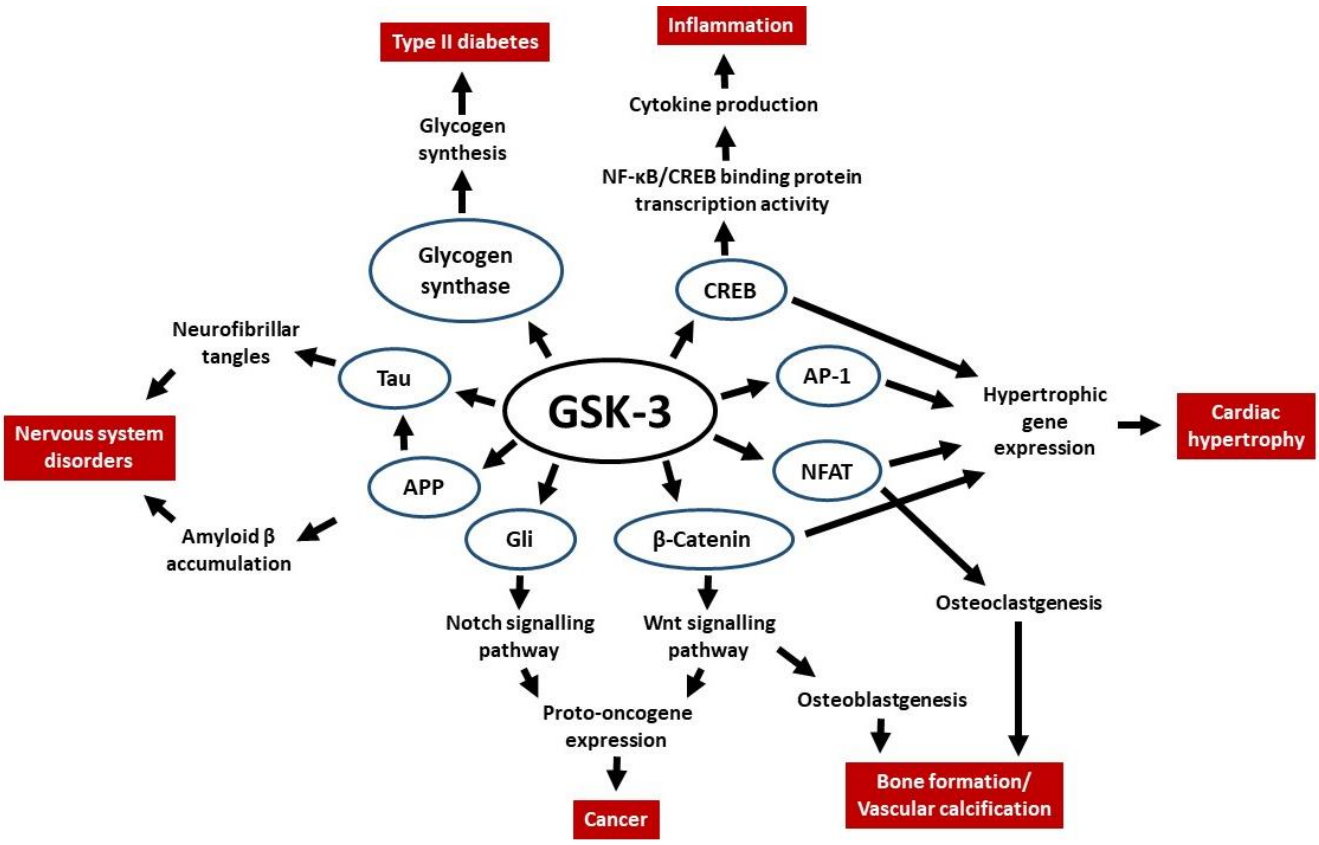


Figure 2.12: Relationship of GSK-3 with various substrates and their role in human disease. GSK-3 substrates: Glycogen synthase, cAMP response element-binding (CREB) protein, Activator protein 1 (AP-1), Nuclear factor of activated T-cells (NFAT), β-catenin, Gli protein, Amyloid precursor protein (APP) and Tau protein. Figure reproduced from (Takahashi-Yanaga, 2013:194) with permission from Elsevier.

GSK-3 inhibitor development has been aided by the information gained from the available crystal structure of *Hs*GSK-3β (Dajani *et al.*, 2001:730; ter Haar *et al.*, 2001:593), as well as from cellular studies and animal models (Kaidanovich-Beilin & Woodgett, 2011:11). A wide variety of synthetic as well as natural compounds have been identified as GSK-3 inhibitors (Maqbool & Hoda, 2017:4337; Saraswati *et al.*, 2018:844). Unfortunately, progress towards use of these inhibitors in a clinical setting is slow mainly due to toxicological challenges (Bhat *et al.*, 2018:S551). Due to its involvement in various cellular processes, GSK-3 inhibition could theoretically result in various unwanted side effects ranging from hypoglycaemia to tumorigenesis (Eldar-Finkelman & Martinez, 2011:9; Martinez *et al.*, 2011:2). Several GSK-3 inhibitors have failed in preclinical or clinical trials due to toxicity issues (Bhat *et al.*, 2018:S550).

Lithium, a drug used for many decades to treat bipolar disorder, was identified as an inhibitor of GSK-3 (Klein & Melton, 1996:8455; Stambolic *et al.*, 1996:1665). Long-term use of lithium has been demonstrated to be safe and effective with no expected adverse effects observed, thus indicating that long-term GSK-3 inhibition can be tolerated (Jope & Roh, 2006:4). However, lithium only inhibits ±25% of the total GSK-3 activity at

therapeutic doses (Martinez *et al.*, 2011:2). Therefore, partial inhibition of GSK-3 may be ideal to restore the elevated GSK-3 activity seen in pathological conditions, to physiological levels (Beurel *et al.*, 2015:121; Eldar-Finkelman & Martinez, 2011:9).

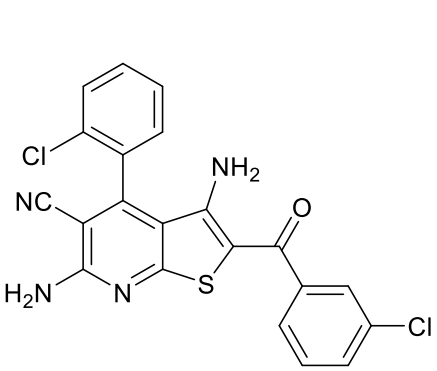
One strategy to achieve partial GSK-3 inhibition is to develop allosteric inhibitors (e.g. substrate-competitive inhibitors) instead of ATP-competitive inhibitors (Eldar-Finkelman & Martinez, 2011:10). In general, allosteric inhibitors demonstrate relatively weak affinities for their targets but are more selective which limits off-target effects (Eldar-Finkelman & Martinez, 2011:9). The L803-mts peptide is an example of a substrate-competitive inhibitor that is highly selective towards GSK-3 (Plotkin *et al.*, 2003:978). This peptide demonstrated therapeutic activity by improving glucose homeostasis in a type II diabetes model (Kaidanovich-Beilin & Eldar-Finkelman, 2006:22) and reducing depressive behaviour in a mild traumatic brain injury model (Shapira *et al.*, 2007:573). Another strategy involves developing inhibitors that only target the specific GSK-3 pool of interest (Bhat *et al.*, 2018:S551). This can be achieved by conjugating the inhibitor to a moiety that specifically binds to the organ/ cell type of interest, or encapsulating the inhibitor in a drug delivery system that has a preference for a specific organ/ cell type (Bhat *et al.*, 2018:S551). Recently, a nanoparticle delivery system carrying a *Hs*GSK-3β inhibitor (AZD2858) was developed for healing bone fractures (Wang *et al.*, 2017:9446). The nanoparticle was functionalised with peptides that specifically target tartrate-resistant acid phosphatase present on bone resorptive surfaces (Wang *et al.*, 2017:9446). This allowed greater accumulation of AZD2858 at the fracture site and enhanced fracture healing in a mouse model (Wang *et al.*, 2017:9451). These strategies show promise for future development of GSK-3 inhibitors.

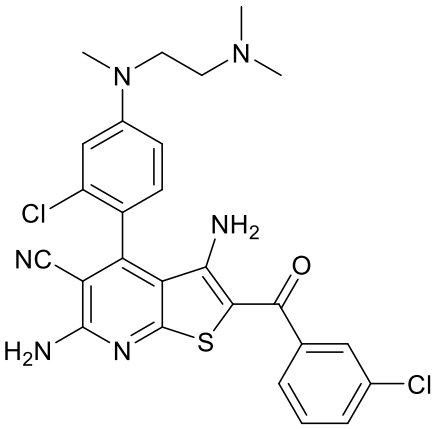
2.3.3. *Pf*GSK-3

*Pf*GSK-3 is one of three GSK-3-related kinases identified for the *P. falciparum* parasite (Ward *et al.*, 2004:4). Comparing the *Pf*GSK-3 sequence to that of the two *Hs*GSK-3 isoforms, revealed a high level of conservation (Droucheau *et al.*, 2004:188). The overall sequence of *Pf*GSK-3 displayed 38.0% identity and 62.4% similarity to *Hs*GSK-3α, and 43.1% identity and 58.9% similarity to *Hs*GSK-3β. Comparing the catalytic site of *Pf*GSK-3 with the two human isoforms, *Pf*GSK-3 displayed 56.1% identity and 75.3% similarity to *Hs*GSK-3α, and 58.2% identity and 76.0% similarity to *Hs*GSK-3β. Droucheau *et al.* (2004:188) also generated a tridimensional structure model of *Pf*GSK-3 using the *Pf*GSK-3 sequence and the available crystal structure of *Hs*GSK-3β. The modelled structure of *Pf*GSK-3 was almost identical to that of *Hs*GSK-3β, as was expected, due to the high degree of sequence similarity. The regions involved in the regulation of enzyme activity and substrate binding were highly conserved between the two enzymes (Droucheau *et al.*, 2004:193). The amino acid residues that comprise the active site, the phosphate-primed substrate-binding site, and the tyrosine phosphorylation site (Tyr216) of *Hs*GSK-3β, are all conserved in *Pf*GSK-3. Only the serine phosphorylation site (Ser9), responsible for inhibition of *Hs*GSK-3β, was absent from *Pf*GSK-3.

*Pf*GSK-3 is present throughout the erythrocytic stages, with maximal levels expressed during the early trophozoite stage (Droucheau *et al.*, 2004:193). After expression, *Pf*GSK-3 is rapidly transported to the cytoplasm of the erythrocyte where it associates with membranous structures known as Maurer's clefts (Droucheau *et al.*, 2004:193). Although the exact biological functions of *Pf*GSK-3 remain to be elucidated, it has been genetically validated as essential for survival of asexual erythrocytic parasites (Solyakov *et al.*, 2011:3). This makes *Pf*GSK-3 an ideal target for new antimalarial drug design, however the high level of conservation between *Pf*GSK-3 and *Hs*GSK-3 poses a challenge to the development of *Pf*GSK-3 selective inhibitors.

Droucheau *et al.* (2004:192) were the first to investigate whether selective *Pf*GSK-3 inhibition was possible. They tested the activity of a few established GSK-3 inhibitors on recombinant *Pf*GSK-3 and rat GSK-3β. These inhibitors either potently inhibited both enzymes (staurosporine, hymenialdisine and indirubin-3'-monoxime) or were more selective towards mammalian GSK-3β (paullone class of inhibitors). Fugel *et al.* (2013:266) reported the same results when testing a variety of reference kinase inhibitors on recombinant *Pf*GSK-3 and porcine GSK-3. Based on these findings they concluded that the structures of the plasmodial and the mammalian enzymes differ enough for species selectivity to be achievable. Indeed, studies have found certain structural differences between the active site of the plasmodial and the mammalian enzymes, which could be exploited for selective drug design (Kruggel & Lemcke, 2009a:890; Osolodkin *et al.*, 2011:733). Fugel *et al.* (2013:271) further demonstrated that selective inhibition of the *Pf*GSK-3 enzyme is indeed possible. They designed a novel series of inhibitors based on hit structures that were identified during a high-throughput screening campaign. This series of inhibitors shared the same 3-amino-4-arylthieno[2,3-*b*]pyridine motif as the parent scaffold. Certain compounds from this series were highly selective towards *Pf*GSK-3 compared to the GSK-3 orthologues of other species (human and pathogenic microorganisms), as well as other human protein kinases. The most active compound of the series (**19**, Figure 2.13) inhibited *Pf*GSK-3 at a submicromolar concentration and demonstrated *in vitro* antiplasmodial activity when tested against erythrocytic stage *P. falciparum* parasites.





	19	**20**
***Pf*GSK-3 IC_{50}**	0.48 µM[a] (0.24 µM)[b]	0.72 µM[b]
***Hs*GSK-3β IC_{50}**	3.3 µM[a] (9.08 µM)[b]	40.2 µM[b]
***Pf*NF54-Luc EC_{50}**	5.5 µM	1.2 µM
Kinetic solubility	1.5 µM	4.8 µM

Figure 2.13: Chemical structures and biological data of the most active compounds from the 4-phenylthieno[2,3-*b*]pyridine class. [a] IC_{50} values determined using a radiometric assay (Fugel *et al.*, 2013:272); [b] IC_{50} values determined using the Kinase Glo Plus assay (Masch *et al.*, 2019:8).

Masch *et al*. (2019:8) further optimised the aqueous solubility and antiplasmodial activity of compound **19**. By means of homology modelling they identified the *para*-position of the 4-phenyl ring as the optimal position to attach an additional aliphatic polar side chain. The analogues with an alkylamino group attached to this position, such as compound **20** (Figure 2.13), retained *Pf*GSK-3 selectivity while demonstrating improved solubility and *in vitro* antiplasmodial activity.

Although *Pf*GSK-3 selective inhibitors are currently limited to the 4-phenylthieno[2,3-*b*]pyridine class, the experimental data from these studies combined with that of various homology modelling studies (Balasaheb Aher & Roy, 2015:960; Fugel *et al.*, 2013:273; Kruggel & Lemcke, 2009b:328) will greatly aid future inhibitor development.

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Chapter 3: An update on development of small-molecule plasmodial kinase inhibitors

molecules

MDPI

Review

An Update on Development of Small-Molecule Plasmodial Kinase Inhibitors

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Abstract: Malaria control relies heavily on the small number of existing antimalarial drugs. However, recurring antimalarial drug resistance necessitates the continual generation of new antimalarial drugs with novel modes of action. In order to shift the focus from only controlling this disease towards elimination and eradication, next-generation antimalarial agents need to address the gaps in the malaria drug arsenal. This includes developing drugs for chemoprotection, treating severe malaria and blocking transmission. Plasmodial kinases are promising targets for next-generation antimalarial drug development as they mediate critical cellular processes and some are active across multiple stages of the parasite's life cycle. This review gives an update on the progress made thus far with regards to plasmodial kinase small-molecule inhibitor development.

Keywords: malaria; *Plasmodium*; kinases; kinase inhibitors; antimalarial agents

1. Introduction

Although significant progress has been made with regards to worldwide malaria control and eradication, this infectious disease continues to have devastating effects, especially in developing countries. An estimated 228 million malaria cases and 405,000 malaria-related deaths were reported globally in 2018 [1]. The ongoing coronavirus (COVID-19) pandemic could also have a profound negative impact on the progress made thus far in the fight against malaria [2]. Patients presenting with fever or requiring malaria treatment are now less likely to visit health care facilities out of fear of contracting COVID-19 [3]. Lockdown periods have disrupted the supply of malaria rapid diagnostic tests, antimalarial drugs and other interventions [3]. A number of antimalarial drugs (e.g., artemisinin, chloroquine and hydroxychloroquine) have also been repurposed for COVID-19 treatment, despite a lack of scientific evidence and approval [3]. This has resulted in a shortage of these drugs and, in the long-run, an increased risk of drug resistance due to improper use of antimalarial monotherapies [3].

Apart from the potential impact of the pandemic, recurring antimalarial drug resistance poses a major threat to malaria control and elimination. *P. falciparum*, the *Plasmodium* species responsible for the majority of malaria-related deaths, is resistant to most antimalarial drugs, including the current first-line artemisinin-based combination therapies [1,4]. The second most common human malaria species, *P. vivax*, has developed widespread resistance to chloroquine [5]. Malaria parasites can also develop cross-resistance to antimalarial drugs from the same chemical class or with the same mode of action, which further exacerbates the problem [6].

The life cycle of the malaria parasite is complex; it consists of an asexual stage which occurs in the human host and a sexual stage which occurs in the mosquito vector [7]. Existing antimalarial drugs are highly stage-specific, with the majority targeting blood-stage parasites for treatment of

symptomatic malaria [7]. In order to eliminate and eventually eradicate malaria, the focus needs to shift from mainly providing curative treatment to blocking disease transmission [8]. Achieving this goal requires a new generation of cost-effective antimalarial agents that are safe and well-tolerated in a wide range of recipients, including vulnerable populations such as pregnant women and infants [8]. Since drug development is expensive and can take up to 15 years before reaching the market, it is important to have clear guidelines [8]. The Medicines for Malaria Venture (MMV) published defined criteria for the types of individual molecules (target candidate profiles (TCPs)) and drug formulations (target product profiles (TPP)) that would be ideal for new malaria therapy [8]. TPP-1 focusses on treating malaria infections and includes a combination of molecules with blood-stage activity (TCP-1), transmission-blocking activity (TCP-5) and activity against relapse causing liver stages (TCP-3) [8]. Alternatively, TPP-1 could also consist of rapid-acting TCP-1 molecules when treating severe/complicated malaria [8]. TPP-2 focusses on chemoprevention of travelers to endemic regions or during epidemics and includes molecules with TCP-1 and hepatic schizont activity (TCP-4) [8].

Plasmodial kinases have been explored as targets for next-generation antimalarial agents due to their involvement in various critical cellular processes throughout the life cycle of the parasite [9–11]. The *P. falciparum* kinome is predicted to encode 85 to 99 protein kinase genes [12,13] as well as a small number of lipid kinase genes. Overall, the *P. falciparum* kinome displays significant divergence from the eukaryotic kinome. The 65 plasmodial kinases that cluster within established eukaryotic protein kinase (ePK) groups (CAMK, AGC, CMGC, CK1 and TKL groups) often display structural and functional characteristics that are not seen in their mammalian counterparts [9,13]. The plasmodial kinome also contains protein kinases that have no mammalian orthologues (orphan kinases) or display homology with more than one of the established ePK groups (composite or hybrid kinases) [9]. These differences can be exploited for selective antimalarial drug development.

Progress on plasmodial kinase inhibitor development up until the beginning of 2018 was discussed in a detailed review by Cabrera and co-workers [14]. Herein, we give an overview thereof, as well as discussing additional kinases and new research related to plasmodial kinase inhibitor development.

2. Calcium-Dependent Protein Kinases (CDPKs)

Enzymes from the classical Ca^{2+}/calmodulin-dependent protein kinase (CaMK) group seem to be rare in the *P. falciparum* kinome [15]. However, the kinome contains calcium-dependent protein kinases (CDPKs) which have a C-terminal calmodulin-like domain that is highly homologous to the CaMK group [16]. CDPKs comprise a unique family of serine/threonine kinases only found in plants, protozoans (including apicomplexan parasites) and some algae [17]. These enzymes play an important role in calcium signalling during the various life stages of the *Plasmodium* parasite [15]. Seven members of the CDPK family (*Pf*CDPK1 to *Pf*CDPK7) have been identified in *P. falciparum* [16]. *Pf*CDPK1 is expressed at all stages of the *Plasmodium* parasite life cycle. During asexual parasite development, *Pf*CDPK1 plays a role in parasite motility [18,19], microneme secretion and subsequent erythrocyte invasion [20], as well as merozoite egress from mature schizonts [19]. Previous studies have shown that *Pf*CDPK1 is likely to be essential for asexual development [19,21,22]; however, the parasite might have other mechanisms in place to compensate for loss of *Pf*CDPK1 activity [21,23,24]. During the sexual development of the parasite, *Pf*CDPK1 activity is indispensable for gametogenesis and subsequent infection of the mosquito vector [21]. In addition, the *P. berghei* homologue (*Pb*CDPK1) is also involved in ookinete development [25].

*Pf*CDPK2, *Pf*CDPK3 and *Pf*CDPK4 are all predominantly expressed during the sexual stage of the parasite development [26–28]. *Pf*CDPK2 and *Pf*CDPK4 are essential for male gametocyte exflagellation and transmission to the mosquito vector [26,27]. In addition, *Pf*CDPK4 is also required for sporozoite invasion of hepatocytes [29]. Although the exact function of *Pf*CDPK3 is not yet known, its *P. berghei* orthologue (*Pb*CDPK3) is expressed in ookinetes where it regulates motility required for invading the midgut of the mosquito [30,31].

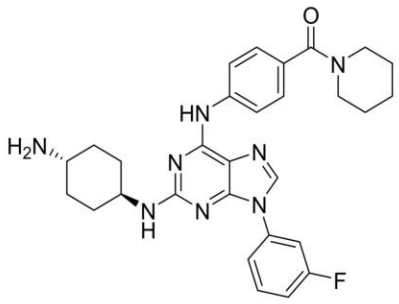
*Pf*CDPK5 and *Pf*CDPK7 are expressed during the asexual erythrocytic stage. *Pf*CDPK5 acts synergistically with *P. falciparum* protein kinase G (*Pf*PKG) to regulate microneme secretion which is required for merozoites to egress mature schizonts [32,33]. Although *Pf*CDPK5 is essential, the parasite is able to compensate for loss of *Pf*CDPK5 activity through hyperactivation of *Pf*PKG [32]. While CDPK7 is not essential to parasite viability, it still plays an important role in the development of the erythrocytic parasite. The growth rate of CDPK7 knockout parasites is significantly reduced due to a delay in maturation of ring-stage parasites to trophozoites and the release of fewer merozoites from each schizont [34]. Little is currently known about CDPK6 of *P. falciparum*. The *P. berghei* orthologue (*Pb*CDPK6) signals to sporozoites when to stop migration and initiate invasion of hepatocytes [35].

2.1. Inhibitor Development for the CDPK Group

The CDPKs are promising drug targets for the development of new antiplasmodial agents as there are no CDPK orthologues in the human host [16]. A unique structural feature of many parasitic CDPKs is the small gatekeeper residue at the hinge region [36]. A small gatekeeper residue results in enlargement of the hydrophobic pocket that accommodates the ATP purine group in the ATP-binding site [36]. Various medicinal chemistry campaigns have developed small-molecule inhibitors termed bumped kinase inhibitors (BKIs) that contain a bulky C3-aryl substituent that can occupy this enlarged hydrophobic pocket [36]. Most mammalian kinases have larger gatekeeper residues that block access to the bulky substituent of BKIs, therefore allowing better selectivity towards the parasitic kinases [36]. Amongst *P. falciparum* CDPKs, *Pf*CDPK4 has the smallest gatekeeper, which is a serine residue, followed by *Pf*CDPK1 with a medium threonine gatekeeper residue [37]. *Pf*CDPK2 (methionine), *Pf*CDPK3 (methionine) and *Pf*CDPK5 (leucine) all have bulky gatekeeper residues [37]. *P. falciparum* CDPK inhibitor development has mainly focussed on *Pf*CDPK1 and *Pf*CDPK4, and most of these inhibitors are BKIs [14].

2.1.1. *Pf*CDPK1

High-throughput screening campaigns have identified various scaffolds as *Pf*CDPK1 inhibitors (Figure 1), including 2,3,9-trisubstituted purines (**1**) [19], indolizines (**2**) [38] and imidazopyridazines (**3**–**5**) [23,38–42]. Of all these scaffolds, imidazopyridazines have been studied more intently.



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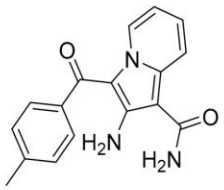
*Pf*CDPK1 IC_{50}: 17 nM

*Pf*3D7 blood stage EC_{50}: 230 nM



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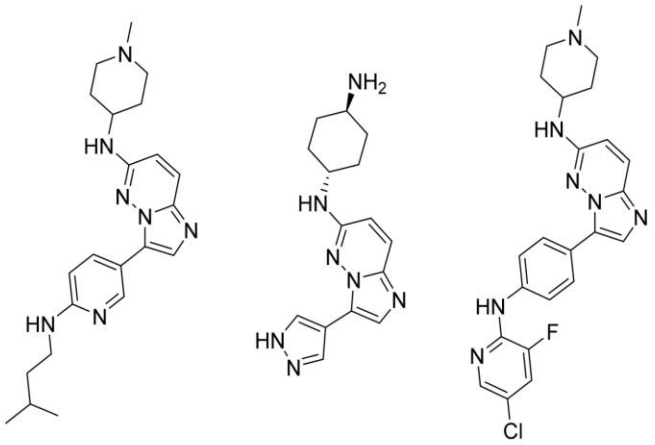
*Pf*CDPK1 K_i: 262 nM

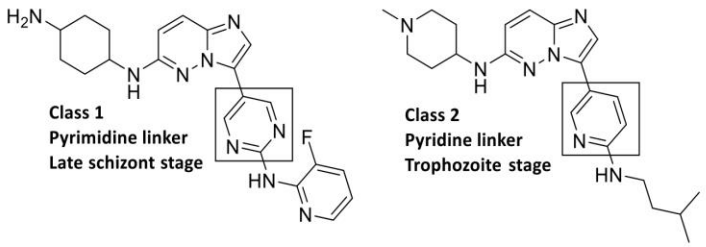
Figure 1. *Cont.*



	3	**4**	**5**
*Pf*CDPK1 IC_{50}	13 nM	70 nM	12 nM
*Pf*3D7 blood stage EC_{50}	40 nM	103 nM	80 nM
In vivo *Pb* mouse model (% parasitemia reduction, oral dose)	46% 50 mg/kg Once daily, 4 days	ND	51% 50 mg/kg Once daily, 4 days

Figure 1. The different scaffolds identified as inhibitors of *Pf*CDPK1. IC_{50}: half-maximal inhibitory concentration; *Pf*3D7: *Plasmodium falciparum* 3D7 strain; EC_{50}: half-maximal effective concentration; K_i: inhibitory constant; *Pb*: *Plasmodium berghei*; ND: not determined.

Imidazopyridazines are generally potent *Pf*CDPK1 inhibitors, with some compounds demonstrating low micromolar to submicromolar activity against recombinant *Pf*CDPK1 and *P. falciparum* erythrocytic parasites [23,38–42]. However, discrepancies between the enzymatic and whole-cell activities are generally reported for these compounds, which may be due to off-target activity or permeability issues [38,39]. Considerable effort was made to improve the selectivity and ADME (absorption, distribution, metabolism, excretion) profiles of imidazopyridazines, which resulted in compounds with well-balanced *Pf*CDPK1 potency, permeability and in vitro activity against *P. falciparum* erythrocytic stage parasites [40–42]. Despite these efforts, studies still reported only modest in vivo activity in a *P. berghei* mouse model [39–41]. Further exploration of the mechanism of action of imidazopyridazines demonstrated that these compounds could be grouped into two classes based on the type of aromatic linker between the core and the R2 substituent (Figure 2) [23]. Class 1 compounds (**6**) had a pyrimidine linker and inhibited *P. falciparum* parasite growth at the late schizont stage, while class 2 compounds (**7**) had a non-pyrimidine linker and inhibited the trophozoite stage of *P. falciparum*. Two additional parasitic targets were also identified for imidazopyridazines: class 1 compounds inhibited *Pf*PKG and class 2 compounds inhibited *Pf*HSP90 (a chaperone protein of *P. falciparum*). These results suggest that the activity of imidazopyridazines against erythrocytic *P. falciparum* parasites is primarily due to inhibition of *Pf*PKG and *Pf*HSP90, rather than *Pf*CDPK1 inhibition.



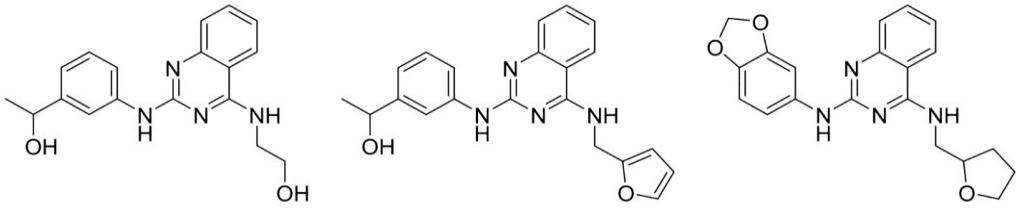
	6	7
*Pf*CDPK1 IC_{50}	0.008 μM	0.074 μM
*Pf*PKG IC_{50}	0.002 μM	>1 μM
*Pf*HSP90 K_D	ND	6.170 μM
*Pf*3D7 blood stage EC_{50}	0.034 μM	0.215 μM

Figure 2. Class 1 and 2 imidazopyridazine-based *Pf*CDPK1 inhibitors. K_D: equilibrium dissociation constant. ND: not determined.

More recently, Flaherty and co-workers [43] designed a hydrocarbon constrained peptide that mimics the C-terminal helical region of the *Pf*CDPK1 junction domain (J-domain). The autoinhibitory J-domain is located between the catalytic domain and the calmodulin-like domain and blocks the active site by acting as a pseudosubstrate when the kinase is in its inactive state. By mimicking the activity of the J-domain, the constrained peptide inhibits *Pf*CDPK1 by locking the kinase in its inactive state. Uptake of the constrained peptide by *P. falciparum*-infected erythrocytes was highly stage-specific, as late-stage schizont erythrocytes demonstrated increased uptake relative to ring-stage and early trophozoite erythrocytes. The constrained peptide inhibited recombinant *Pf*CDPK1 in the low micromolar range (IC_{50}: 3.5 μM) and caused a significant decrease in parasitemia at concentrations of ≥10 μM.

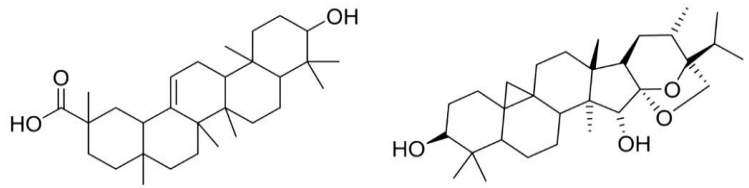
Lima and co-workers [44] designed and developed shape-based and machine learning models of *Pf*CDPK1, *Pf*CDPK4 and *Pf*PK6. These models were used for virtual screening of drug-like molecules to identify potent inhibitors with activity against multiple *P. falciparum* kinases. The computational hits were then evaluated in vitro against drug-sensitive (3D7) and multidrug-resistant (Dd2) *P. falciparum* erythrocytic parasites. Quinazoline derivatives (compounds **8**–**10**, Figure 3) inhibited the growth of both drug-sensitive and multidrug-resistant *P. falciparum* strains in the nanomolar range. Compounds **8** and **10** also demonstrated good in vivo inhibition of *P. berghei* ookinete formation at a concentration of 10 μM. Molecular docking studies indicated that compound **8** was able to interact with *Pf*CDPK1, *Pf*CDPK4 and *Pf*PK6, thus highlighting its potential as a multi-kinase inhibitor.

Another virtual screening campaign against a *Pf*CDPK1 homology model (*Pb*CDPK1 crystal structure, PBD ID: 3Q5I), identified 18 compounds from the MyriaScreen Diversity Library II that complement the *Pf*CDPK1 ATP-binding site [45]. Two of these compounds, **11** (ST092793) and **12** (S344699) (Figure 4), significantly inhibited recombinant *Pf*CDPK1 and demonstrated in vitro activity against *P. falciparum* erythrocytic parasites. Interestingly, isothermal titration calorimetry and fluorescence spectroscopy showed that **11** preferentially binds to the inactive conformation of *Pf*CDPK1, thereby locking the enzyme in this state throughout the erythrocytic stage.



	8	**9**	**10**
*Pf*3D7 blood stage EC_{50}	0.35 μM	0.43 μM	0.39 μM
*Pf*Dd2 blood stage EC_{50}	0.70 μM	0.48 μM	0.40 μM
Pb ookinete inhibition	70.02%	8.59%	51.81%

Figure 3. Quinazoline derivatives as inhibitors of multiple plasmodial kinases.



	11	**12**
*Pf*CDPK1 K_D	51.9 μM	200.0 μM
*Pf*CDPK1 IC_{50}	33.8 μM	42.6 μM
*Pf*3D7 blood stage EC_{50}	9.5 μM	17.8 μM

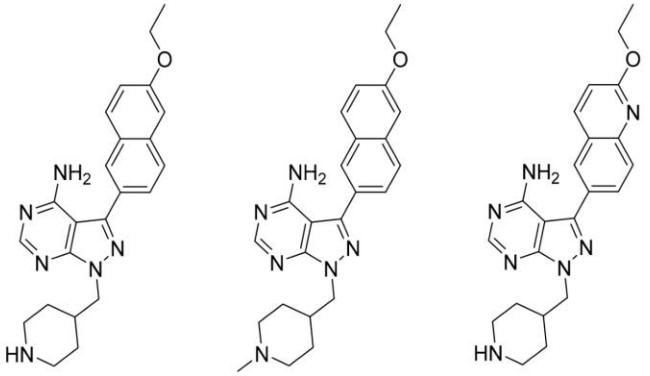
Figure 4. Structures and biological data of compounds **11** and **12**.

Overall, the results from these studies suggest that *Pf*CDPK1 may not be the most suitable target for *P. falciparum* blood-stage infections. It seems as though the pathways and/or enzymes that are able to compensate for the loss of *Pf*CDPK1 activity [21,23,24] greatly affect the potency of *Pf*CDPK1 inhibitors in vivo. Greater success may be achieved if future drug development focusses on *Pf*CDPK1 as a potential transmission-blocking target.

2.1.2. *Pf*CDPK4

As *Pf*CDPK4 is essential for sexual stage development of *P. falciparum*, it is a promising drug target for developing new transmission-blocking antimalarial drugs. The scaffolds explored thus far for *Pf*CDPK4 inhibition include phenothiazines, pyrazolopyrimidines, imidazopyrazines and 5-aminopyrazole-4-carboxamide derivatives [46–50].

Based on pyrazolopyrimidine BKIs designed for *Toxoplasma gondii* CDPK1 (*Tg*CDPK1) and *Cryptosporidium parvum* CDPK1 (*Cp*CDPK1), a series of pyrazolopyrimidine derivatives (e.g., compounds **13**–**15**, Figure 5) with potent activity against *Pf*CDPK4 were designed [49,50].



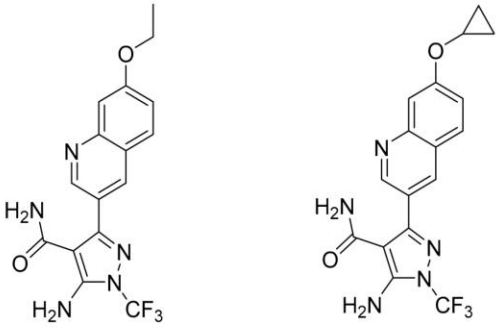
	13	**14**	**15**
*Pf*CDPK4 IC_{50}	4 nM	10 nM	2 nM
*Pf*NF54 blood stage EC_{50}	2 μM	8 μM	ND
*Pf*NF54 exflagellation EC_{50}	35 nM	47 nM	<40 nM

Figure 5. Pyrazolopyrimidine derivatives as inhibitors of *Pf*CDPK4. *Pf*NF54: *Plasmodium falciparum* NF54 strain.

Minimal off-target activity was observed for some of these compounds when tested against human Src and Abl tyrosine kinases, which both have one of the smallest gatekeeper residues (threonine) in the human kinome [49,50]. However, when screened against human non-kinase targets, compound **14** also inhibited the human ether-a-go-go related gene potassium channel (hERG) which is critical for cardiac repolarisation [51]. Pyrazolopyrimidine compounds have been shown to block exflagellation of male gametocytes in *P. falciparum* parasites [49,50] and in *P. berghei*-infected mice [49] within the nanomolar range. When *Anopheles stephensi* mosquitoes were allowed to feed on *P. berghei*-infected mice treated with compound **13** (10 mg/kg, intraperitoneally), oocyst formation was blocked in the mosquito midgut. Similarly, infective sporozoite formation was inhibited in *Anopheles stephensi* mosquitoes that fed on *Pf*NF54-infected human blood containing 3 μM of compound **13** [49]. *P. falciparum* parasites expressing *Pf*CDPK4 with a mutated gatekeeper (small serine residue changed to a large methionine residue, S147M), were insensitive to pyrazolopyrimidine-based compounds and demonstrated normal exflagellation, which confirms *Pf*CDPK4 to be the target of these inhibitors [48,49]. Substituting the pyrazolopyrimidine scaffold with an imidazopyrazine core generally resulted in similar *Pf*CDPK4

selectivity and potency [50]. In silico studies revealed the structure-activity relationships of these pyrazolopyrimidine and imidazopyrazine compounds with the *Pf*CDPK4 target [52].

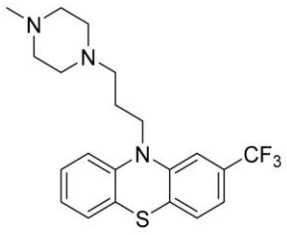
Another scaffold used for *Tg*CDPK1 inhibitor development [53], 5-aminopyrazole-4-carboxamide, was also shown to potently inhibit *Pf*CDPK4 in the nanomolar range [47]. The most active 5-aminopyrazole-4-carboxamide derivatives (compounds **16** and **17**, Figure 6) demonstrated potent inhibition of *P. falciparum* male gametocyte exflagellation at a concentration of 0.1 μM. The in vitro inhibition was much higher than the enzymatic assay predicted, which may indicate multiple targets for these compounds. In terms of selectivity over human kinases, these inhibitors demonstrated high selectivity over Src kinase and hERG.



	16	**17**
*Pf*CDPK4 IC_{50}	37 nM	82 nM
*Pf*NF54 blood stage EC_{50}	9 μM	>20 μM
*Pf*NF54 exflagellation EC_{50}	89%	84%

Figure 6. 5-Aminopyrazole-4-carboxamide derivatives as inhibitors of *Pf*CDPK4.

Members of the phenothiazine class were identified as possible non-ATP-competitive inhibitors of *Pf*CDPK4 [46]. Trifluoperazine (TFP) (**18**, Figure 7) was the most active of this class, with a binding affinity (K_d) of 134.5 μM and K_i value of 150 μM for *Pf*CDPK4. The discrepancy between the enzymatic activity of TFP and the reported in vitro activity (EC_{50}: 1.9 μM) against *P. falciparum* indicates that this compound modulates multiple targets.



18

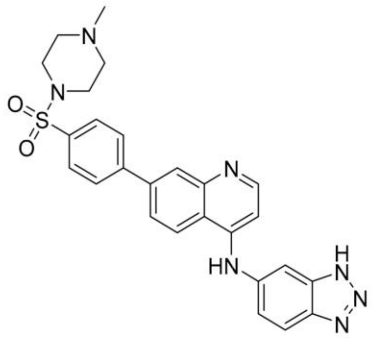
*Pf*CDPK4 K_i	150.0 μM
Pf blood stage EC_{50}	1.9 μM

Figure 7. Structure and biological data of trifluoperazine (TFP).

Homology modelling indicated that TFP possibly binds to the calmodulin-like domain of *Pf*CDPK4 which prevents repositioning of the autoinhibitory J-domain upon binding of Ca^{2+}, thereby locking the kinase in its inactive state.

2.1.3. *Pf*CDPK5

To date, only one study has been published on inhibitor development for *Pf*CDPK5. Rout and Mahapatra predicted the three-dimensional structure of *Pf*CDPK5 through homology modelling using *P. berghei* CDPK1 as a template [54]. Possible inhibitors of *Pf*CDPK5 were then identified through virtual screening of five different sets of compounds with known antimalarial activity. MMV687246 (**19**, Figure 8), from the Malaria box assembled by The Medicines for Malaria Venture, demonstrated the highest binding affinity for *Pf*CDPK5 and was suggested as a possible lead for future experimental validation and inhibitor design.



19

*Pf*CDPK5 binding affinity	−11.6 kcal/mol

Figure 8. Structure and biological data of MMV687246.

3. AGC Group

Three of the five malarial kinases that cluster within this group, namely adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), cyclic guanosine monophosphate (cGMP)-dependent protein kinase G (PKG) and protein kinase B (PKB) have been characterised [13]. cAMP-, cGMP- and calcium-mediated signalling pathways are closely linked within the malaria parasite. In merozoites, a rise in cytosolic cAMP levels leads to activation of PKA and an increase in cytosolic calcium levels via induction of the Epac (exchange protein directly activated by cAMP) pathway [55]. When activated by cGMP, PKG regulates phosphoinositide metabolism which produces inositol (1,4,5)-triphosphate (IP_3), a messenger molecule that signals the release of intracellular calcium [56]. The release of calcium in turn activates stage-specific effector pathways, including CDPK signalling [29,56]. In contrast to the other two kinases, PKB is activated by calmodulin in a calcium-dependent manner [57]. Phospholipase C has been identified as the upstream regulator responsible for releasing the calcium required for PKB activation [57].

During the asexual parasite stages, *Pf*PKA, *Pf*PKG and *Pf*PKB regulate different factors required for parasite invasion and egress. *Pf*PKA phosphorylates the *P. falciparum* apical membrane antigen 1 (*Pf*AMA1) which is critical for tight junction formation between the parasite and the host cell during erythrocyte invasion [58–60]. *Pf*PKA has also been implicated in regulation of parasite motility [61], microneme secretion [55], anion channel conductance at the erythrocytic plasma membrane [62] and the cell cycle of the intraerythrocytic parasite [63]. However, Patel and co-workers [60] reported that events prior to invasion, such as egress, rise in cytosolic calcium levels and microneme secretion, can all occur in the absence of cAMP and *Pf*PKA. Apart from merozoite invasion, they also did not observe

any other critical role for cAMP and *Pf*PKA in the erythrocytic life cycle. *Pf*PKG controls invasion and egress of sporozoites (liver-stage parasites) [29,64] and merozoites (blood-stage parasites) [65–67] by regulating parasite motility and microneme secretion. *Pf*PKB also plays a role in merozoite invasion of erythrocytes by regulating parasite motility [68]. During the sexual parasite stages, *Pf*PKG regulates gametogenesis, which involves male gamete exflagellation and rounding up of female gametes, and ookinete motility required for mosquito midgut invasion [56,69,70]. Both *Pf*PKA and *Pf*PKG have been validated as essential kinases, *Pf*PKA being essential for blood-stage parasites [58,60] and *Pf*PKG being essential for multiple life cycle stages [67,69,71]. *Pf*PKB is regarded as likely essential to blood-stage parasite survival [22].

3.1. Inhibitor Development for the AGC Group

3.1.1. *Pf*PKG

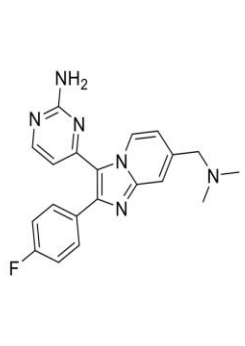
In terms of inhibitor development, *Pf*PKG is one of the plasmodial kinases that has been studied extensively thus far. It is regarded as a very attractive antimalarial drug target as its inhibition offers simultaneous prophylactic, curative and transmission-blocking potential [72]. As *Pf*PKG is an essential enzyme for multiple life cycle stages, there is a relatively low risk of the parasite developing high-grade resistance to *Pf*PKG-selective inhibitors [72]. The PKG enzyme is also highly conserved in all human malaria species, with an overall sequence identity of 90–92% and identical catalytic site and gatekeeper residues [73]. Thus, PKG inhibitors have the potential to be active against multiple malaria species. Although human PKG orthologues (cytosolic PKG-Iα and PKG-Iβ, membrane-associated PKG-II) exist, selective inhibitor development is still possible as there is significant structural divergence between plasmodial and mammalian PKGs [74].

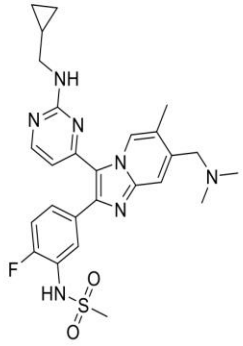
A variety of scaffolds have been explored for *Pf*PKG inhibitor development, with the imidazopyridines [71,75,76] and thiazoles [73,77,78] being the most advanced inhibitors of *Pf*PKG. Compound **20** (Figure 9), a PKG inhibitor of *Eimeria tenella* [79], was used as a lead compound to develop potent imidazopyridine inhibitors of *Pf*PKG [71,75,76]. Compound **21** (Figure 9) was the most potent of a series of imidazopyridines synthesised by Baker and co-workers [71], with an IC_{50} value of 0.16 nM against *Pf*PKG and an EC_{50} value of 2.1 nM against *P. falciparum* blood-stage parasites. Compound **21** demonstrated no toxicity in vitro or in vivo, high selectivity over human kinases and moderate metabolic stability in vitro. Oral administration of compound **21** to *P. falciparum*-infected mice (twice-daily dose of 100 mg/kg) over a period of four days reduced parasitemia below detectable levels. Apart from blood-stage efficacy, compound **21** also inhibited transmission of mature *P. falciparum* gametocytes to *Anopheles stephensi* mosquitoes (IC_{50}: 41.3 nM).

Subsequent studies focussed on improving the ADME properties of this chemical class while retaining potent inhibitory activity. By means of structure-activity relationship and modelling studies, Large and co-workers [75,76] systematically varied the substituents of compound **20** and explored other bicyclic cores. Compounds **22** [75] and **23** [76] (Figure 9) retained the potent *Pf*PKG and in vitro antimalarial activity of compound **20**. Compound **22** also retained the LogD and lipophilic ligand efficiency of compound **20**, and showed improved permeability, but demonstrated poor stability in mouse liver microsomes [75]. Compound **23** demonstrated an excellent balance of activity and physicochemical properties. In addition to good LogD and LLE, compound **23** showed improved microsomal stability and excellent selectivity over human kinases, including the two human PKG orthologues (PKG1α and PKG1β) [76].

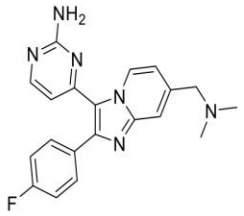
A 2,3-diaryl-pyrrole inhibitor of PKG developed for *Eimeria tenella* (compound **24**, Figure 10) [80] was shown to be a potent inhibitor of *Pf*PKG (IC_{50}: 3.5 nM) [81]. However, compound **24** only demonstrated modest in vitro activity against *P. falciparum* and failed to reduce parasitemia in a *P. berghei* mouse model [81]. A scaffold-hopping approach performed on compound **24** lead to the identification of thiazoles (compound **25**) as *Pf*PKG inhibitors [78]. Substitution of the thiazole scaffold was optimised to improve the enzymatic and in vitro activity of compound **25**, which lead to compound

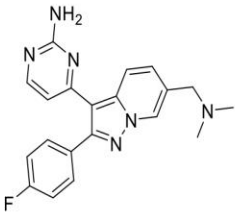
26 (IC_{50}: 2 nM, EC_{50}: 113 nM) [78]. Compound **26** demonstrated excellent selectivity over human kinases, good permeability and metabolic stability in mouse and human liver microsomes.

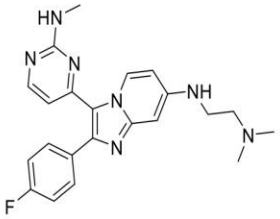




	20	**21**
*Pf*PKG IC_{50}	3.1 nM	0.16 nM
*Pf*3D7 blood stage EC_{50}	395.0 nM	2.10 nM
Pf transmission-blocking IC_{50}	507.3 nM	41.3 nM
In vivo *Pf* mouse model (% parasitemia reduction, oral dose)	ND	99.9% 100 mg/kg Twice daily, 4 days

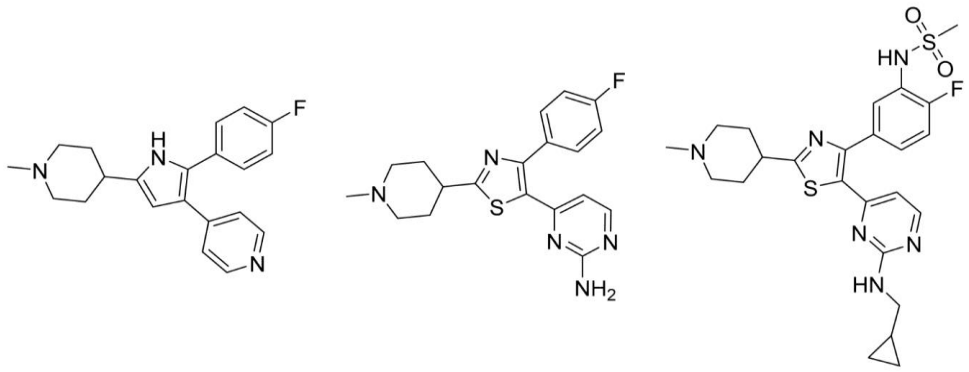






	20	**22**	**23**
*Pf*PKG pI_{50}	8.7	8.4	8.6
LLE	6.3	5.9	6.4
LogD	2.4	2.5	2.2
MLM	52%	22%	93%

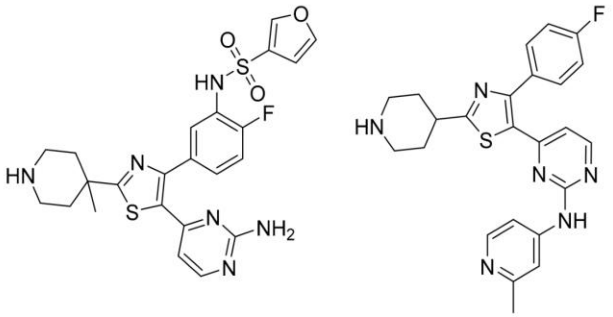
Figure 9. Imidazopyridine-based compounds as inhibitors of *Pf*PKG. pI_{50}: negative log of the IC_{50} value in molar; LLE: lipophilic ligand efficiency; LogD: distribution coefficient; MLM: % remaining after 30 min incubation with mouse live microsomes.



	24	**25**	**26**
*Pf*PKG IC_{50}	3.5 nM	17 nM	2 nM
*Pf*3D7 blood stage EC_{50}	490 nM	1.8 μM	113 nM

Figure 10. 2,3-Diaryl-pyrrole and thiazole compounds as inhibitors of *Pf*PKG.

During a high-throughput screening campaign, Penzo and co-workers [73] also identified several thiazole derivatives, such as compound **27** (Figure 11), with nanomolar potencies against *Pf*PKG [73]. The in vitro activity of the most potent compounds was studied at 48 and 72 h in wild-type and transgenic (*Pf*PKG gatekeeper mutant, T618Q) *P. falciparum* blood-stage parasites, and the EC_{50} values against the two strains were very similar—indicating that the thiazole derivatives also inhibit targets other than *Pf*PKG. Additional targets identified for compound **27** included CDPK1, CDPK4, CDK-related kinase (Pfcrk-5), NIMA-related kinase (*Pf*nek-1), CK1 and an unnamed putative protein kinase (Pf3D7_0926100). Compound **27** also demonstrated good solubility, no toxicity against HepG2 cells, and selectivity over the human PKG orthologue (PKGIα), human lymphocyte-specific protein tyrosine kinase (LCK) and human Aurora B kinase. Besides blood-stage activity, compound **27** also had potent activity against male and female gametes.

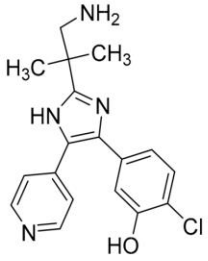


	27	**28**
*Pf*PKG IC_{50}	0.8 nM	4.4 nM
*Pf*3D7 blood stage IC_{50} at 48 h	0.19 μM	0.13 μM
*Pf*3D7 blood stage IC_{50} at 72 h	0.17 μM	0.09 μM
*Pf*NF54 male gamete IC_{50}	0.5 μM	0.3 μM
*Pf*NF54 female gamete IC_{50}	0.8 μM	0.4 μM

Figure 11. Thiazole derivatives as inhibitors of multiple plasmodial kinases.

Matralis and co-workers [77] specifically focussed on developing a series of thiazole derivatives with a fast-killing profile similar to that of artemisinins. Compound **28** (Figure 11) was the most potent in this series, with in vitro nanomolar activity against *Pf*PKG, *P. falciparum* blood-stage parasites and gametocytes. It also possessed good physicochemical properties. Despite good selectivity over human enzymes, ion channels and receptors, this compound showed activity towards hERG. Parasite reduction ratio studies demonstrated that compound **28** had fast-killing properties similar to those of artesunate. CDPK1, CDPK4 and serine/arginine protein kinase 2 (SRPK2, also known as CLK2) were identified as additional targets of compound **28**. The fast-killing activity of compound **28** was mainly attributed to SRPK2 inhibition. This study demonstrates the potential of simultaneously targeting *Pf*PKG and SRPK2 to develop fast-killing drugs with curative and transmission-blocking activity.

Vanaerschot and co-workers [72] identified *Pf*PKG as the primary target of the Medicines for Malaria Venture compound MMV030084 (**29**, Figure 12). MMV030084 had an IC_{50} value of 0.4 nM against recombinant *Pf*PKG, and docking studies using the *Pf*PKG crystal structure (PDB ID: 5DYK) showed a strong interaction between MMV030084 and the ATP-binding site. When evaluated against the different life cycle stages of the parasite, MMV030084 inhibited sporozoite invasion of hepatocytes, merozoite egress from mature schizonts, and male gamete exflagellation. MMV030084 inhibited liver cell invasion by *P. berghei* parasites (IC_{50}: 199 nM) with minimal toxicity against the host cells (CC_{50}: 41.5 µM). The development of *P. falciparum* blood-stage parasites was halted at the schizont stage when treated with MMV030084 (drug-sensitive strain IC_{50}: 109 nM; multidrug-resistant strain IC_{50}: 120 nM). Male gamete exflagellation of the *P. falciparum* NF54 strain was inhibited by MMV030084 with an IC_{50} value of 141 nM. In vitro MMV030084 resistance selection studies identified *P. falciparum* tyrosine kinase-like 3 (*Pf*TKL3) as an MMV030084-resistance mediator, which allows merozoite egress from erythrocytes when mutated. No mutation was identified for *Pf*PKG itself.

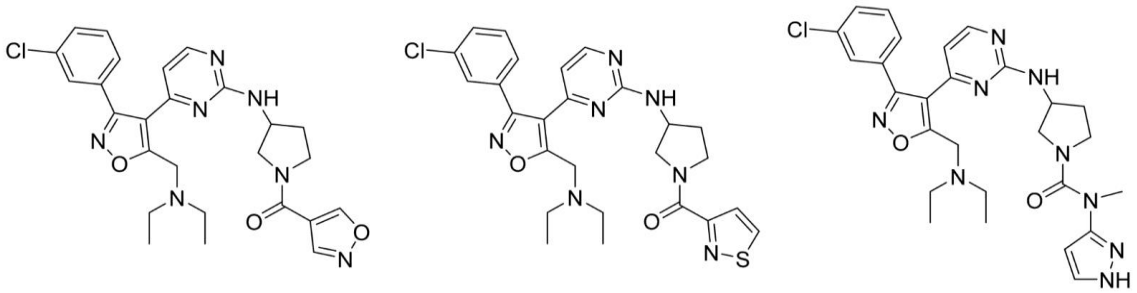


29

*Pf*PKG IC_{50}	0.4 nM
*Pf*3D7 blood stage IC_{50}	109 nM
*Pf*Dd2 blood stage IC_{50}	120 nM
Pb liver stage IC_{50}	199 nM
*Pf*NF54 male gamete IC_{50}	141 nM

Figure 12. Structure and biological data of MMV030084.

A screening campaign by Mahmood and co-workers [82] identified isoxazole-based inhibitors with *Pf*PKG activity and selectivity over human PKG. Optimisation of this scaffold led to compounds **30**–**32** (Figure 13) with IC_{50} values <20 nM against *Pf*PKG. Further evaluation of the physicochemical properties and in vitro activity of these compounds against the whole-cell parasite has not yet been reported.

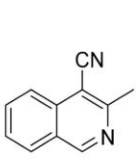


	30	**31**	**32**
*Pf*PKG IC_{50}	13 nM	15 nM	10 nM

Figure 13. Isoxazole-based inhibitors of *Pf*PKG.

3.1.2. *Pf*PKA and *Pf*PKB

Drug discovery efforts targeting *Pf*PKA and *Pf*PKB are limited. Buskes and co-workers [83] attempted to develop *Pf*PKA inhibitors using the commercially available PKA inhibitor 3-methylisoquinoline-4-carbonitrile (**33**, Figure 14) as a starting point. They studied the interactions of compound **33** and a series of substituted isoquinolines (e.g., **34**, Figure 14) with a *Pf*PKA homology model. In vitro evaluation of this series demonstrated low micromolar activity against drug-sensitive (3D7) and -resistant (W2) *P. falciparum* strains. However, biochemical evaluation of this series showed minimal activity against PKA [84]. It was suggested that these compounds likely inhibit another kinase that is involved in parasitic cytokinesis and erythrocyte invasion [84].



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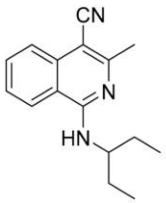
Rat PKA IC_{50}: 30 nM



34

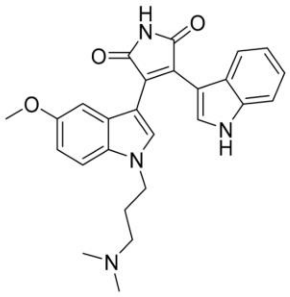
*Pf*3D7 blood stage EC_{50}: 1.0 μM

*Pf*W2 blood stage EC_{50}: 0.7 μM

Residual human PKA activity: 92%

Figure 14. 3-Methylisoquinoline-4-carbonitrile derivatives and their biological data.

As *Pf*PKB shares high sequence homology with the catalytic sites of mammalian PKB and protein kinase C (PKC), established PKB and PKC inhibitors were tested for activity against *Pf*PKB. Go 6983 (**35**, Figure 15), a PKC inhibitor, was shown to inhibit *Pf*PKB (IC_{50}: ±1 μM) and significantly reduce *P. falciparum* parasite growth at the late schizont stage [85]. The mammalian PKB inhibitor A443654 (**36**, Figure 15) inhibited *Pf*PKB with an IC_{50} value of 200 nM [57]. Incubation of *P. falciparum* schizonts with A443654 did not affect the morphology or the number of schizonts but dramatically reduced the number of ring-stage parasites formed after invasion. This indicates that A443654 blocks invasion, which corroborates the function of *Pf*PKB in erythrocytic invasion. A peptide inhibitor that corresponds to the pseudosubstrate motif of the *Pf*PKB N-terminal effectively inhibited *Pf*PKB activity and also reduced the formation of *P. falciparum* ring-stage parasites [57].



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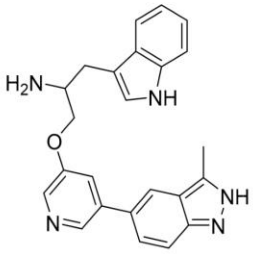


36

*Pf*PKB IC_{50}	1 μM	200 nM

Figure 15. Structures and biological data of Go 6983 and A443654.

Crystal structures of *Pf*PKA's regulatory subunit (PDB ID: 5K8S, 5KBF and 5T3N) [86] and methods for expression and purification of active recombinant *Pf*PKAr (regulatory subunit) [86] and *Pf*PKAc (catalytic subunit) [87] are available for future drug development efforts for this target. The crystal structure of *Pf*PKB is not yet available; however, the full-length *Pf*PKB gene has been successfully expressed and purified as an active recombinant protein [57,68,85].

4. CMGC Group

The CMGC kinase family consists of cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), glycogen synthase kinase-3 (GSK-3) and CDK-like kinases (CLKs).

4.1. Cyclin-Dependent Kinases (CDKs)

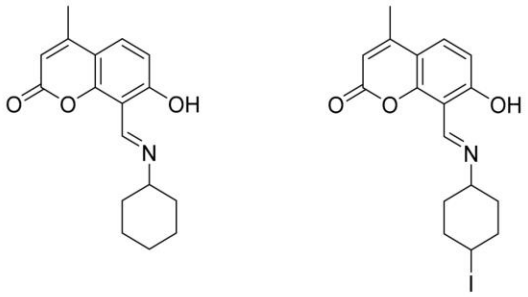
Eukaryotic cyclins and CDKs are essential regulators of the cell cycle [88]. Several CDK homologues have been identified in the *P. falciparum* parasite, namely protein kinase 5 (*Pf*PK5), protein kinase 6 (*Pf*PK6), MO15-related kinase (*Pf*mrk) and the CDK-related kinases (*Pf*crk-1, *Pf*crk-3, *Pf*crk-4, *Pf*crk-5) [88,89].

*Pf*PK5 is most related to mammalian CDK1 and CDK5 [90] and demonstrates sensitivity to mammalian CDK1/CDK2 inhibitors [91]. It has been proposed that *Pf*PK5 is likely involved in the regulation of DNA replication (S-phase of the cell cycle) during erythrocytic schizogony [90,92,93]. *Pf*mrk, a putative homologue of mammalian CDK7, is predominantly expressed in gametocytes and to a lesser extent in the asexual stages (trophozoites and schizonts) [94]. *Pf*mrk is localised in the nucleus of the parasite and is presumably involved in the regulation of DNA replication [95]. *Pf*crk-1 and *Pf*crk-3 display maximal homology to mammalian CDKs involved in transcriptional control [13,96]. *Pf*crk-1 is mainly expressed in gametocytes [97]; however, the *P. berghei* orthologue (*Pb*crk-1) was found to be essential for erythrocytic schizogony [98]. *Pf*crk-3 has been demonstrated to be essential to erythrocytic parasites and presumably regulates gene expression via interaction with chromatin modification enzymes [96]. *Pf*crk-4 is another essential enzyme for asexual proliferation and also plays a critical role in ookinete formation and early oocyst development [22,99]. Two atypical CDKs, namely *Pf*PK6 and *Pf*crk-5, demonstrate both CDK and mitogen-activated protein kinase (MAPK) homology [100,101]. Nuclear and cytoplasmic localisation have been reported for *Pf*PK6 in trophozoite and schizont parasites [100]. Unlike *Pf*PK5 and *Pf*mrk which are activated by various cyclins in vitro [102,103], *Pf*PK6 appears to be a cyclin-independent kinase [100]. *Pf*crk-5 is a cyclin-dependent enzyme localised in the nuclear periphery [101]. Although parasites lacking *Pf*crk-5 are viable, they display decreased erythrocytic proliferation due to a lower number of merozoites released per schizont [101].

4.1.1. CDK Inhibitor Development

*Pf*PK5

Developing *Pf*PK5-selective inhibitors is quite challenging due to the high degree of homology between *Pf*PK5 and the human CDKs. Eubanks and co-workers [104] demonstrated that a target-based screening approach was more effective to identify *Pf*PK5-selective inhibitors than chemically modifying existing CDK inhibitors. The 4-methylumbelliferone analogues, compounds **37** and **38** (Figure 16), demonstrated a 2-fold binding affinity for *Pf*PK5 over human CDK2 (*Hs*CDK2). No significant toxicity was observed against human hepatoma cell lines (HuH7 and HepG2) for either compound. However, both compounds failed to inhibit drug-resistant *P. falciparum* (Dd2 strain) asexual parasite growth in vitro, most likely due to poor physicochemical properties.



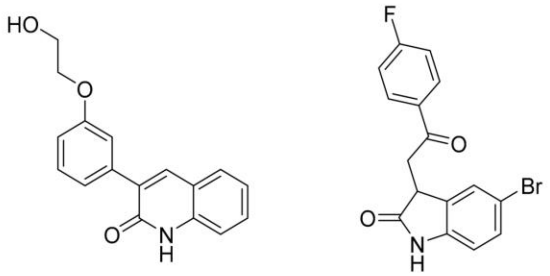
	37	**38**
*Pf*PK5 $K_{d(app)}$	5 μM	11 μM
*Hs*CDK2 $K_{d(app)}$	14 μM	28 μM
*Pf*Dd2 blood stage EC_{50}	>20 μM	>20 μM

Figure 16. 4-Methylumbelliferone inhibitors of *Pf*PK5 and their biological data. $K_{D(app)}$: apparent dissociation constant.

*Pf*mrk

Scaffolds studied for *Pf*mrk inhibition include quinolinones [105], oxindoles [106], chalcones [107], flavonoids [108] and sulfonamide-based compounds [109–111].

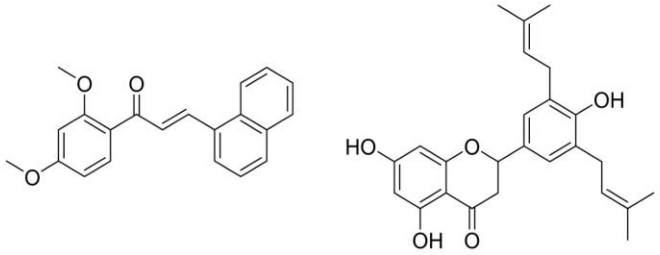
A series of quinolinones (e.g., compound **39**, Figure 17) demonstrated *Pf*mrk activity with IC_{50} values ranging from 18 to 539 μM [105]. Neither antimalarial evaluation against whole-cell parasites nor molecular docking has been performed for this chemical class. Based on the structure of commercially available indirubin-3′-monoxime, a moderate inhibitor of *Pf*mrk and in vitro *P. falciparum* parasite growth, oxindole-based compounds were explored as inhibitors of *Pf*mrk [106]. Several oxindoles selectively inhibited *Pf*mrk in the low micromolar range, with the most potent being compound **40** (Figure 17). None of the oxindoles demonstrated any significant in vitro activity against whole-cell parasites. Homology modelling showed that these compounds had the same orientation in the active site of *Pf*mrk as in human CDK2, but with additional contact points which might be responsible for the *Pf*mrk specificity.



	39	**40**
*Pf*mrk IC_{50}	18 μM	1.4 μM
*Pf*D6 blood stage EC_{50}	ND	>14.4 μM
*Pf*W2 blood stage EC_{50}	ND	>14.4 μM

Figure 17. Quinolinone and oxindole derivatives as inhibitors of *Pf*mrk.

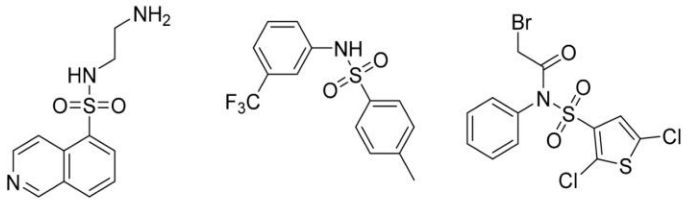
The chalcone and sulfonamide-based scaffolds were identified by means of a three-dimensional structure-activity relationship (3D-QSAR) pharmacophore model [109,112]. Compound **41** (Figure 18) was the most active compound of a series of chalcones tested against *Pf*mrk (IC_{50}: 1.3 μM) [107]. However, a weak correlation was observed between the *Pf*mrk activity and the in vitro activity against drug-sensitive (D6) and -resistant (W2) *P. falciparum* strains. As several mechanisms of action have been reported for the antimalarial activity of chalcones [113–115], Geyer and co-workers [107] proposed that *Pf*mrk inhibition might be an additional mechanism demonstrated by some chalcones. Several flavonoids isolated from *Erythrina* sp., were evaluated for activity against *Pf*mrk [108]. The most potent flavonoid, Abyssinone V (**42**), had an IC_{50} value of 0.038 μM. Despite potent *Pf*mrk activity, the flavonoids demonstrated similar in vitro antimalarial activity to that of the chalcones, presumably due to the lower permeability of the flavonoids.



	41	**42**
*Pf*mrk IC_{50}	1.3 μM	0.04 μM
*Pf*D6 blood stage EC_{50}	4.9 μM	2.4 μM
*Pf*W2 blood stage EC_{50}	4.6 μM	3.6 μM

Figure 18. Structures and biological data of chalcone and flavonoid-based inhibitors of *Pf*mrk.

Isoquinoline sulfonamides were generally weak inhibitors of *Pf*mrk, except compound **43** (Figure 19) which demonstrated an IC_{50} value of 0.7 μM [111]. However, compound **43** failed to inhibit drug-sensitive (D6) and -resistant (W2) *P. falciparum* parasite growth. Thiophene sulfonamides exemplified by **44** and **45** (Figure 19) were found to be potent inhibitors of *Pf*mrk with IC_{50} values in the submicromolar range [110]. However, all of these compounds, except compound **45**, are also potent inhibitors of human CDK7. Thiophene sulfonamides demonstrated minimal cytotoxicity and moderate in vitro activity against a multidrug-resistant *P. falciparum* (W2) strain.



	43	**44**	**45**
*Pf*mrk IC_{50}	0.7 μM	0.7 μM	0.6 μM
*Pf*D6 blood stage EC_{50}	>72 μM	ND	ND
*Pf*W2 blood stage EC_{50}	>72 μM	31.7 μM	23.3 μM

Figure 19. Sulfonamide-based inhibitors of *Pf*mrk.

*Pf*PK6 and CDK-Related Kinases

To our knowledge, no medicinal chemistry campaigns have focussed on drug development for *Pf*PK6 and the CDK-related kinases (*Pf*crk-1 to -5). Homology models have been developed for *Pf*PK6 [116,117] and *Pf*crk-4 [99], which can be used for virtual screening of small-molecule inhibitors against these targets. Methods for expression and purification of active recombinant *Pf*PK6 [100], *Pf*crk-1 (kinase domain) [118], *Pf*crk-3 [96] and *Pf*crk-5 [101] are also available in the literature.

4.2. Mitogen-Activated Protein Kinases (MAPKs)

The *P. falciparum* kinome encodes two MAPK homologues, namely *Pf*map-1 and *Pf*map-2. Interestingly, *Pf*map-2 is essential for the asexual stages of *P. falciparum* parasites [119]; however, the *P. berghei* orthologue (*Pb*map-2) is only essential for male exflagellation in the mosquito midgut [120]. *Pf*map-1 also plays an important role during asexual development; however, parasites are able to compensate for loss of *Pf*map-1 activity by upregulating *Pf*map-2 [119].

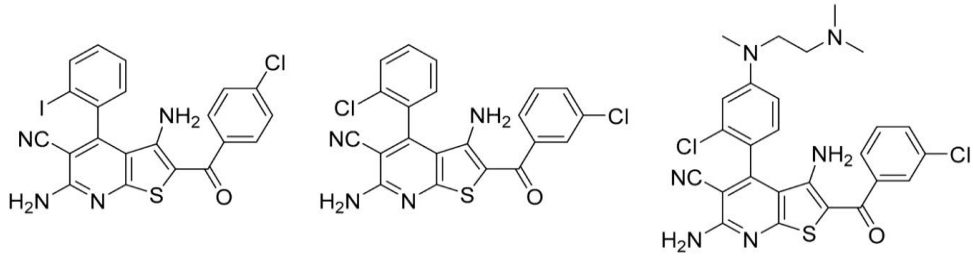
Human p38 MAPK inhibitors have been shown to inhibit drug-sensitive (HB3) and -resistant *P. falciparum* strains in vitro; however, antiplasmodial activity has yet to be attributed to plasmodial MAPK inhibition [121].

4.3. Glycogen Synthase Kinase-3 (GSK-3)

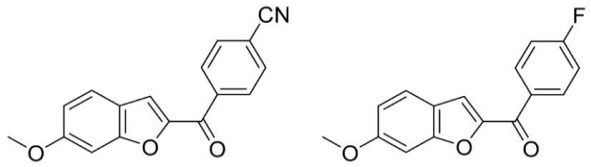
*Pf*GSK-3 is one of three GSK3-related kinases identified in the *P. falciparum* parasite [13]. Although *Pf*GSK-3 is expressed throughout the erythrocytic stage, it is predominantly expressed during the early trophozoite stage [122]. After expression, *Pf*GSK-3 is rapidly transported to the cytoplasm of the erythrocyte where it appears to associate with membranous structures known as Maurer's clefts [122]. The exact biological functions of *Pf*GSK-3 remain to be elucidated; however, it has been demonstrated to be essential for the survival of asexual erythrocytic parasites [22].

*Pf*GSK-3 Inhibitor Development

Despite the high degree of homology between *Pf*GSK-3 and mammalian GSK-3, significant structural differences exist that can be exploited for *Pf*GSK-3-selective inhibition [122–125]. Fugel and co-workers [123] designed a novel series of 4-phenylthieno [2,3-*b*]pyridine based on structures of hits (e.g., compound **46**, Figure 20) identified during a high-throughput screening campaign. Compounds from this series, such as compound **47** (Figure 20), selectively inhibited *Pf*GSK-3 (IC_{50}: 0.48 μM) over human GSK-3 (GSK-3α IC_{50}: >100 μM; GSK-3β IC_{50}: 3.3 μM) and demonstrated broad selectivity when tested against two panels of mammalian kinases. Compound **47** also had in vitro activity against erythrocytic *P. falciparum* (NF54-Luc) parasites (EC_{50}: 5.5 μM). Masch and co-workers [126] improved the solubility and antimalarial activity of the 4-phenylthieno[2,3-*b*]pyridine compounds by attaching an additional aliphatic polar side chain to the *para*-position of the 4-phenyl ring. Compared to compound **47**, compound **48** (Figure 20) exhibited 4.5-fold higher antiplasmodial activity against erythrocytic *P. falciparum* (NF54-Luc) parasites (EC_{50}: 1.2 μM), as well as improved solubility (**47**: 4.8 μM; **48**: 1.5 μM). Recently, we identified a series of benzofuran-based compounds as potent and selective inhibitors of *Pf*GSK-3 ([127], submitted for publication). The most promising benzofurans, compounds **49** and **50** (Figure 20), inhibit *Pf*GSK-3 in nanomolar concentrations and demonstrate 316-fold and 175-fold selectivity for *Pf*GSK-3 over human GSK-3.



	46	**47**	**48**
*Pf*GSK-3 IC_{50}	1.6 μM	0.5 μM	0.7 μM
Mammalian GSK-3β IC_{50}	>100 μM	3.3 μM	40.2 μM
*Pf*NF54 blood stage EC_{50}	ND	5.5 μM	1.2 μM



	49	**50**
*Pf*GSK-3 IC_{50}	0.5 nM	1.7 nM
Human GSK-3β IC_{50}	155.0 nM	294.0 nM

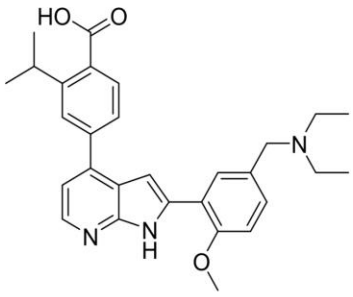
Figure 20. 4-Phenylthieno[2,3-*b*]pyridine and benzofuran-based inhibitors of *Pf*GSK-3.

4.4. CDK-Like Protein Kinases (CLKs)

Four members of the CLK family have been identified in *P. falciparum*, *Pf*CLK-1 to -4 [13]. All four enzymes are essential to the asexual erythrocytic parasites [22,128] as they regulate mRNA splicing through phosphorylation of serine/arginine-rich (SR) proteins [129]. *Pf*CLK1 and *Pf*CLK2 exhibit homology to the yeast SR protein, Sky1p, and are expressed throughout the erythrocytic stage and in gametocytes [128]. Both kinases are localised within the nucleus of the parasite, with *Pf*CLK2 also present in the cytoplasm [128]. *Pf*CLK3 is a closely related homologue of human pre-mRNA processing factor 4B (PRP4 or PRPF4B) [130] which regulates mRNA splicing through phosphorylation of proteins associated with the spliceosome complex [131]. *Pf*CLK4, also known as SR protein-specific kinase 1 (SRPK1), is expressed in erythrocytic parasites and abundantly in gametocytes [132,133]. *Pf*CLK4 negatively regulates mRNA splicing by phosphorylating a putative plasmodial SR protein (*Pf*SR1) in vitro [132,134]. Both *Pf*CLK4 and *Pf*SR1 are localised inside the nucleus of ring and early trophozoite stage parasites. As erythrocytic development progresses, the two proteins are exported to the nuclear periphery (mature trophozoites) and finally to the cytoplasm (schizonts and gametocytes) [132,134].

CLK Inhibitor Development

A high-throughput screening campaign identified TCMDC-135051 (**51**, Figure 21) as a highly potent and selective inhibitor of *Pf*CLK3 [135]. TCMDC-135051 demonstrated selectivity for *Pf*CLK3 over closely related human kinases (CLK2 and PRPF4B), the closest related plasmodial kinase (*Pf*CLK1) and two other plasmodial kinases (*Pf*PKG and *Pf*CDPK1). *Pf*CLK3 mutations were observed in *P. falciparum* parasites with reduced sensitivity to TCMDC-135051, which indicated that *Pf*CLK3 was the primary target of this compound. A recombinant *Pf*CLK3 variant with a G449P mutation and parasites expressing the G449P mutant *Pf*CLK3 both demonstrated reduced TCMDC-135051 sensitivity, confirming that the antimalarial activity of TCMDC-135051 was due to *Pf*CLK3 inhibition. TCMDC-135051 inhibition resulted in downregulation of 425 essential *P. falciparum* genes and upregulation of certain genes involved in RNA processing, which is consistent with the proposed mRNA splicing role of *Pf*CLK3. TCMDC-135051 was active against multiple stages of the parasite's life cycle, including liver-stage sporozoites, blood-stage parasites, gametocyte development and subsequent transmission to the mosquito vector. TCMDC-135051 also demonstrated activity against CLK3 of *P. vivax* (*Pv*CLK3) and *P. berghei* (*Pb*CLK3), as well as in vitro activity against *P. knowlesi* and *P. berghei* blood-stage parasites. A dose-dependent reduction in parasitemia was observed when TCMDC-135051 was administered intraperitoneally to *P. berghei*-infected mice (twice daily, 5-day period), with the maximal dose (50 mg/kg) reducing parasitemia below detectable levels.



	51
*Pf*CLK3 IC_{50}	40 nM
*Pv*CLK3 IC_{50}	33 nM
*Pb*CLK3 IC_{50}	13 nM
*Pf*Dd2 blood stage EC_{50}	450 nM
Pb liver sporozoites EC_{50}	400 nM
In vivo *Pb* mouse model (% parasitemia reduction, peritoneal dose)	99.9% 50 mg/kg Twice daily, 5 days

Figure 21. Structure and biological data of TCMDC-135051.

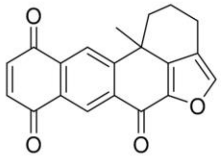
5. Casein Kinase 1 (CK1) Group

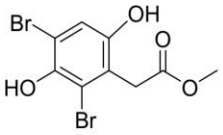
The *P. falciparum* kinome encodes a single CK1 enzyme (*Pf*CK1) that is expressed throughout the erythrocytic stages. *Pf*CK1 is essential for blood-stage parasite survival [22] and is likely involved in cellular processes such as mRNA splicing, protein trafficking and erythrocyte invasion [136,137]. To our knowledge, no medicinal chemistry programs have targeted this enzyme thus far.

6. NIMA- and Aurora-Related Kinases

Four Never in Mitosis, gene A (NIMA)-related kinases or NEKs (*Pf*nek-1 to -4) [12,13] and three Aurora-related kinases (*Pf*ark-1 to -3) [138] have been identified for *P. falciparum*. Both groups of kinases are involved in the regulation of the parasitic cell cycle [88,139]. *Pf*nek-1 and the three Aurora-related kinases are likely essential for erythrocytic schizogony, while *Pf*nek-2 and *Pf*nek-4 are essential for sexual development of the parasite [139].

With regards to inhibitor development, compounds (e.g., **52** and **53**, Figure 22) isolated from marine sponges were found to inhibit *Pf*nek-1 and demonstrate in vitro *P. falciparum* activity [140–142]. Human Aurora kinase inhibitors also demonstrated antiplasmodial activity in vitro; however, the activity is yet to be attributed to plasmodial Aurora-related kinase inhibition [143].





	52	**53**
*Pf*nek-1 IC_{50}	1 μM	1.8 μM
*Pf*FCB1 blood stage EC_{50}	3 μM	12.0 μM

Figure 22. Structures of marine sponge compounds as inhibitors of *Pf*nek-1. *Pf*FCB1: *Plasmodium falciparum* FCB1 strain.

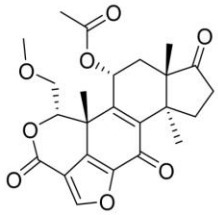
7. Phosphoinositide Lipid Kinases (PIKs)

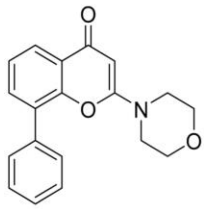
Five *P. falciparum* PIKs have been identified that are phylogenetically divergent from human PIKs [144]. The phosphoinositide 3-kinase (*Pf*PI3K) and phosphatidylinositol 4-kinase (*Pf*PI4K) homologues are both essential for parasite survival and most likely involved in cellular signalling and trafficking [145–147].

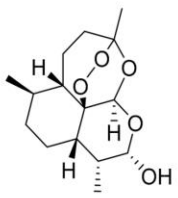
7.1. PIK Inhibitor Development

7.1.1. PI3K

To date, only two compounds have been identified for studying the functions of *Pf*PI3K. The mammalian PI3K inhibitors (Figure 23) wortmannin (**54**) and LY294002 (**55**) both inhibit *Pf*PI3K and blood-stage *P. falciparum* parasite growth [146,147]. *Pf*PI3K was also identified as a target of dihydroartemisinin (**56**, Figure 23) during the early ring stages [148].







	54	**55**	**56**
*Pf*PI3K IC_{50}	1.8 μM	1.8 μM	4 nM
Pf blood stage EC_{50}	2.5 μM	26.0 μM	3 nM

Figure 23. Structures of wortmannin, LY294002 and dihydroartemisinin.

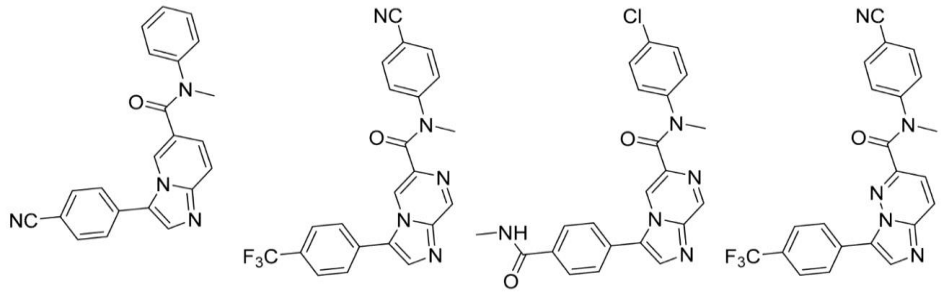
7.1.2. PI4K

Considerable progress has been made with regards to inhibitor development for PI4K, with one candidate progressing into clinical trial evaluation. The imidazopyridine/ pyrazine/ pyridazine class [145,149–152], the aminopyridine/pyrazine class [153–156] and the bipyridine sulfonamide [157] scaffolds have been evaluated for PI4K inhibition.

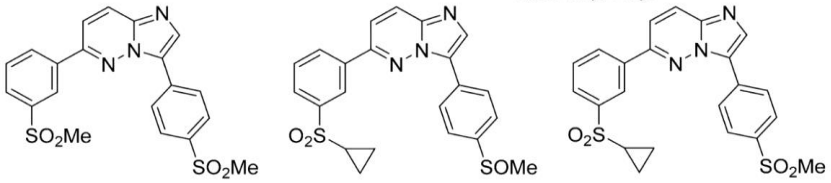
A phenotypic screening identified compound **57** (Figure 24), which demonstrated activity against *P. falciparum* blood-stage parasites but was inactive against *P. yoelii* (*Py*) and *P. cynomolgi* (*Pc*) liver-stage parasites [152]. Liver-stage activity was acquired by replacing the imidazopyridine core of compound **57** with an imidazopyrazine core (KAI407, **58**, Figure 24). From a series of imidazopyrazines, compound **59** (KDU691, Figure 24) had optimal antimalarial activity (blood and liver stages) and physicochemical properties, which translated into in vivo efficacy against *P. berghei*-infected mice [152]. McNamara and co-workers [145] further demonstrated that compound **59** reduced liver- and blood-stage parasites, gametocyte viability and transmission to the mosquito vector for multiple *Plasmodium* species. Plasmodial PI4K was identified as the direct target of imidazopyrazine compounds [145].

Le Manach and co-workers [150] synthesised a series of imidazopyrazines based on structures of hits identified during a screening campaign. Compound **61** (Figure 24) was highly active against drug-sensitive (NF54) and -resistant (K1) *P. falciparum* strains and reduced parasitemia by 98% in *P. berghei*-infected mice (4 × 50 mg/kg). However, compound **61** failed to produce significant in vivo efficacy at lower doses, displayed poor solubility and showed activity towards hERG. Further optimisation of this scaffold led to compound **62** (Figure 24), which was completely curative in *P. berghei*-infected mice (4 × 50 mg/kg) and retained high in vivo efficacy at lower doses [151]. Compound **62** acted as a prodrug which was rapidly metabolised to the highly active sulfone (compound **63**, Figure 24) in vivo.

Compound **64** (Figure 25) was identified during a high-throughput screening campaign against drug-sensitive (NF54) and -resistant (K1) *P. falciparum* strains [155]. Optimisation of the 2-aminopyridine scaffold resulted in compound **65** (MMV390048 or MMV048, Figure 25), which demonstrated in vitro and in vivo activity against the liver, blood and sexual (gametocyte) stages of the parasite [155,158]. Whole-genome screening of MMV048-resistant *P. falciparum* strains and chemoproteomic profiling identified plasmodial PI4K as the target of MMV048 [158]. MMV048 showed high selectivity over human and other plasmodial kinases, a good ADME profile and an acceptable preclinical safety profile in various animal species (mice, rats, dogs and monkeys) [155,158]. Phase I clinical trials for MMV048 were recently completed (ClinicalTrials.gov: NCT02230579; NCT02281344; NCT02554799) [159]. A single oral dose of up to 120 mg was generally well tolerated in healthy volunteers and adverse events were mild to moderate. Treatment with 20 mg of MMV048 initially reduced parasitemia in volunteers with induced *P. falciparum* blood-stage malaria; however, recrudescence occurred 2 to 7 days after treatment. Formulation influences the pharmacodynamic profile of MMV048, with the tablet formulation resulting in significantly less variability than the powder-in-a-bottle formulation. MMV048 progressed to phase 2a clinical trials in 2017, where its activity was evaluated in Ethiopian adults with either uncomplicated *P. falciparum* or *P. vivax* infection (ClinicalTrials.gov: NCT02880241).

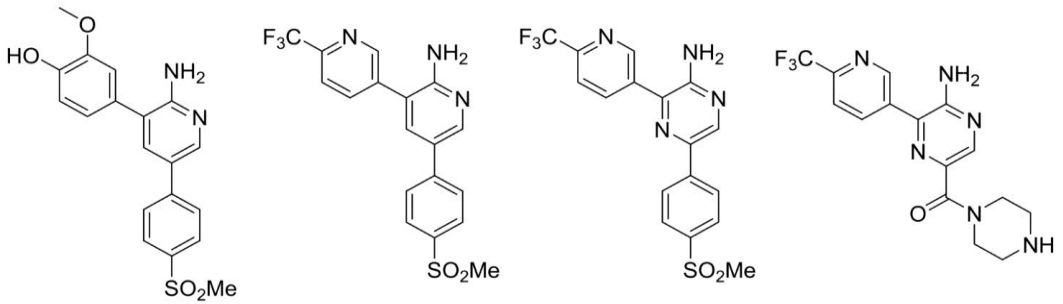


	57	**58**	**59**	**60**
Pf blood stage EC_{50}	0.60 μM	0.06 μM	0.058 μM	0.004 μM
Py liver schizonts EC_{50}	>10 μM	0.16 μM	0.036 μM	0.009 μM
Pc hypnozoites EC_{50}	>10 μM	0.81 μM	0.196 μM	ND
*Pv*PI4K IC_{50}	ND	ND	1.5 nM	ND
In vivo *Pb* mouse model (% parasitemia reduction, oral dose)	ND	ND	99.6% 50 mg/kg Twice daily, 4 days	ND



	61	**62**	**63**
*Pf*NF54 blood stage EC_{50}	7.3 nM	1.1 nM	0.9 nM
*Pf*K1 blood stage EC_{50}	6.3 nM	ND	0.5 nM
In vivo *Pb* mouse model (% parasitemia reduction, oral dose)	98% 50 mg/kg Once daily, 4 days	99.8% 50 mg/kg Once daily, 4 days	ND

Figure 24. Imidazopyridine-, pyrazine- and pyridazine-based compounds as inhibitors of plasmodial PI4K.

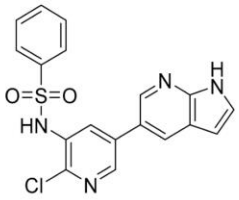


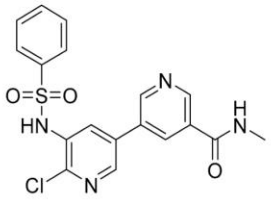
	64	**65**	**66**	**67**
*Pv*PI4K IC_{50}	10 nM	3.4 nM	1.5 nM	23 nM
*Pf*NF54 blood stage EC_{50}	49 nM	28 nM	10.0 nM	5.4 nM
*Pf*K1 blood stage EC_{50}	49 nM	25 nM	8.4 nM	5.2 nM
In vivo *Pb* mouse model				
ED_{90}	ND	1.1 mg/kg	ND	1.0 mg/kg
In vivo *Pf* mouse model				
ED_{90}	ND	0.57 mg/kg	0.12 mg/kg	0.25 mg/kg

Figure 25. Structures and biological data of aminopyridine- and pyrazine-based compounds as inhibitors of plasmodial PI4K.

Younis and co-workers [156] replaced the 2-aminopyridine core of MMV048 with a 2-aminopyrazine ring (compound **66**, Figure 25), which improved the in vitro antimalarial activity but had poor solubility. In an attempt to improve the aqueous solubility, the methyl sulfonyl group of compound **66** was replaced with a piperazinyl carboxamide (UCT943, **67**, Figure 25) [154]. While retaining PI4K selectivity, UCT943 demonstrated improved solubility and potency against all life cycle stages compared to the clinical candidate, MMV048 [154,160]. The in vivo efficacy of UCT943 in a *P. falciparum*-infected NSG mouse model was also 2-fold more potent than that of MMV048 [160]. Gibhard and co-workers [153] also explored the option of using the more soluble sulfoxide analogue of compound **66** as a prodrug to improve drug exposure in vivo. In a *P. falciparum*-infected NSG mouse model, the sulfoxide was rapidly absorbed and converted to its sulfone analogue (compound **66**), which resulted in higher exposure compared to when the sulfone was administered.

Hit compound **68** (Figure 26), discovered during a phenotypic screening campaign, displayed weak in vitro activity against *P. falciparum* blood-stage parasites (EC_{50}: 3.9 μM) but had potent *Pf*PI4K activity (IC_{50}: 7.7 nM) [157]. Systematic optimisation of the bipyridine sulfonamide scaffold led to compound **69** (Figure 26) which was selective for *Pf*PI4K and had potent activity against several drug-sensitive and -resistant *P. falciparum* strains. Compound **69** showed in vivo blood-stage efficacy (99.9% reduction in parasitemia at 80 mg/kg for 7 days) in a *P. yoelii*-infected mouse model and liver-stage efficacy (1 mg/kg, single dose) in a *P. berghei*-infected mouse model.





	68	**69**
*Pf*PI4K IC_{50}	7.7 nM	0.9 nM
*Pf*3D7 IC_{50}	3.9 μM	25.1 nM
In vivo blood stage efficacy *Py* mouse model (% parasitemia reduction, oral dose)	ND	99.9% 80 mg/kg Once daily, 7 days
In vivo liver stage efficacy *Pb* sporozoite-infected mice (Full protection and cure, oral dose)	ND	1 mg/kg Single dose

Figure 26. Bipyridine sulfonamide based compounds as inhibitors of *Pf*PI4K.

In silico screening campaigns against *Pf*PI4K homology models have identified a number of virtual hits that can be used as potential starting points for drug development [149,161,162]. Compounds **70–74** (Figure 27) all have good ADMET properties and form strong interactions with the ATP-binding site of *Pf*PI4K. Compounds **71** and **72** also demonstrated selectivity for *Pf*PI4K over the human PI4KB orthologue [149].

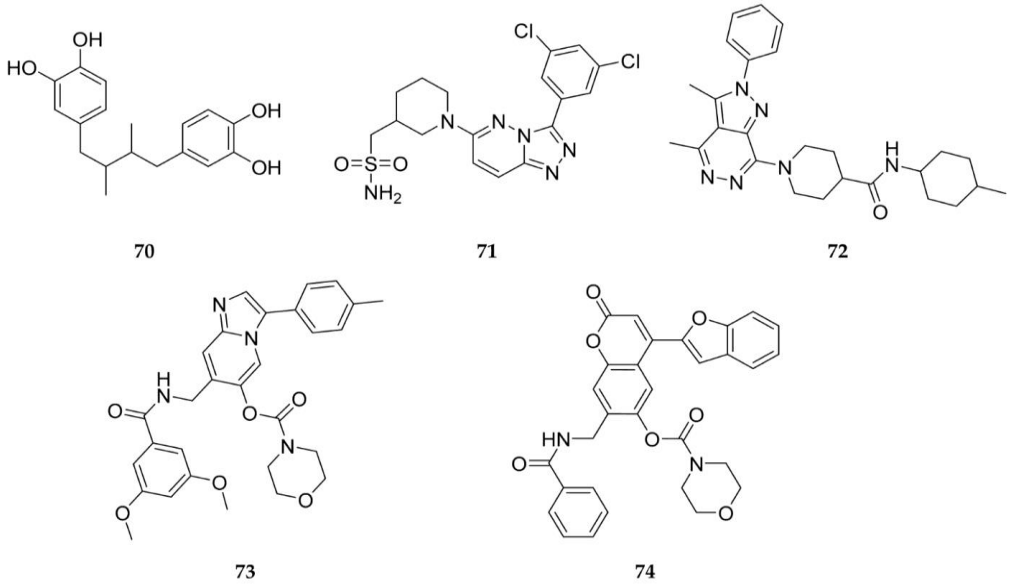


Figure 27. Hits identified during in silico screening against *Pf*PI4K homology models.

8. Orphan Kinases

Orphan kinases are particularly attractive drug targets as they do not have orthologues in the human host [11]. Some of the orphan kinases that have been characterised for *P. falciparum* include protein kinase 7 (*Pf*PK7), protein kinase 9 (*Pf*PK9) and the FIKK family [163].

*Pf*PK7 is a composite kinase that displays homology to the mitogen-activated protein kinase (MAPKK) family in its C-terminal region and fungal PKA homology in its N-terminal region [164]. Despite its homology, *Pf*PK7 is unlikely to be a functional MAPKK orthologue as the typical MAPKK activation site is absent and it is unable to phosphorylate the two MAPK orthologues (*Pf*map-1 and *Pf*map-2) in vitro [164]. *Pf*PK7 is expressed in asexual liver- and blood-stage parasites as well as in gametocytes and is localised in the cytoplasm [164]. Disruption of the *pfpk7* gene decreases the growth rate of erythrocytic parasites and drastically reduces the parasite's ability to produce oocysts during the sexual stage [165].

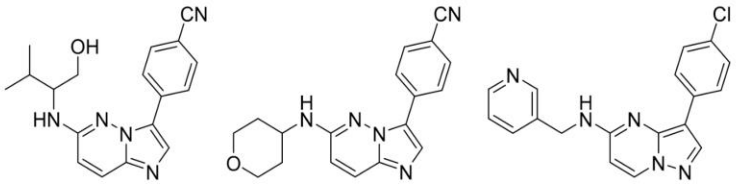
*Pf*PK9 clusters at the base of the CDPK and AGC family branches but does not associate with either of the two groups [13]. *Pf*PK9 is essential for *P. falciparum* parasite viability [22,166] and is expressed during the late ring stages as well as the schizont stage where it exhibits maximal expression [167]. During the ring stages, *Pf*PK9 is localised to the parasitophorous vacuolar membrane, which acts as the interface between the parasite and the cytoplasm of the erythrocyte. As the parasite matures into schizonts, the localisation of *Pf*PK9 shifts to the parasite's plasma membrane [167]. This suggests that *Pf*PK9 is involved in signal transduction between the cytosol of the parasite and the intraerythrocytic environment. Thus far, only one downstream target has been identified for *Pf*PK9, namely E2 ubiquitin-conjugating enzyme 13 (*Pf*UBC13) [167]. *Pf*UBC13 is an orthologue of eukaryotic UBC13 which is involved in the attachment of lysine 63 (K63)-linked polyubiquitin chains to target proteins. This modulates the activity of various cellular processes such as DNA repair and immune responses.

The FIKK family, a group of serine/threonine kinases specific to apicomplexan parasites, is named after the phenylalanine (F)–isoleucine (I)–lysine (K)–lysine (K) motif located in the *N*-terminal region of their kinase domains. While most *Plasmodium* species only have a single FIKK kinase, 20 FIKK kinase members have been identified for *P. falciparum* [168]. Although the biological functions of this group of kinases are still unclear, evidence suggests that most FIKK kinases are involved in erythrocyte remodelling during infection [169,170]. Studies have identified nine FIKK kinases that are exported via the Maurer's clefts to the erythrocytic membrane, where remodelling occurs [169,171]. Disruption of individual genes encoding for *Pf*FIKK4.2, *Pf*FIKK7.1 or *Pf*FIKK12 significantly altered erythrocytic membrane rigidity and phosphorylation of certain cytoskeletal membrane proteins [170,172]. One such erythrocytic cytoskeletal protein, dematin, was also identified as a potential substrate for *Pf*FIKK4.1 [173].

8.1. Inhibitor Development for Orphan Kinases

8.1.1. *Pf*PK7

As *Pf*PK7 plays a role in both the erythrocytic and sexual stages, *Pf*PK7 inhibitors could possibly decrease parasite virulence and act as transmission-blocking agents. This possibility makes *Pf*PK7 an interesting target for drug development. A number of established kinase inhibitors, including a MAPKK inhibitor (U0126) and PKA inhibitors (H89 & PKI), had no activity against recombinant *Pf*PK7 (IC_{50} >100 μM) [164]. A high-throughput screening campaign identified imidazopyridines (compounds **75** and **76**, Figure 28) and pyrazolopyrimidine (compound **77**, Figure 28) with *Pf*PK7 and in vitro activity in the low micromolar range [174]. However, these compounds also inhibited a number of other kinases in the low micromolar range.



	75	**76**	**77**
***Pf*PK7 IC_{50}**	6.3 μM	4.3 μM	4.6 μM
***Pf* blood stage GI_{50}**	2.5 μM	1.5 μM	1.0 μM

Figure 28. Imidazopyridine- and pyrazolopyrimidine-based compounds as inhibitors of *Pf*PK7.

The crystal structures of *Pf*PK7 in complex with adenylylimidodiphosphate (an ATP analogue, PBD: 2PML), compound **75** (PBD: 2PMN) and hymenialdisine (PBD: 2PMO) were elucidated. These structures highlighted some atypical features that are specifically relevant to drug discovery: Firstly, an aspartic acid residue (D123) in the hinge region protrudes to block access to the C-terminal domain surface in the ATP-binding site (Figure 29A). This structural impediment explains the inactivity of most established protein kinase inhibitors towards *Pf*PK7. Secondly, a hydrophobic pocket was identified in the back of the ATP-binding site, which can be exploited for designing *Pf*PK7-selective drugs (Figure 29B).

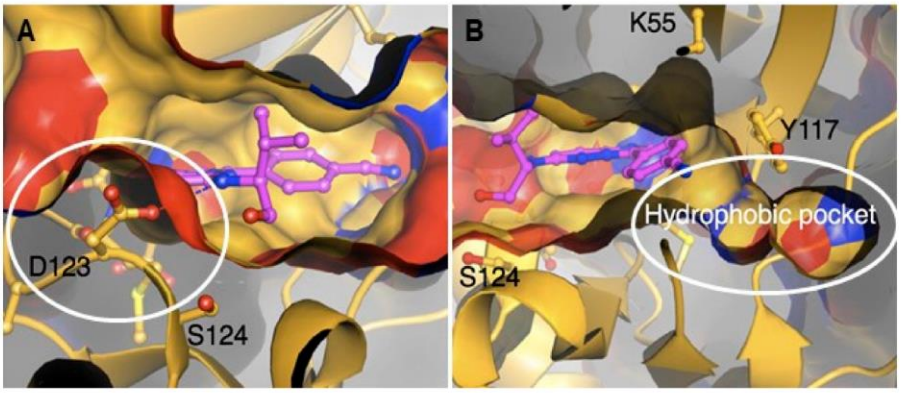
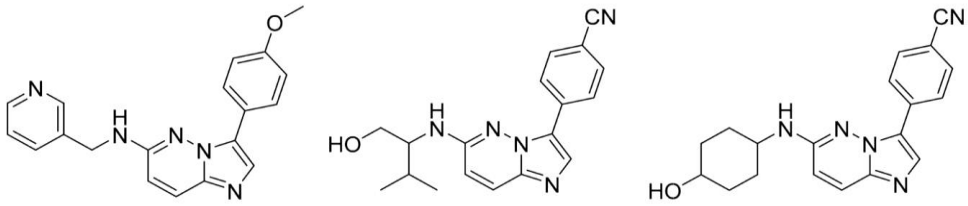


Figure 29. Structure of *Pf*PK7 in complex with compound **75**. (**A**) The protruding aspartic acid residue (D123) blocks access to the C-terminal domain surface of the ATP-binding site. (**B**) The hydrophobic pocket located at the back of the ATP-binding site. (Figure reproduced from [174] with permission from Elsevier.)

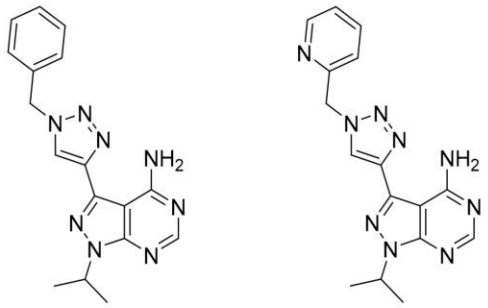
Another high-throughput screening campaign identified a number of imidazopyridazines (e.g., **78**, Figure 30) as weak *Pf*PK7 inhibitors [175]. Using the crystal structure data from the previous study [174], Bouloc and co-workers [175] aimed to improve the potency of the imidazopyridazine scaffold by varying the aryl and amine substituents of **78**. This ultimately led to compounds **79** and **80** which demonstrated improved *Pf*PK7 activity (IC_{50}: 0.28 μM and 0.13 μM, respectively). These compounds also showed antiplasmodial activity against drug-sensitive (3D7) and -resistant (K1) strains of *P. falciparum* without significant cytotoxicity. However, these compounds were also unselective and inhibited several other kinases.



	78	**79**	**80**
*Pf*PK7 IC_{50}	11.6 μM	0.28 μM	0.13 μM
*Pf*3D7 blood stage EC_{50}	ND	2.60 μM	1.03 μM
*Pf*K1 blood stage EC_{50}	ND	6.86 μM	2.65 μM

Figure 30. Imidazopyridazine-based compounds as inhibitors of *Pf*PK7.

Klein and co-workers [176] aimed to design *Pf*PK7 inhibitors that exploit the hydrophobic pocket in the ATP-binding site. They designed two series of pyrazolopyrimidines with 1- and 4-substituted triazole rings at the 3-position of the pyrazole ring. The substituted triazoles were designed with a "bent" geometry that would allow the inhibitor to interact with the hydrophobic pocket. Two compounds (**81** and **82**, Figure 31) demonstrated *Pf*PK7 activity in micromolar concentrations (IC_{50}: 20 and 10 μM, respectively). Docking studies with compound **81** indicated binding interactions similar to those seen with the ATP analogue but with additional interaction of the 4-phenyl-(1,2,3-triazol-1-yl) moiety with the hydrophobic pocket.

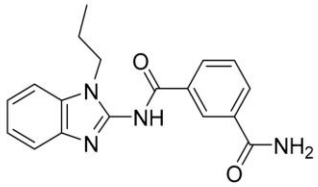


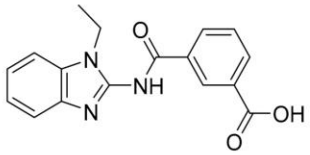
	81	**82**
*Pf*PK7 IC_{50}	20 μM	10 μM

Figure 31. Structures of pyrazolopyrimidine-based compounds with a bent geometry.

8.1.2. *Pf*PK9

Only one study has been published thus far with regards to inhibitor development for *Pf*PK9. Screening of a kinase-targeted library against *Pf*PK9 identified takinib (**83**, Figure 32), which demonstrated low micromolar binding affinity ($K_{d(app)}$: 0.46 μM) for *Pf*PK9 [177]. Takinib is a potent inhibitor (IC_{50}: 9.5 nM, [178]) of human mitogen-activated protein kinase kinase kinase 7 (MAP3K7, or more commonly referred to as TAK1). TAK1's activity is regulated through K63-ubiquitination by human ubiquitin-conjugating enzyme, UBC13 [179]. Treatment of *P. falciparum*-infected erythrocytes with takinib resulted in a dose-dependent reduction in K63-ubiquitin levels, confirming that *Pf*PK9 regulates the activity of *Pf*UBC13 in vivo [167]. In order to achieve *Pf*PK9 selectivity over TAK1, a series of takinib analogues was developed. From this series, compound **84** (Figure 32) was identified as a *Pf*PK9-selective inhibitor ($K_{d(app)}$: 4.1 μM, 8.9-fold less potent than takinib) with antiparasitic activity against liver-stage *P. berghei* parasites (EC_{50} = 43 μM) and no significant hepatocyte cytotoxicity. Compound **84** also showed a similar decrease in K63-linked ubiquitin levels in *P. falciparum*-infected erythrocytes, as seen with takinib. Interestingly, takinib and compound **84** both induced an unusual phenotype in liver-stage parasites. Liver-stage drugs generally decrease the size and/or number of parasites. However, when liver-stage *P. berghei* parasites were treated with 10 μM of either takinib or compound **84**, the parasite size increased while the number of parasites remained unchanged. Treatment with 30 μM of either takinib or compound **84** also increased the parasite size but simultaneously decreased the number of parasites. This suggests a unique mechanism of action for these inhibitors, which has potential in new antimalarial drug development.





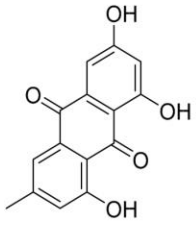
	83	**84**
*Pf*PK9 $K_{d(app)}$	0.5 μM	4 μM
TAK1 IC_{50}	9.5 μM	120 μM
Pb liver stage EC_{50}	7.3 μM	43 μM

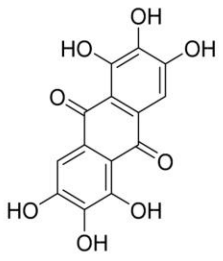
Figure 32. Structures and biological data of takinib and its analogue. $K_{D(app)}$: apparent dissociation constant.

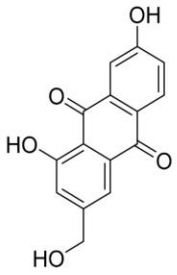
8.1.3. FIKKs

To date, five *P. falciparum* FIKK kinase members have been identified as essential for parasite survival, three of which are exported to the erythrocytic membrane (*Pf*FIKK9.1, *Pf*FIKK10.1 and *Pf*FIKK10.2) and two of which are localised within the parasite (*Pf*FIKK3 and *Pf*FIKK9.5) [171]. The non-exported FIKK8 kinase was also demonstrated to be essential to *P. berghei* erythrocytic parasites [180]. FIKK8 is the only FIKK kinase member that is conserved in all *Plasmodium* species as well as other apicomplexan parasites [181].

Interestingly, FIKK kinases tend to have small gatekeeper residues [180,182], a characteristic that is rarely seen in human serine/threonine kinases but is more common to human tyrosine kinases [183]. Not surprisingly, a screening campaign identified a number of tyrosine kinase inhibitors that block FIKK kinase activity [182,184]. One such tyrosine inhibitor, an anthraquinone named emodin (**85**, Figure 33), demonstrated in vitro activity against various *P. falciparum* strains (IC_{50} values of approximately 13 μM) [185] as well as activity against *Pf*FIKK8 (IC_{50}: 2 μM) and the *P. vivax* orthologue (*Pv*FIKK8; IC_{50}: 1.9 μM) [182,184]. Structurally related analogues of emodin (Figure 33), aloe emodin (**86**) and rufigallol (**87**), did not demonstrate any significant activity against *Pf*FIKK8 [184].







	85	**86**	**87**
*Pf*FIKK8 IC_{50}	2.0 μM	No inhibition	No inhibition
*Pv*FIKK8 IC_{50}	1.9 μM	ND	ND

Figure 33. Structures of emodin, aloe emodin and rufigallol.

9. Summary

Recurring antimalarial drug resistance necessitates the development of new antimalarial drugs with different chemical scaffolds and modes of action. Plasmodial kinases were identified as promising

targets for next-generation antimalarial drug development. To date, significant progress has been made towards characterisation and small-molecule inhibitor development for plasmodial kinases. Various plasmodial kinases have been validated as essential for one or multiple stages of the parasite's life cycle. Therefore, targeting plasmodial kinases could result in new drugs for chemoprevention and transmission-blocking, which will contribute to malaria elimination.

Plasmodial kinase inhibitors have been successfully identified by means of phenotypic and target-based screening approaches. The expression of several plasmodial kinases as active recombinant enzymes has facilitated crystallography and target-based medicinal chemistry efforts. Knowledge gained from developing human kinase inhibitors has also significantly contributed to plasmodial kinase inhibitor development. A number of potential scaffolds were identified through either phenotypic or target-based screening of human kinase inhibitor libraries.

Inhibitor promiscuity is always a concern when dealing with a conserved group of targets such as the kinases. Therefore, it is important to cross-screen potential plasmodial kinase inhibitors against human kinase panels. Inhibitors that target multiple plasmodial kinases could be beneficial as this ability would limit the risk of drug resistance, provided that this promiscuity does not also affect host kinases. Overall, studies targeting plasmodial kinases have demonstrated that selectivity over host kinases is an achievable goal.

Although a significant amount of work still needs to be done in terms of fully understanding the functions, stage specificity and interactions of plasmodial kinases, this group shows promise for future antimalarial drug development.

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Chapter 4: Exploration of benzofuran-based compounds as potent and selective *Plasmodium falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) inhibitors

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Exploration of benzofuran-based compounds as potent and selective *Plasmodium falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) inhibitors

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ABSTRACT

Plasmodium falciparum glycogen synthase kinase-3 (*Pf*GSK-3) has been identified as a potential target for the development of novel drugs against multi-drug resistant malaria. A series of benzofuran-based compounds was synthesised and evaluated as inhibitors of recombinantly expressed and purified *Pf*GSK-3 and human glycogen synthase kinase-3 beta (*Hs*GSK-3β). Of this series, five compounds (**5k**, **5m**, **5p**, **5r**, **5s**) preferentially inhibited *Pf*GSK-3, with four of these compounds exhibiting IC_{50} values in the sub-micromolar range (0.00048–0.440 μM). Evaluation of the structure-activity relationships required for *Pf*GSK-3 selective inhibition indicated that a C6-OCH_3 substitution on ring A is preferred, while the effect of the ring B substituent on activity, in decreasing order is: C4′-CN > C4′-F > C3′-OCH_3 > C3′,4′-diCl. To date, development of *Pf*GSK-3 inhibitors has been limited to the 4-phenylthieno[2,3-*b*]pyridine class. Chalcone-based scaffolds, such as the benzofurans described herein, are promising new hits which can be explored for future design of *Pf*GSK-3 selective inhibitors.

1. Introduction

Protein kinases have been exploited as targets for the design and development of small molecules to treat various types of cancers for decades [1,2]. Inspired by the success achieved in oncology, protein kinases are currently being exploited as viable drug targets in other diseases; including diabetes, arthritis and immune disorders [3–5]. The completion of the *Plasmodium falciparum* genome sequencing project in 2002 [6] further stimulated an interest in exploiting the parasite's protein kinases for the development of novel drugs against multi-drug resistant malaria [7–11].

Despite significant progress made in reducing malaria morbidity and mortality over the last decade, this infectious disease continues affecting people worldwide. In 2018, an estimated 228 million malaria cases and 405 000 malaria-related deaths were reported globally [12]. Developing countries, such as sub-Saharan Africa, are more severely affected by malaria due to poverty, limited access to adequate health care and preventative measures, and recurring resistance to insecticides and antimalarial drugs [13]. Sub-Saharan Africa accounted for 93% of all malaria cases and 94% of deaths reported for 2018 [12]. Pregnant women and young children are particularly vulnerable to infection and developing severe malaria, as pregnancy reduces immunity and young children have not yet acquired partial immunity against the disease. In 2018 alone, a staggering 272 000 children under the age of five, died of this disease [12].

Among the five *Plasmodium* species affecting humans, *P. falciparum* is responsible for the most lethal form of malaria and is also the most prevalent species in sub-Saharan Africa [12]. *P. falciparum* is resistant to several antimalarial drugs including current first-line artemisinin-based combination therapies (ACTs) [14]. Artemisinin resistance is prevalent across Southeast Asia, however, resistance has now also emerged in Rwanda which can significantly compromise malaria control in sub-Saharan Africa [15]. In order to combat multi-drug resistant malaria an urgent need exists to develop new antimalarial drugs with novel modes of action.

Plasmodium kinases are attractive targets for new antimalarial drugs, as they are involved in key signalling pathways throughout the life cycle of the parasite [7]. Of the 65 *Plasmodium* kinases that cluster within known eukaryotic protein kinase families, 36 are considered to be essential for the erythrocytic stages of the parasite [16,17]. *P. falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) is one such essential kinase and has been suggested as a potential drug target [6,18]. *Pf*GSK-3 was identified as one of three GSK-3-related kinases in the *P. falciparum* parasite [17], and shares a high degree of sequence homology with the

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two human GSK-3 isoforms (*Hs*GSK-3α and *Hs*GSK-3β) [18]. As the crystal structure of *Pf*GSK-3 is yet to be determined, a tridimensional structure model generated from the *Pf*GSK-3 sequence and the crystal structure of *Hs*GSK-3β [19], showed a high degree of structural similarity between the two enzymes [18].

As *Hs*GSK-3 is involved in numerous cellular processes, the extent to which *Hs*GSK-3 inhibition can be tolerated without presenting unwanted side effects, is still debated on [20–23]. Therefore, inhibitors targeting the plasmodial enzyme would have to be highly selective in order to prevent simultaneous inhibition of *Hs*GSK-3 and the potential side effects this may cause. Previous studies have tested various established kinase inhibitors such as staurosporine, indirubin-3′-monoxime, hymenialdisine and its derivatives, and members of the paullone family against both enzymes to determine whether *Pf*GSK-3 selective inhibition would be possible [18,24–26]. The majority of these inhibitors (staurosporine, indirubin-3′-monoxime and hymenialdisine derivatives) were unselective, inhibiting both *Pf*GSK-3 and mammalian GSK-3β in the nanomolar range [18,24–26]. However, the paullone inhibitors were more selective towards mammalian GSK-3β which indicated that species selectivity could be achieved [18,25,26]. Furthermore, certain key structural differences were identified in the active sites of these two enzymes that can be exploited for selective drug design [27,28].

Fugel and co-workers [25] were the first to design a series of novel 3,6-diamino-4-(2-halophenyl)-2-benzoylthieno[2,3-*b*]pyridine-5-carbonitrile derivatives that were highly selective towards *Pf*GSK-3. The most active compound of this series (**1**, Fig. 1) inhibited *Pf*GSK-3 at a sub-micromolar concentration (IC_{50}: 0.48 μM) and demonstrated *in vitro* antiplasmodial activity (EC_{50}: 5.5 μM) when tested against the erythrocytic stages of *P. falciparum*. Masch and co-workers [29] further optimised compound **1** by attaching various substituents to the *para* position of the 4-phenyl ring. Compound **2** (Fig. 1) was the most promising of the series as it retained *Pf*GSK-3 selectivity (*Pf*GSK-3 IC_{50}: 0.72 μM; *Hs*GSK-3β IC_{50}: 40.2 μM), and showed improved aqueous solubility (4.8 μM) and *in vitro* antiplasmodial activity (EC_{50}: 1.2 μM) compared to compound **1** (solubility: 1.5 μM; EC_{50}: 5.5 μM). The structural core of these inhibitors, 3-amino-4-arylthieno[2,3-*b*]pyridine motif, is based on a well-known chalcone scaffold (highlighted in blue, **1**, Fig. 1). This suggested the potential of using chalcone-like scaffolds in the design of new *Pf*GSK-3 inhibitors.

Herein we explore an alternative scaffold, benzofurans (**3**, Fig. 1), which is somewhat structurally similar to the thieno[2,3-*b*]pyridine motif. A series of benzofuran-based compounds (**5a-v**) with a variety of polar and lipophilic substituents on both ring A (R_1, R_2, R_3) and ring B (R_1', R_2'), were synthesised and evaluated as potential inhibitors of recombinantly expressed and purified *Pf*GSK-3 and *Hs*GSK-3β, in order to gain further insight into the structure-activity relationship around this scaffold for the design of potent and selective *Pf*GSK-3 inhibitors.

2. Experimental section

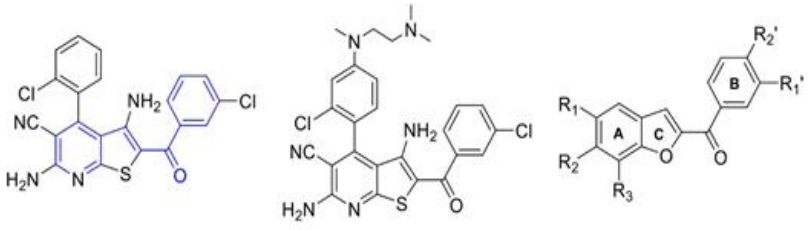
2.1. Chemistry

All starting materials for chemical synthesis were purchased from Sigma-Aldrich and used without further purification. Progress of reactions were monitored by thin layer chromatography (TLC) using

Silica gel $60F_{254}$ aluminium sheets (Merck) and a UV254 fluorescent indicator. The proton (^1H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz and 151 MHz, respectively. Either deuterated chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO-d_6) was used as solvent and tetramethylsilane (TMS) as internal reference for NMR analysis. All chemical shifts (δ) are reported in parts per million (ppm) in relation to the solvent signal ($CDCl_3$ residual peak at 7.26 ppm and DMSO-d_6 residual peak at 2.50 ppm for ^1H NMR). Spin multiplicities are reported as follows: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet) and m (multiplet). Coupling constants (J) are reported in Hz. High resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF-Q II mass spectrometer using atmospheric pressure chemical ionisation (APCI) in positive mode. High performance liquid chromatography (HPLC) analyses were done using an Agilent 1100 HPLC system. Melting points (mp) were determined using a Buchi B545 melting point apparatus and values are reported as obtained. Characterisation data for all compounds are available in the Supplementary material.

2.1.1. Synthesis of compounds 3a and 4a

*2.1.1.1. 2-hydroxy-4,5-dimethoxybenzaldehyde (**3a**).* 2,4,5-Trimethoxybenzaldehyde (15.0 g, 76 mmol) was added to DCM (100 ml) and the mixture was cooled on ice. BBr_3 (19.2 g, 76 mmol) was then added dropwise and the solution was stirred while on ice for approximately 10 min before stirring at room temperature for 5 h. Upon reaction completion as suggested by TLC analysis, the reaction mixture was again cooled on ice and HCl (32%, 40 ml) was slowly added to the mixture while stirring. The resulting precipitate was collected by filtration. The filtrate was extracted with DCM (3x 100 ml), the combined organic extracts were dried over $MgSO_4$ and solvent removed *in vacuo*. The resultant crude from the organic extracts were pooled with the dried precipitate to yield compound **3a** as a dark green solid (2.6 g, 91%), which was used without further purification. mp: 99.8–102.3 °C; ^1H



1

***Pf*GSK-3 IC_{50}:** 0.48 μM

***Hs*GSK-3β IC_{50}:** 3.3 μM

***Pf*NF54-Luc EC_{50}:** 5.5 μM

2

***Pf*GSK-3 IC_{50}:** 0.72 μM

***Hs*GSK-3β IC_{50}:** 40.2 μM

***Pf*NF54-Luc EC_{50}:** 1.2 μM

Solubility: 4.8 μM vs 1.5 μM for compound **1**

3

Fig. 1. *Pf*GSK-3 selective inhibitors (**1** and **2**) belonging to the 4-phenylthieno[2,3-*b*]pyridine class, synthesised by Fugel *et al.* [25] and Masch *et al.* [29]. The structural core of these compounds is based on a well-known chalcone scaffold (**1**, highlighted in blue). Another chalcone-based scaffold, benzofurans (**3**), was explored for the design of potent and selective *Pf*GSK-3 inhibitors during the present study. IC_{50}: half-maximal inhibitory concentration; *Pf*NF54-Luc: transgenic *Plasmodium falciparum* NF54 strain expressing the luciferase gene; EC_{50}: half-maximal effective concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NMR (600 MHz, $CDCl_3$) δ 11.41 (s, 1H), 9.70 (s, 1H), 6.91 (s, 1H), 6.47 (s, 1H), 3.94 (s, 3H), 3.88 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 194.01, 159.38, 157.26, 142.97, 113.18, 112.88, 100.17, 56.46, 56.38. APCI-HRMS *m/z* calculated for $C_9H_{11}O_4$ (MH^+): 183.0652, found: 183.0667. Purity (HPLC): 90.81%.

*2.1.1.2. 2-bromo-3′-methoxyacetophenone (**4a**).* $CuBr_2$ (14.88 g, 66.62 mmol) was added to a solution of 3′-methoxyacetophenone (5.00 g, 33.29 mmol) in ethyl acetate (75 ml) and DCM (75 ml). The reaction mixture was stirred at 85 °C for 24 h. Upon completion as suggested by TLC analysis, the reaction mixture was cooled to room temperature and then filtered through a bed of silica. The filtrate was concentrated to yield compound **4a** as a green solid (6.98 g, 91%), which was used without further purification. mp: 53.7–54.9 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.60 (d, J = 7.8 Hz, 1H), 7.50–7.45 (m, 2H), 7.27–7.24 (m, 1H), 4.95 (s, 2H), 3.82 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 191.57, 159.44, 135.35, 130.04, 121.18, 119.84, 113.28, 55.42, 34.36. APCI–HRMS *m/z* calculated for $C_9H_{10}BrO_2$ (MH^+): 228.9859, found: 228.9862. Purity (HPLC): 56.08%.

2.1.2. Synthesis of compounds 5a-r

*2.1.2.1. 1-benzofuran-2-yl(phenyl)methanone (**5a**).* Anhydrous K_2CO_3 (1.70 g, 12.28 mmol) was added to a mixture of 2-bromoacetophenone (0.82 g, 4.09 mmol) and salicylaldehyde (0.44 ml, 4.09 mmol) in PEG-400 (5 ml) and the mixture was stirred at 100 °C. Upon reaction completion as indicated by TLC analysis, the reaction mixture was cooled to room temperature and quenched with deionised water (10 ml). In cases where a precipitate was formed, it was collected by filtration. When no precipitate formed upon addition of water, the mixture was extracted with ethyl acetate (3x 20 ml). The combined organic extracts were washed with brine (20 ml), dried over $MgSO_4$, concentrated and recrystallized from a suitable solvent [either ethanol (**5a-q**) or methanol (**5r-s**)] to yield the title compound **5a** as a yellow solid (67%): mp: 90.3–91.8 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.08–8.02 (m, 2H), 7.73 (d, J = 7.8 Hz, 1H), 7.68–7.61 (m, 2H), 7.58–7.47 (m, 4H), 7.37–7.31 (m, 1H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 184.44, 156.02, 152.22, 137.25, 132.92, 129.46, 128.56, 128.41, 127.02, 124.01, 123.34, 116.60, 112.60. APCI-HRMS *m/z* calculated for $C_{15}H_{11}O_2$ (MH^+): 223.0754, found: 223.0763. Purity (HPLC): 95.88%.

*2.1.2.2. (5-chloro-1-benzofuran-2-yl)(phenyl)methanone (**5b**).* Prepared as for **5a** from 2-bromoacetophenone (0.30 g, 1.51 mmol) and 5-chlorosalicylaldehyde (0.24 g, 1.51 mmol) with anhydrous K_2CO_3 (0.63 g, 4.25 mmol) to yield compound **5b** as white crystals (66%): mp: 142.8–143.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04–7.99 (m, 2H), 7.70–7.60 (m, 2H), 7.58–7.49 (m, 3H), 7.46–7.41 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 184.10, 154.21, 153.30, 136.84, 133.16, 129.60, 129.45, 128.65, 128.61, 128.19, 122.59, 115.45, 113.65. APCI-HRMS *m/z* calculated for $C_{15}H_{10}ClO_2$ (MH^+): 257.0364, found: 257.0364. Purity (HPLC): 99.1%.

*2.1.2.3. (7-bromo-5-chloro-1-benzofuran-2-yl)(phenyl)methanone (**5c**).* Prepared as for **5a** from 2-bromoacetophenone (0.30 g, 1.50 mmol) and 3-bromo-5-chlorosalicylaldehyde (0.36 g, 1.50 mmol) with anhydrous K_2CO_3 (0.63 g, 4.25 mmol) to yield compound **5c** as dark beige crystals (43%): mp: 143.4–144.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.12–8.07 (m, 2H), 7.67–7.59 (m, 3H), 7.57–7.51 (m, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 183.25, 154.10, 151.71, 136.37, 133.42, 130.82, 130.04, 129.69, 129.46, 128.66, 121.69, 115.30, 105.61. APCI-HRMS *m/z* calculated for $C_{15}H_9BrClO_2$ (MH^+): 334.9469, found: 334.9465. Purity (HPLC): 97.35%.

*2.1.2.4. (5-chloro-1-benzofuran-2-yl)(4-chlorophenyl)methanone (**5d**).* Prepared as for **5a** from 2,4′-dichloroacetophenone (0.30 g, 1.59 mmol) and 5-chlorosalicylaldehyde (0.25 g, 1.59 mmol) with anhydrous K_2CO_3 (0.66 g, 4.76 mmol) to yield compound **5d** as white crystals (66%): mp: 191.3–191.8 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.03–7.96 (m, 2H), 7.69 (d, J = 2.0 Hz, 1H), 7.57–7.41 (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.64, 154.21, 153.18, 139.74, 134.99, 130.93, 129.76, 128.97, 128.84, 128.09, 122.63, 115.38, 113.64. APCI-HRMS *m/z* calculated for $C_{15}H_9Cl_2O_2$ (MH^+): 290.9974, found: 290.9963. Purity (HPLC): 100%.

*2.1.2.5. (7-bromo-5-chloro-1-benzofuran-2-yl)(4-chlorophenyl)methanone (**5e**).* Prepared as for **5a** from 2,4′-dichloroacetophenone (0.30 g, 1.59 mmol) and 3-bromo-5-chlorosalicylaldehyde (0.37 g, 1.59 mmol) with anhydrous K_2CO_3 (0.66 g, 4.76 mmol) to yield compound **5e** as beige crystals (42%): mp: 164.9–165.0 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.12–8.07 (m, 2H), 7.64 (q, J = 1.8 Hz, 2H), 7.57 (s, 1H), 7.52 (d, J = 8.5 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 181.81, 153.99, 151.69, 140.08, 134.51, 131.19, 130.97, 130.20, 129.04, 128.55, 121.73, 115.26, 105.58. APCI-HRMS *m/z* calculated for $C_{15}H_8BrCl_2O_2$ (MH^+): 368.9079, found: 368.9052. Purity (HPLC): 99.76%.

*2.1.2.6. 1-benzofuran-2-yl(4-chlorophenyl)methanone (**5f**).* Prepared as for **5a** from 2,4′-dichloroacetophenone (0.30 g, 1.59 mmol) and salicylaldehyde (0.17 ml, 1.59 mmol) with anhydrous K_2CO_3 (0.66 g, 4.76 mmol) to yield compound **5f** as beige crystals (65%): mp: 150.0–151.4 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.10–7.98 (m, 2H), 7.72 (d, J = 7.7 Hz, 1H), 7.62 (d, J = 8.3 Hz, 1H), 7.60–7.46 (m, 4H), 7.33 (t, J = 7.4 Hz, 1H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.94, 155.99, 152.04, 139.42, 135.36, 130.91, 128.87, 128.55, 126.87, 124.09, 123.34, 116.48, 112.54. APCI-HRMS *m/z* calculated for $C_{15}H_{10}ClO_2$ (MH^+): 257.0364, found: 257.0339. Purity (HPLC): 100%.

*2.1.2.7. [6-(diethylamino)benzofuran-2-yl](phenyl)methanone hydrochloride (**5g**).* [6-(diethylamino)benzofuran-2-yl](phenyl)methanone was prepared as for **5a** from 2-bromoacetophenone (0.52 g, 2.59 mmol) and 4-(diethylamino)salicylaldehyde (0.5 g, 2.59 mmol) with anhydrous K_2CO_3 (1.07 g, 7.76 mmol) to yield a yellow oil. The oil was dissolved in ethanol (10 ml), and acetyl chloride (5 ml) was added dropwise to the mixture while stirring at room temperature. The title compound (**5g**) was collected by filtration as a yellow solid (50%): mp: 215.4–217.1 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.26 (s, 1H), 8.12–8.07 (m, 2H), 7.93 (d, J = 8.5 Hz, 1H), 7.81 (dd, J = 8.4, 1.1 Hz, 1H), 7.68 (dd, J = 10.6, 4.3 Hz, 1H), 7.63 (s, 1H), 7.57 (t, J = 7.8 Hz, 2H), 3.63 (d, J = 7.2 Hz, 4H), 1.32 (t, J = 7.2 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 183.58, 155.20, 154.82, 136.95, 136.30, 133.59, 129.65, 128.76, 128.42, 125.17, 118.63, 114.90, 108.29, 54.07, 10.44. APCI-HRMS *m/z* calculated for $C_{19}H_{20}NO_2$ (MH^+): 294.1489, found: 294.1517. Purity (HPLC): 100%.

*2.1.2.8. (4-chlorophenyl)(7-methoxy-1-benzofuran-2-yl)methanone (**5h**).* Prepared as for **5a** from 2,4′-dichloroacetophenone (0.50 g, 2.64 mmol) and *o*-vanillin (0.40 g, 2.64 mmol) with anhydrous K_2CO_3 (1.10 g, 7.93 mmol) to yield compound **5h** as beige crystals (82%): mp: 189.9–190.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.08–8.03 (m, 2H), 7.55 (s, 1H), 7.53–7.48 (m, 2H), 7.27 (ddd, J = 15.7, 10.5, 4.4 Hz, 2H), 6.96 (dd, J = 7.8, 0.8 Hz, 1H), 4.02 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.56, 152.45, 146.08, 145.72, 139.42, 135.26, 131.06, 128.87, 128.49, 124.75, 116.27, 114.97, 109.63, 56.07. APCI-HRMS *m/z* calculated for $C_{16}H_{12}ClO_3$ (MH^+): 287.0469, found: 287.0473. Purity (HPLC): 100%.

*2.1.2.9. (4-chlorophenyl)(6-methoxy-1-benzofuran-2-yl)methanone (**5i**).* Prepared as for **5a** from 2,4′-dichloroacetophenone (0.50 g, 2.64 mmol) and 2-hydroxy-4′-methoxybenzaldehyde (0.40 g, 2.64 mmol) with anhydrous K_2CO_3 (1.10 g, 7.93 mmol) to yield compound **5i** as pink crystals (54%): mp: 186.7–187.4 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98–7.93 (m, 2H), 7.56 (d, J = 8.7 Hz, 1H), 7.50–7.44 (m, 3H), 7.07 (d, J = 1.9 Hz, 1H), 6.95 (dd, J = 8.7, 2.2 Hz, 1H), 3.87 (s, 3H); ^{13}C NMR

(151 MHz, $CDCl_3$) δ 182.42, 161.37, 157.68, 151.62, 139.05, 135.71, 130.74, 128.80, 123.67, 120.24, 117.26, 114.70, 95.54, 55.74. APCI-HRMS *m/z* calculated for $C_{16}H_{12}ClO_3$ (MH^+): 287.0469, found: 287.0498. Purity (HPLC): 100%.

2.1.2.10. (4-chlorophenyl)(5-methoxy-1-benzofuran-2-yl)methanone ***(5j)***. Prepared as for **5a** from 2,4′-dichloroacetophenone (0.30 g, 1.59 mmol) and 2-hydroxy-5′-methoxybenzaldehyde (0.20 ml, 1.59 mmol) with anhydrous K_2CO_3 (0.66 g, 4.76 mmol) to yield compound **5j** as pink crystals (65%): mp: 190.6–191.1 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04–7.97 (m, 2H), 7.53–7.46 (m, 4H), 7.14–7.07 (m, 2H), 3.84 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.78, 156.71, 152.78, 151.22, 139.36, 135.38, 130.94, 128.85, 127.39, 118.78, 116.45, 113.22, 103.83, 55.84. APCI-HRMS *m/z* calculated for $C_{16}H_{12}ClO_3$ (MH^+): 287.0469, found: 287.0499. Purity (HPLC): 100%.

2.1.2.11. (3,4-dichlorophenyl)(6-methoxy-1-benzofuran-2-yl)methanone ***(5k)***. Prepared as for **5a** from 2-bromo-3′,4′-dichloroacetophenone (0.88 g, 3.29 mmol) and 2-hydroxy-4′-methoxybenzaldehyde (0.50 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5k** as brown crystals (52%): mp: 162.5–163.7 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.21 (d, J = 2.0 Hz, 1H), 8.00 (dd, J = 8.3, 2.0 Hz, 1H), 7.93 (d, J = 8.3 Hz, 1H), 7.88 (s, 1H), 7.80 (d, J = 8.7 Hz, 1H), 7.44 (d, J = 1.6 Hz, 1H), 7.09 (dd, J = 8.7, 1.6 Hz, 1H), 3.93 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 180.89, 161.78, 157.80, 150.95, 137.77, 136.00, 132.14, 131.50, 131.23, 129.58, 124.91, 120.51, 119.20, 115.19, 96.23, 56.39. APCI-HRMS *m/z* calculated for $C_{16}H_{11}Cl_2O_3$ (MH^+): 321.0080, found: 321.0062. Purity (HPLC): 100%.

2.1.2.12. (6-methoxy-1-benzofuran-2-yl)(phenyl)methanone ***(5l)***. Prepared as for **5a** from 2-bromoacetophenone (0.65 g, 3.29 mmol) and 2-hydroxy-4′-methoxybenzaldehyde (0.50 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5l** as beige crystals (57%): mp: 103.8–104.6 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00–7.96 (m, 2H), 7.62–7.47 (m, 4H), 7.44 (d, J = 0.7 Hz, 1H), 7.08 (d, J = 1.9 Hz, 1H), 6.94 (dd, J = 8.7, 2.2 Hz, 1H), 3.87 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 183.90, 161.23, 157.65, 151.80, 137.53, 132.58, 129.26, 128.46, 123.63, 120.34, 117.33, 114.53, 95.59, 55.72. APCI-HRMS *m/z* calculated for $C_{16}H_{13}O_3$ (MH^+): 253.0859, found: 253.0861. Purity (HPLC): 100%.

2.1.2.13. (4-fluorophenyl)(6-methoxy-1-benzofuran-2-yl)methanone ***(5m)***. Prepared as for **5a** from 2-bromo-4′-fluoroacetophenone (0.71 g, 3.29 mmol) and 2-hydroxy-4′-methoxybenzaldehyde (0.50 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5m** as a light brown solid (90%): mp: 161.1–163.1 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.11–8.05 (m, 2H), 7.58 (d, J = 8.7 Hz, 1H), 7.48 (d, J = 0.9 Hz, 1H), 7.24–7.18 (m, 2H), 7.10 (d, J = 1.9 Hz, 1H), 6.97 (dd, J = 8.7, 2.2 Hz, 1H), 3.90 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.22, 165.52 (d, J = 254.3 Hz), 161.32, 157.66, 151.79, 133.70 (d, J = 3.1 Hz), 131.97 (d, J = 9.1 Hz), 123.66, 120.31, 117.08, 115.68 (d, J = 21.8 Hz), 114.67, 95.61, 55.77. APCI-HRMS *m/z* calculated for $C_{16}H_{12}FO_3$ (MH^+): 271.0765, found: 271.0743. Purity (HPLC): 98.28%.

2.1.2.14. (4-bromophenyl)(6-methoxy-1-benzofuran-2-yl)methanone ***(5n)***. Prepared as for **5a** from 2,4′-dibromoacetophenone (0.91 g, 3.29 mmol) and 2-hydroxy-4′-methoxybenzaldehyde (0.50 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5n** as a light brown solid (85%): mp: 200.9–202.4 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.94–7.87 (m, 2H), 7.71–7.61 (m, 2H), 7.59 (d, J = 8.7 Hz, 1H), 7.48 (d, J = 0.9 Hz, 1H), 7.10 (d, J = 1.9 Hz, 1H), 6.98 (dd, J = 8.7, 2.2 Hz, 1H), 3.90 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.60, 161.43, 157.74, 151.63, 136.19, 131.82, 130.88, 127.71, 123.72, 120.28, 117.34, 114.76, 95.59, 55.79. APCI-HRMS *m/z* calculated for $C_{16}H_{12}BrO_3$ (MH^+): 330.9964, found: 330.9965. Purity (HPLC): 96.52%.

2.1.2.15. (6-methoxy-1-benzofuran-2-yl)(4-methoxyphenyl)methanone ***(5o)***. Prepared as for **5a** from 2-bromo-4′-methoxyacetophenone (0.75 g, 3.29 mmol) and 2-hydroxy-4′-methoxybenzaldehyde (0.50 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5o** as a beige solid (86%): mp: 151.0–151.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.12–8.03 (m, 2H), 7.57 (d, J = 8.7 Hz, 1H), 7.46 (d, J = 0.7 Hz, 1H), 7.10 (d, J = 1.9 Hz, 1H), 7.04–6.93 (m, 3H), 3.90 (d, J = 9.5 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.46, 163.38, 160.97, 157.42, 152.25, 131.76, 130.13, 123.48, 120.42, 116.25, 114.34, 113.79, 95.67, 55.75, 55.53. APCI-HRMS *m/z* calculated for $C_{17}H_{15}O_4$ (MH^+): 283.0965, found: 283.0974. Purity (HPLC): 100%.

2.1.2.16. 4-[(6-methoxy-1-benzofuran-2-yl)carbonyl]benzonitrile ***(5p)***. Prepared as for **5a** from 2-bromo-4′-cyanoacetophenone (0.74 g, 3.29 mmol) and 2-hydroxy-4′-methoxybenzaldehyde (0.50 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5p** as a beige solid (80%): mp: 183.1–186.7 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.14–8.08 (m, 2H), 7.86–7.80 (m, 2H), 7.60 (d, J = 8.7 Hz, 1H), 7.52 (d, J = 0.9 Hz, 1H), 7.09 (d, J = 1.9 Hz, 1H), 6.99 (dd, J = 8.7, 2.2 Hz, 1H), 3.91 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 181.93, 161.81, 158.00, 151.27, 140.93, 132.32, 129.76, 123.91, 120.18, 118.09, 118.02, 115.88, 115.12, 95.53, 55.82. APCI-HRMS *m/z* calculated for $C_{17}H_{12}NO_3$ (MH^+): 278.0812, found: 278.0801. Purity (HPLC): 98.24%.

2.1.2.17. (4-chlorophenyl)(5,6-dimethoxy-1-benzofuran-2-yl)methanone ***(5q)***. Prepared as for **5a** from 2,4′-dichloroacetophenone (0.52 g, 2.74 mmol) and compound **3a** (0.50 g, 2.74 mmol) with anhydrous K_2CO_3 (1.14 g, 8.23 mmol) to yield compound **5q** as white crystals (85%): mp: 183.8–184.0 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01–7.97 (m, 2H), 7.54–7.46 (m, 3H), 7.11 (s, 1H), 7.07 (s, 1H), 3.98 (s, 3H), 3.95 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 182.06, 152.03, 151.98, 151.78, 147.94, 139.00, 135.81, 130.82, 128.79, 119.09, 117.34, 102.69, 95.13, 56.36, 56.35. APCI-HRMS *m/z* calculated for $C_{17}H_{14}ClO_4$ (MH^+): 317.0575, found: 317.0574. Purity (HPLC): 100%.

2.1.2.18. (6-Methoxybenzofuran-2-yl)(3-methoxyphenyl)methanone ***(5r)***. Prepared as for **5a** from compound **4a** (0.75 g, 3.29 mmol) and 2-hydroxy-4-methoxybenzaldehyde (0.50 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5r** as a light grey solid (52%): mp: 81.4–81.5 °C; 1H NMR (600 MHz, $CDCl_3$) δ 7.58 (dd, J = 14.9, 8.1 Hz, 2H), 7.50 (s, 1H), 7.47 (s, 1H), 7.43 (t, J = 7.9 Hz, 1H), 7.18–7.14 (m, 1H), 7.10 (s, 1H), 6.96 (dd, J = 8.6, 2.0 Hz, 1H), 3.89 (m, 6H); 13C NMR (151 MHz, CDCl3) δ 183.77, 161.43, 159.81, 157.84, 151.93, 138.96, 129.61, 123.80, 121.99, 120.50, 119.09, 117.54, 114.70, 114.01, 95.79, 55.90, 55.66. APCI-HRMS *m/z* calculated for $C_{17}H_{15}O_4$ (MH^+): 283.0965, found: 283.0946. Purity (HPLC): 99.40%.

2.1.2.19. (7-Methoxybenzofuran-2-yl)(3-methoxyphenyl)methanone ***(5s)***. Prepared as for **5a** from 2-hydroxy-3-methoxybenzaldehyde (0.50 g, 3.29 mmol) and compound **4a** (0.75 g, 3.29 mmol) with anhydrous K_2CO_3 (1.36 g, 9.86 mmol) to yield compound **5s** as a brown solid (46%): mp: 80.1–86.8 °C; 1H NMR (600 MHz, CDCl3) δ 7.81–7.73 (m, 1H), 7.66 (d, J = 0.8 Hz, 1H), 7.62 (t, J = 6.7 Hz, 1H), 7.57–7.48 (m, 1H), 7.44–7.29 (m, 3H), 7.05 (dd, J = 13.0, 7.0 Hz, 1H), 4.12 (s, 3H), 3.97 (s, 3H); 13C NMR (151 MHz, $CDCl_3$) δ 183.83, 159.83, 152.65, 146.29, 145.92, 138.53, 129.66, 128.76, 124.78, 122.35, 119.61, 116.56, 115.14, 114.03, 109.83, 56.30, 55.64. APCI-HRMS *m/z* calculated for $C_{17}H_{15}O_4$ (MH^+): 283.0965, found: 283.0970. Purity (HPLC): 98.03%.

2.1.3. Synthesis of compounds 5t-v

2.1.3.1. (4-chlorophenyl)(7-hydroxy-1-benzofuran-2-yl)methanone ***(5t)***.

$AlCl_3$ (0.87 g, 6.49 mmol) was added to a 100 ml round bottom flask containing **5h** (0.62 g, 2.17 mmol) and toluene (5 ml). The mixture was stirred at 100 °C. Upon reaction completion as suggested by TLC analysis, the mixture was cooled to room temperature and quenched with deionised water (10 ml). The mixture was then extracted with ethyl acetate (3x 20 ml). The combined organic extracts were washed with brine (20 ml), dried over $MgSO_4$, concentrated and purified using silica gel column chromatography (petroleum ether: ethyl acetate, 5:1) to yield compound **5t** as a beige solid (29%): mp: 213.4–214.5 °C; ^{1}H NMR (600 MHz, DMSO) δ 10.42 (s, 1H), 8.06–7.99 (m, 2H), 7.76 (s, 1H), 7.70–7.63 (m, 2H), 7.24 (dd, J = 7.8, 1.0 Hz, 1H), 7.16 (t, J = 7.8 Hz, 1H), 6.97 (dd, J = 7.7, 1.0 Hz, 1H); ^{13}C NMR (151 MHz, DMSO) δ 182.29, 151.05, 144.88, 143.32, 137.97, 135.45, 131.13, 128.86, 128.61, 124.96, 117.75, 114.02, 113.81. APCI-HRMS *m/z* calculated for $C_{15}H_{10}ClO_3$ (MH^+): 273.0313, found: 273.0304. Purity (HPLC): 98.58%.

*2.1.3.2. (4-chlorophenyl)(6-hydroxy-1-benzofuran-2-yl)methanone (**5u**).* Prepared as for **5s** from compound **5i** (0.41 g, 1.43 mmol) with $AlCl_3$ (0.57 g, 4.28 mmol) to yield compound **5u** as a beige solid (27%): mp: 212.2–212.9 °C; ^{1}H NMR (600 MHz, DMSO) δ 10.27 (s, 1H), 7.99–7.95 (m, 2H), 7.71 (d, J = 0.8 Hz, 1H), 7.68–7.62 (m, 3H), 7.06–7.02 (m, 1H), 6.91 (dd, J = 8.6, 2.1 Hz, 1H); ^{13}C NMR (151 MHz, DMSO) δ 181.98, 160.02, 157.78, 150.73, 138.01, 136.33, 131.32, 129.25, 124.95, 119.49, 118.99, 115.23, 97.95. APCI-HRMS *m/z* calculated for $C_{15}H_{10}ClO_3$ (MH+): 273.0313, found: 273.0326. Purity (HPLC): 100%.

*2.1.3.3. (4-chlorophenyl)(5-hydroxy-1-benzofuran-2-yl)methanone (**5v**).* Prepared as for **5s** from compound **5j** (0.28 g, 0.96 mmol) with $AlCl_3$ (0.39 g, 2.89 mmol) to yield compound **5v** as a yellow solid (29%): mp: 215.8–217.1 °C; ^{1}H NMR (600 MHz, DMSO) δ 9.57 (s, 1H), 8.04–7.98 (m, 2H), 7.71–7.64 (m, 3H), 7.58 (d, J = 8.9 Hz, 1H), 7.12 (d, J = 2.4 Hz, 1H), 7.04 (dd, J = 8.9, 2.5 Hz, 1H); ^{13}C NMR (151 MHz, DMSO) δ 182.65, 154.64, 152.16, 150.32, 138.34, 135.99, 131.51, 129.30, 128.08, 119.11, 117.69, 113.21, 107.23. APCI-HRMS *m/z* calculated for $C_{15}H_{10}ClO_3$ (MH^+): 273.0313, found: 273.0309. Purity (HPLC): 95.49%.

2.2. Biology

2.2.1. Cloning of HsGSK-3β and PfGSK-3

The full length open reading frame of *Hs*GSK-3β (1263 bp; Ensembl Genome ID: ENST00000264235.12) and *Pf*GSK-3 (1323 bp; Ensembl Genome ID: PF3D7_0312400) gene sequences were purchased from GeneArt (Thermo Scientific, Waltham, MA). The *Hs*GSK-3β and *Pf*GSK-3 sequences are available in the Supplementary material (Figure A and B).

The *Hs*GSK-3β gene was subcloned into the pET32a(+) expression vector using restriction enzyme directional cloning and verified by Sanger sequencing. Purified plasmid DNA was used to transform Origami™ cells (Novagen, Madison, WI) already containing the pGro7 chaperone expression plasmid (Takara, Madison, WI).

The *Pf*GSK-3 gene was amplified by PCR using 5′-ATG AAA AAT TGG CCT-3′ as the forward primer and 5′-ACT TTC TAT GAT AAC GTG CGT-3′ as the reverse primer (IDT, Coralville, IA). Amplification reactions were prepared according to the Platinum™ *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) instruction manual (annealing step: 51.6 °C for 30 s; total of 30 PCR cycles). The *Pf*GSK-3 gene was then cloned into the pBAD/Thio-TOPO® expression vector (pBAD/TOPO® ThioFusion™ Expression Kit; Invitrogen, Carlsbad, CA), by direct insertion of the PCR product. The cloning reaction was transformed into chemically competent TOP10 One Shot® *E. coli* cells included in the kit. The orientation of the inserts and the fidelity of the constructs were verified by Sanger sequencing.

2.2.2. Expression of recombinant HsGSK-3β and PfGSK-3

A single colony of Origami™ cells co-transformed with the pGro7 and pET32a(+)-*Hs*GSK-3β plasmids, was inoculated into 5 ml of LB medium with carbenicillin (50 μg/ml) and chloramphenicol (20 μg/ml). The culture was incubated overnight at 37 °C and 225 rpm. The overnight culture was transferred into 100 ml of LB medium with carbenicillin (50 μg/ml), chloramphenicol (20 μg/ml), and 0.5 mg/ml L-arabinose (for expression of the pGro7 chaperone proteins). The culture was incubated at 37 °C and 225 rpm, until an OD_{600} of ±0.5 was reached. Expression of the *Hs*GSK-3β protein was then induced with 1 mM IPTG for 4 h at 30 °C and 225 rpm.

A single colony of TOP10 *E. coli* transformed with pBAD/Thio-*Pf*GSK-3 was inoculated into 5 ml of LB medium containing carbenicillin (100 μg/ml). The culture was incubated overnight at 37 °C and 225 rpm. The overnight culture was transferred into 500 ml of LB medium with carbenicillin (100 μg/ml) and cultured at 37 °C and 225 rpm, until an OD_{600} of ±0.5 was reached. Expression was then induced with 0.02% L-arabinose for 3 h at 37 °C and 225 rpm.

2.2.3. Extraction and purification of recombinant HsGSK-3β and PfGSK-3

Bacterial cells were harvested by centrifugation (5000 xg, 10 min, room temperature) and the proteins were extracted using B-PER™ Complete Bacterial Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) according to the supplier's procedure. Briefly, the bacterial pellet was resuspended in the required amount of B-PER™ Reagent (with added protease inhibitors; Complete Ultra Protease Inhibitor Mini Tablets; Merck, Darmstadt, Germany), and incubated at room temperature with gentle rocking for 15 min. The lysate was then centrifuged (16 000 xg, 20 min, 4 °C) and the soluble fraction collected for purification. The recombinant His-tag proteins were then purified by immobilized metal affinity chromatography using Protino® Ni-TED 2000 Packed Columns (Macherey-Nagel Inc., Düren, Germany). Briefly, the entire soluble protein fraction was loaded onto a pre-equilibrated column. For the *Hs*GSK-3β protein, the column was washed twice with LEW buffer, before eluting the protein with Elution buffer (250 mM imidazole). For the *Pf*GSK-3 protein, the column was washed once with LEW buffer and then three times with diluted Elution buffer (5 mM imidazole), before eluting the protein with Elution buffer (250 mM imidazole, 5% glycerol).

Amicon® Ultra-15 centrifugal devices with a 50 kDa molecular weight cut-off (Merck, Darmstadt, Germany), were used for further purification and concentration of both recombinant proteins, as well as for exchanging the elution buffer with storage buffer. Storage buffer consisted of 50 mM Tris-HCl pH 8.0 (with added protease inhibitors, Complete Ultra Protease Inhibitor Mini Tablets, Roche); 150 mM NaCl; 0.5 mM EDTA; 0.25 mM DTT; 25% glycerol. SDS-PAGE analysis with Coomassie brilliant blue staining was used to monitor the expression and purification procedures. To confirm the presence of the His-tag proteins, western blot analysis was performed using a 6x His-tag monoclonal antibody (Thermo Fisher Scientific, Madison, WI). Protein concentrations were determined using the Qubit® protein assay kit (Life Technologies, Carlsbad, CA) with the Qubit® 2.0 fluorometer. Both recombinant proteins were stored in aliquots at −80 °C.

2.2.4. ADP-Glo™ kinase assay

Activity of the recombinant *Hs*GSK-3β and *Pf*GSK-3 were measured using the ADP-Glo™ Kinase Assay Kit (Promega, Madison, WI). The assay was optimised by determining the optimal substrate (GS-1 peptide: YRRAAVPPSPSLSRHSSPHQpSEDEEE, where pS is a phosphorylated serine; GenScript, Piscataway, NJ) and enzyme concentrations to use for each reaction. To determine the optimal substrate concentration, serial twofold dilutions of substrate in kinase reaction buffer (40 mM Tris-HCl pH 8.0; 20 mM $MgCl_2$; 0.1 mg/ml BSA; 50 μM DTT) were made, and the reactions were performed using 30 ng of enzyme and 10 μM of ATP per 30 μl reaction. The control reactions consisted of the same serial dilutions of substrate but without any added enzyme. The optimal

substrate concentrations were calculated to be 0.8 mg/ml for *Hs*GSK-3β and 0.6 mg/ml for *Pf*GSK-3, as these concentrations resulted in the largest difference in luminescence between the reaction wells and the control wells. To determine the optimal enzyme concentration, serial twofold dilutions of enzyme in kinase reaction buffer were made, and the reactions were performed using the optimal concentration of substrate and 10 μM of ATP per 20 μl reaction. The optimal enzyme concentration was within the linear range of the kinase titration curve (luminescence vs enzyme concentration) and generated a signal-to-background ratio of 10 (luminescence of reaction/ luminescence of reaction without kinase). The optimal enzyme concentrations were 8 ng and 32 ng for *Hs*GSK-3β and *Pf*GSK-3, respectively.

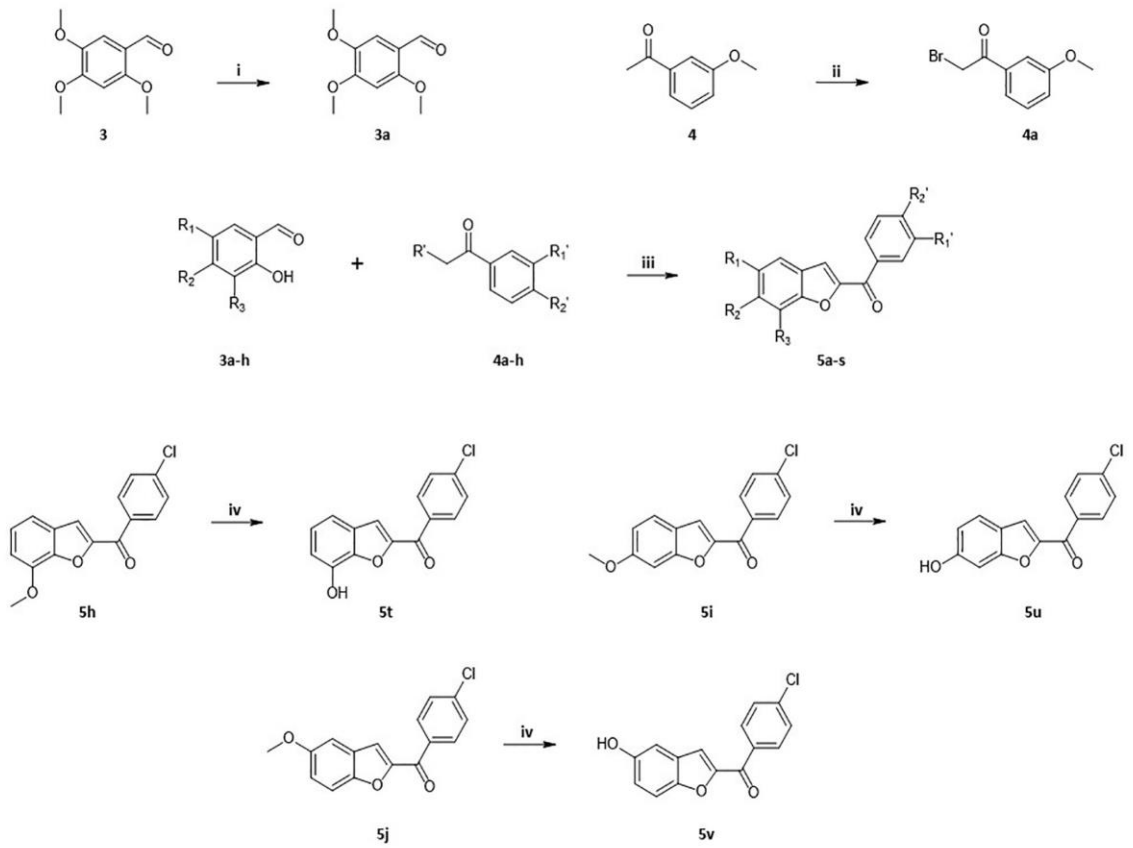
All test compounds were screened at a concentration of 10 μM (1% final DMSO concentration). The compounds were dissolved in 100% DMSO, diluted with kinase reaction buffer, and incubated with the kinase for 10 min at room temperature (23 °C), before adding the substrate and ATP to the reaction. The 20 μl kinase reaction consisted of the following: 4 μl of test compound (10 μM, 1% DMSO), 8 μl of enzyme (8 ng *Hs*GSK-3β or 32 ng *Pf*GSK-3) and 8 μl of ATP/substrate mix (ATP: 10 μM; substrate: 0.8 mg/ml for *Hs*GSK-3β or 0.6 mg/ml for *Pf*GSK-3) in kinase reaction buffer. The reaction was incubated at 30 °C for 30 min and then equilibrated to room temperature for 30 min. 5 μl of the kinase reaction was then transferred to a solid white 384-well plate (Brand-*plates*®; Merck, Darmstadt, Germany), 5 μl of ADP-Glo™ Reagent was added, and incubated for 40 min at room temperature. 10 μl of Kinase Detection Reagent was then added and incubated for 30 min at room temperature. Luminescence was measured using a SpectraMax® iD3 multi-mode microplate reader (integration time: 0.5 s per well), and the accompanying SoftMax Pro 7 software (Molecular Devices, San Jose, CA).

For compounds showing inhibitory activity at 10 μM, dose-response curves were performed. Using Graphpad Prism Software, the sigmoidal dose-response curves were obtained by plotting luminescence against the logarithm of the compounds' concentrations. The IC_{50} values were then calculated using nonlinear regression. All reactions were performed in triplicate, and data was normalised to a negative control (reaction without kinase). Indirubin-3′-monoxime, a known inhibitor of both *Hs*GSK-3β and *Pf*GSK-3 [18,25], was included as a positive control. The intra- and inter-assay CV values are available in the Supplementary material (Table A).

3. Results and discussion

3.1. Chemistry

Except for reagents **3a** and **4a**, all other reagents were purchased from commercial suppliers and were used as supplied. The reagents (**3a** & **4a**) and target compounds (**5a-v**) were synthesised according to the reactions shown in Scheme 1. 2-Hydroxy-4,5-dimethoxybenzaldehyde (**3a**) was obtained by *ortho*-demethylation of 2,4,5-trimethoxybenzaldehyde using BBr_3 as demethylation agent in DCM [30]. The selective demethylation at the *ortho*-position is a result of the *ortho*-directing effect of the carbonyl group [31]. The selective α-bromination of 3′-methoxyacetophenone was achieved using $CuBr_2$ in ethyl acetate/DCM (1:1) to yield 2-bromo-3′-methoxyacetophenone (**4a**) [32,33]. Compounds **5a-v** were prepared using Rap-Stoermer condensation reaction



Scheme 1. Synthesis of reagents **3a** & **4a**, and compounds **5a-v**. Reagents and conditions: **i**) BBr_3, ice bath (10 min), room temperature (5 h); **ii**) $CuBr_2$, ethyl acetate/DCM (1:1), 85 °C (24 h); **iii**) K_2CO_3, PEG-400, 100 °C (1–2 h); **iv**) $AlCl_3$, toluene, 100 °C (2–3 h).

between salicylaldehydes and α-haloketones in PEG-400 [34]. Compounds **5t-v** were prepared by demethylation of compounds **5h-j** with $AlCl_3$ in toluene [35].

Reagents and compounds were obtained in fair to good yields (42–91%), except for compounds **5t-v** which had poor yields (27–29%). The structures and purity of the target compounds were verified by ^1H NMR, ^{13}C NMR, MS, melting point and HPLC analyses.

3.2. Expression and purification of recombinant HsGSK-3β and PfGSK-3

Recombinant *Hs*GSK-3β was expressed from pET32a(+) as a thioredoxin-histidine-fusion of approximately 64 kDa. Co-expression of the pGro7 chaperone proteins (*gro*ES and *gro*EL) with recombinant *Hs*GSK-3β, facilitated the protein folding process and thereby increased the recovery of soluble *Hs*GSK-3β. The C-terminal histidine-tag allowed for easy detection and purification using nickel-affinity chromatography.

*Pf*GSK-3 was expressed using a similar method to the one described by Droucheau and co-workers [18], however, the present study expressed the full length *Pf*GSK-3 protein instead of a truncated version were the *N*-terminal is omitted. The pBAD/Thio-TOPO® system was used as it tightly regulates expression, thus preventing "leaky" expression of potentially toxic proteins such as *Pf*GSK-3. The recombinant *Pf*GSK-3 protein (approximately 68 kDa) was expressed with an *N*-terminal thioredoxin fusion protein for increased solubility, and a C-terminal histidine-tag for purification with nickel-affinity chromatography.

The *Pf*GSK-3 protein required additional purification due to the presence of several other proteins that co-eluted with *Pf*GSK-3 (Fig. 2). The washing protocol of the Protino® Ni-TED column was adapted as follows: instead of washing only with LEW buffer, the column was also washed with diluted Elution buffer (containing only 5% imidazole). As a result, some of the previously co-eluted proteins could be washed away before eluting the *Pf*GSK-3 protein. As a second purification step, the Amicon® Ultra-15 centrifugal device was used to remove all proteins smaller than 50 kDa. Despite the additional measures to purify the *Pf*GSK-3 protein, an additional protein (>50 kDa) still remained. Droucheau and co-workers [18] also reported the presence of another protein after *Pf*GSK-3 purification. They concluded that this additional protein was likely a proteolytic degradation product of *Pf*GSK-3, and used the *Pf*GSK-3 protein without further purification.

3.3. ADP-Glo™ kinase assay

A series of benzofuran derivatives (**5a-v**) was screened against recombinant *Pf*GSK-3 and *Hs*GSK-3β using the ADP-Glo™ Kinase Assay. The compounds that showed inhibitory activity at a concentration of 10 μM, were evaluated further by determining the IC_{50} values (Table 1). Of the seven compounds that showed inhibitory activity during the initial screening, one compound was selective towards *Hs*GSK-3β (**5u**), one compound was unselective inhibiting both *Pf*GSK-3 and *Hs*GSK-3β (**5v**), and five compounds were selective towards *Pf*GSK-3 (**5k**, **5m**, **5p**, **5r**, **5s**).

Compound **5p** was the most active compound of the series with an IC_{50} value of 0.00049 μM and a 316-fold selectivity for *Pf*GSK-3 versus *Hs*GSK-3β. This was closely followed by the activity of compound **5m** which exhibits an IC_{50} value of 0.00168 μM with a 175-fold selectivity towards *Pf*GSK-3. Compared to the reference inhibitor, indirubin-3′-monoxime (IC_{50}: 0.250 μM), compound **5p** and **5m** are approximately 510-fold and 148-fold more potent, respectively. The most selective *Pf*GSK-3 inhibitor of the series was compound **5r**, with a 559-fold selectivity for *Pf*GSK-3 versus *Hs*GSK-3β. As mentioned previously, *Pf*GSK-3 inhibitors would have to be very selective in order to prevent simultaneous inhibition of *Hs*GSK-3β and the possible ensuing side effects [21,22].

Comparing the activity of compound **5p** and **5m** to that of the 4-phenylthieno[2,3-*b*]pyridine inhibitors (**1** and **2**, Fig. 1), the benzofurans developed in this study were more potent *Pf*GSK-3 inhibitors, demonstrating lower IC_{50} values than the 4-phenylthieno[2,3-*b*]

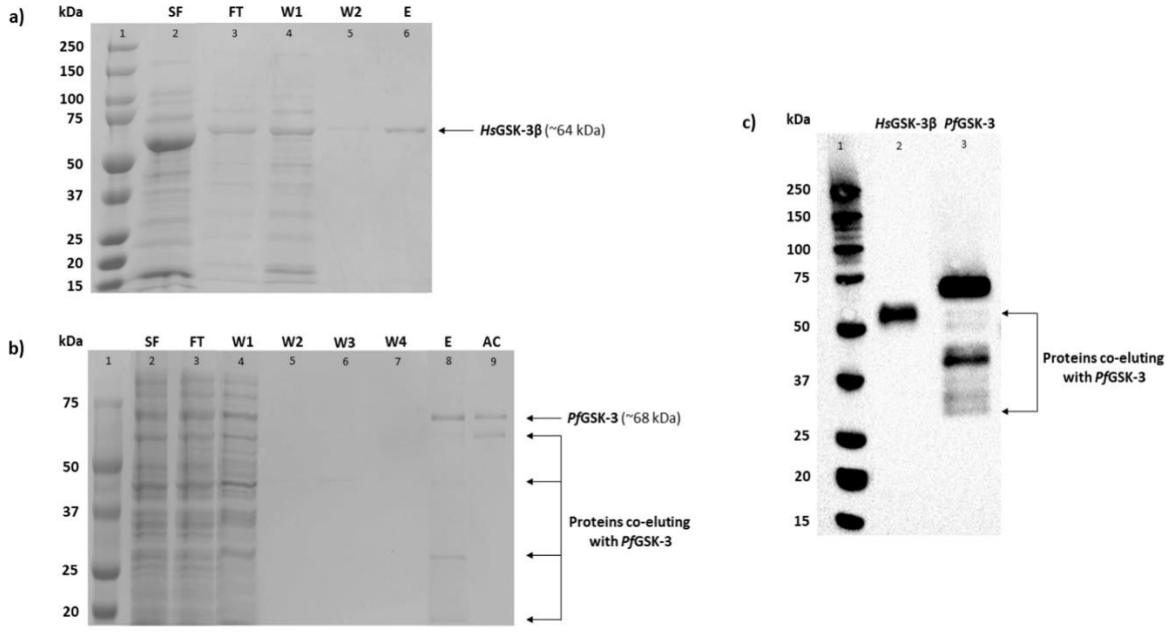
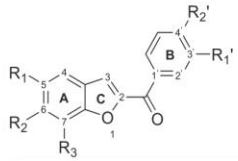


Fig. 2. 10% SDS-PAGE analysis of the different fractions collected during purification of recombinant *Hs*GSK-3β (**a**) and *Pf*GSK-3 (**b**). For *Hs*GSK-3β (**a**): Lane 1: protein ladder (kDa); SF: soluble fraction loaded onto the Protino® Ni-TED column; W1-W2: column wash with LEW buffer; E: column elution fraction. For *Pf*GSK-3 (**b**): Lane 1: protein ladder (kDa); SF: soluble fraction loaded onto the Protino® Ni-TED column; W1: column wash with LEW buffer; W2-W4: column wash with 5% imidazole buffer; E: column elution fraction; AC: concentrate collected from the Amicon® centrifugal device. Western blot analysis (**c**) of the column elution fractions of *Hs*GSK-3β and *Pf*GSK-3.

Table 1
Structures and IC_{50} values of benzofuran derivatives assayed against recombinant *Hs*GSK-3β and *Pf*GSK-3.



#	Ring A			Ring B		IC_{50} ± SD (μM) [a]		SI [b]
	5	6	7	3′	4′			
	R_1	R_2	R_3	$R_{1'}$	$R_{2'}$	*Hs*GSK-3β	*Pf*GSK-3	
5a	H	H	H	H	H	–	–	–
5b	Cl	H	H	H	H	–	–	–
5c	Cl	H	Br	H	H	–	–	–
5d	Cl	H	H	H	Cl	–	–	–
5e	Cl	H	Br	H	Cl	–	–	–
5f	H	H	H	H	Cl	–	–	–
5g	H	Diethylamino	H	H	H	–	–	–
5h	H	H	OCH_3	H	Cl	–	–	–
5i	H	OCH_3	H	H	Cl	–	–	–
5j	OCH_3	H	H	H	Cl	–	–	–
5k	H	OCH_3	H	Cl	Cl	25.817 ± 0.354	1.692 ± 0.850	15
5l	H	OCH_3	H	H	H	–	–	–
5m	H	OCH_3	H	H	F	0.294 ± 0.014	0.00168 ± 0.00005	175
5n	H	OCH_3	H	H	Br	–	–	–
5o	H	OCH_3	H	H	OCH_3	–	–	–
5p	H	OCH_3	H	H	CN	0.155 ± 0.005	0.00049 ± 0.00010	316
5q	OCH_3	OCH_3	H	H	Cl	–	–	–
5r	H	OCH_3	H	OCH_3	H	70.997 ± 0.506	0.127 ± 0.010	559
5s	H	H	OCH_3	OCH_3	H	33.960 ± 1.054	0.440 ± 0.113	77
5t	H	H	OH	H	Cl	–	–	–
5u	H	OH	H	H	Cl	0.137 ± 0.002	12.560 ± 0.352	0
5v	OH	H	H	H	Cl	5.449 ± 0.170	5.103 ± 0.580	1
Indirubin-3′-monoxime						0.005 ± 0.001 (0.005–0.05) [c]	0.250 ± 0. 080 (0.065) [d]	–

[a] The IC_{50} values were determined in triplicate and expressed as the mean ± standard deviation (SD) in μM. IC_{50} values were only determined for those compounds that exhibited inhibitory activity during the initial screening at 10 μM concentration.
[b] The selectivity index (SI) is the selectivity for *Pf*GSK-3 and is given as the ratio of (*Hs*GSK-3β IC_{50})/ (*Pf*GSK-3 IC_{50}).
[c] Literature value obtained from [37] for recombinant *Hs*GSK-3β.
[d] Literature value obtained from [18] for a truncated form of recombinant *Pf*GSK-3.

pyridines. However, as Masch and co-workers [29] pointed out, comparison of IC_{50} values is only relevant when the same assay is used to generate the data. Fugel and co-workers [25] used a radiometric kinase assay, while Masch and co-workers [29] compared a radiometric assay to the Kinase-Glo® Plus Luminescent Assay (Promega, Madison, WI). Although, the ADP-Glo™ Kinase Assay used during this study is similar to the Kinase-Glo® Plus Assay, the two assays differ in that they quantify different aspects of the kinase reaction. The Kinase-Glo® Plus Assay quantifies the remaining ATP after the kinase reaction has taken place, and the resulting luminescent signal is inversely proportional to the kinase activity. The ADP-Glo™ Kinase Assay quantifies ADP generated during the kinase reaction, and the luminescent signal is directly proportional to the kinase activity. An advantage of the ADP-Glo™ Kinase Assay used in this study is that much lower levels of ADP formation can be detected, which is beneficial when working with low enzyme concentrations or kinases with a low enzyme turnover rate [36]. The choice of kinase assay is therefore an important aspect that should be carefully considered and standardised to enable comparison of the IC_{50} values in future studies.

Analysing the structure-activity relationships (SARs) of the benzofuran derivatives with both *Pf*GSK-3 and *Hs*GSK-3β, revealed some interesting trends. The *Pf*GSK-3 selective compounds (**5k**, **5m**, **5p**, **5r**, **5s**) all have a C6-OCH_3 substitution on ring A, except for compound **5s** which has a C7-OCH_3 substitution. Active compounds seem to favour the substitution of ring A with a single OCH_3 group at position C6, for when the position of the OCH_3 group is changed (**5j**: C5-OCH_3) or an additional OCH_3 group is added to ring A (**5q**: C5,6-diOCH_3), the compound loses its activity. Likewise, when substituents other than OCH_3 are present at position C6 of ring A, the compound either loses its activity (**5g**: C6-diethylamino group), or becomes more selective towards *Hs*GSK-3β (**5u**: C6-OH). A loss of activity is also observed when ring A is substituted with Cl and Br groups (**5b** & **5d**: C5-Cl; **5c** & **5e**: C5-Cl, C7-Br), or when ring A is unsubstituted (**5a** & **5f**). Substituting ring A with an OH group results in unselective activity (**5v**: C5-OH) or loss of activity (**5t**: C7-OH).

The activity of the compounds also seems to be influenced by the substitution pattern of ring B. The compounds with C6-OCH_3 substitution on ring A are active and selective towards *Pf*GSK-3 when ring B is substituted with a C3′,4′-diCl (**5k**); C4′-F (**5m**); C4′-CN (**5p**); or C3′-OCH_3 (**5r**) group. However, when ring B is unsubstituted (**5l**) or substituted with a Cl (**5i**), Br (**5n**) or OCH_3 (**5o**) group at position C4′, the compounds were inactive. Likewise, the activity of compounds with C7-OCH_3 substitution on ring A is also influenced by the substitution pattern of ring B. This is clearly demonstrated when comparing compounds **5s** and **5h**. The *Pf*GSK-3 selective compound **5s** is substituted at position C3′ (OCH_3 group) of ring B, while the inactive compound **5h** is substituted at position C4′ (Cl group). The effect of substituting ring B with other types of substituents at position C3′ and C4′ still has to be determined.

4. Conclusion

With the continuing threat of multi-drug resistant malaria, an urgent need exists to develop novel antimalarial drugs. Herein, a series of

benzofuran derivatives was evaluated as potent and selective *Pf*GSK-3 inhibitors. Five compounds demonstrated selectivity for *Pf*GSK-3 over *Hs*GSK-3β, with IC_{50} values ranging from 0.00049 to 1.692 μM. Compound **5p** and **5m** were the most potent *Pf*GSK-3 inhibitors of the series (IC_{50}: 0.00049 μM and 0.00168 μM, respectively), while compound **5r** was the most selective inhibitor with a 559-fold selectivity towards *Pf*GSK-3. The SARs of these compounds provide valuable insight with regards to the use of chalcone-based scaffolds for the design of *Pf*GSK-3 selective inhibitors. The results warrant further exploration of the benzofuran class of inhibitors with regards to their interaction with the *Pf*GSK-3 active site (molecular docking), *in vitro* activity against blood stage *P. falciparum* parasites, toxicity, and selectivity towards other human protein kinases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.104839.

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Chapter 5: Conclusion

5.1. Introduction

Effective malaria control relies heavily on the limited number of available antimalarial drugs. The ability of the *Plasmodium* parasite to develop resistance to these drugs necessitates the continuous generation of new antimalarial drugs with novel modes of action (Mishra *et al.*, 2017:1301). Plasmodial protein kinases show promise as targets for next-generation antimalarial drugs due to their involvement in several essential processes throughout the various stages of the parasite's life cycle (Doerig, 2004:158; Doerig *et al.*, 2010:605). Although significant progress has been made towards drug discovery for this group, medicinal chemistry efforts for some essential plasmodial kinases have been limited (Cabrera *et al.*, 2018:8068). For instance, *Pf*GSK-3 is a plasmodial kinase that is essential for survival of the erythrocytic stages of *P. falciparum* (Solyakov *et al.*, 2011:3). Although some *Pf*GSK-3 selective inhibitors have been identified (Fugel *et al.*, 2013:271; Masch *et al.*, 2019:10), these inhibitors all belong to the 4-phenylthieno[2,3-*b*]pyridine class. The present study explored benzofurans as an alternative scaffold for the design of potent and selective *Pf*GSK-3 inhibitors.

5.2. Findings and contributions

5.2.1. Chapter 3 – Review on small-molecule plasmodial kinase inhibitors

Although not listed as a specific objective, the first contribution of this study was a review article published in *Molecules* (Moolman *et al.*, 2020). The last extensive review published on this topic was in 2018 by Cabrera *et al*. (2018). Since then several new studies have been published on plasmodial kinase inhibitor development, including kinases that were targeted previously (e.g. *Pf*CDPK1, *Pf*PKG, *Pf*GSK-3 and *Pf*PI4K) and kinases targeted for the first time (e.g. *Pf*CDPK5, *Pf*PK5 and *Pf*CLK3). The current article provides an overview of the work summarised by Cabrera and co-workers, as well as discussing new research, and additional plasmodial kinases. This article provides other researchers in the field of antimalarial drug development with valuable information on:

- the different types of scaffolds identified thus far through phenotypic- and target-based screening approaches. Here the reader can identify which scaffolds show promise as starting points for which plasmodial kinases, how the scaffolds have been modified in previous studies, as well as the successes and problems highlighted for specific scaffolds.
- the possible functions, life cycle stage specificity, and compensatory mechanisms that have been identified for certain plasmodial kinases. The reader can see which plasmodial kinases have been validated as essential to parasite survival as well as the level of validation achieved. This includes

genetic validation – where potential essentiality is confirmed by disrupting gene function or expression, phenotypic validation – where a chemical compound inhibits a kinase target and also demonstrates an effect on the parasite, *in vivo* validation – where the chemical compound reduces parasitaemia in a malaria mouse model, and clinical validation – where the drug demonstrates efficacy in malaria patients.

- the availability of crystal structures or homology models, methods for expressing recombinant plasmodial kinases, as well as biological assays to screen inhibitors.
- certain essential plasmodial kinases that are yet to be targeted by drug discovery programs or that require further characterisation.

Overall, this review highlights the gaps that still exist in plasmodial kinase inhibitor development and provides information that can be used as a starting point for future medicinal chemistry efforts.

5.2.2. Chapter 4 – Evaluation of benzofuran derivatives as inhibitors of *Pf*GSK-3

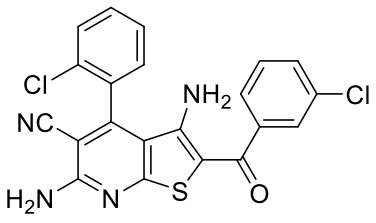
The first part of this study focused on the design, synthesis and characterisation of benzofuran derivatives (objectives 1 and 2). A series of 22 benzofuran derivatives were synthesised and characterised through NMR, MS, HPLC and mp analyses. The benzofuran derivatives were designed to carry a variety of polar and lipophilic substituents on the C5, C6 and C7 positions of ring A, and the C3'and C4' positions of ring B. The second part of this study involved the expression and purification of active recombinant *Hs*GSK-3β and *Pf*GSK-3 (objective 3). The method described by Wang *et al*. (1994:14567) was adapted slightly for the expression of *Hs*GSK-3β – the pGro7 chaperone proteins, *gro*ES and *gro*EL, were co-expressed with *Hs*GSK-3β to facilitate the protein folding process and thereby increase recovery of soluble *Hs*GSK-3β. *Pf*GSK-3 was expressed using a similar method to the one described by Droucheau *et al.* (2004:184). However, the present study expressed the full-length *Pf*GSK-3 protein while Droucheau *et al.* (2004:182) expressed a truncated version of the *Pf*GSK-3 protein (N-terminal was omitted). The purification process for *Pf*GSK-3 required extensive optimisation due to the co-elution of several other proteins with recombinant *Pf*GSK-3. Droucheau *et al.* (2004:189) also reported the presence of other proteins after purification and concluded that it was likely proteolytic degradation products of *Pf*GSK-3. The present study optimised a purification method for *Pf*GSK-3 (Annexure C, p.192) which included:

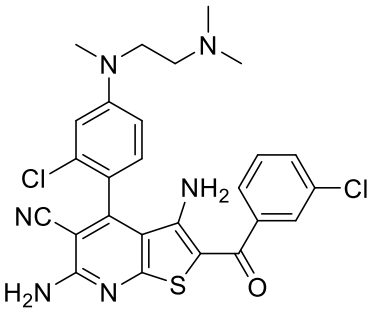
- Adding protease inhibitors to the extraction reagent to prevent proteolytic degradation.
- Adapting the wash protocol of the Protino® Ni-TED columns – instead of only washing the column with LEW (Lysis-Equilibration-Wash) buffer, the column was also washed with diluted Elution buffer containing 5% imidazole. This allowed some of the co-eluting proteins to be washed away before eluting the *Pf*GSK-3 protein.

- Using an Amicon® Ultra-15 centrifugal device as a second method of purification. Since a very prominent band was visible between 37-50 kDa on the Western blot (Chapter 4, Figure 2c), the Amicon® device was used to remove all proteins smaller than 50 kDa.

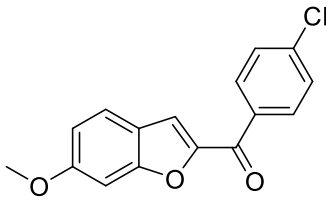
Although these additional measures significantly reduced the number of proteins co-eluting with *Pf*GSK-3, one other protein still remained (Chapter 4, Figure 2b).

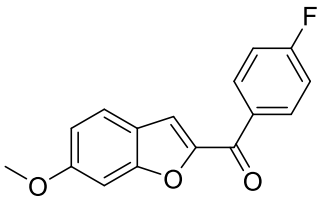
The third part of this study involved screening of the benzofuran derivatives against *Hs*GSK-3β and *Pf*GSK-3 using the ADP-Glo™ Kinase Assay (objective 4 and 5). Of the seven compounds that demonstrated activity, five compounds (**5k**, **5m**, **5p**, **5r**, **5s**) selectively inhibited *Pf*GSK-3 in the submicromolar to micromolar range (IC_{50}: 0.00048 – 1.692 µM). Compound **5p** was the most active compound of the series (*Pf*GSK-3 IC_{50}: 0.00049 µM, *Hs*GSK-3β IC_{50}: 0.155 µM), followed closely by compound **5m** (*Pf*GSK-3 IC_{50}: 0.00168 µM, *Hs*GSK-3β IC_{50}: 0.294 µM). Both compounds **5p** and **5m** demonstrated high selectivity towards *Pf*GSK-3 (316- and 175-fold, respectively). Compound **5r** (*Pf*GSK-3 IC_{50}: 0.127 µM, *Hs*GSK-3β IC_{50}: 70.997 µM) was the most selective inhibitor of the series with 559-fold selectivity towards *Pf*GSK-3. Compared to the 4-phenylthieno[2,3-*b*]pyridine inhibitors (**19** and **20**, Figure 5.1), the benzofurans (**5p**, **5m**, **5r**) demonstrated greater inhibitory activity (i.e. lower IC_{50} values) and higher selectivity towards *Pf*GSK-3. However, Masch *et al.* (2019:7) observed that comparison of IC_{50} values is only relevant if the same assay was used. Unfortunately, the data available for *Pf*GSK-3 were generated using three different assays. Fugel *et al.* (2013:272) used a radiometric assay, while Masch *et al.* (2019:7) compared a radiometric assay to the Kinase-Glo® Plus luminescent assay. The Kinase-Glo® Plus assay is very similar to the ADP-Glo™ Kinase assay used during this study, however, the two assays quantify different aspects of the kinase reaction (Tanega *et al.*, 2009:609). The ADP-Glo™ Kinase assay was specifically chosen for this study as it can detect low levels of ADP formation, which is beneficial when working with low enzyme concentrations or kinases with a low enzyme turnover rate (Tanega *et al.*, 2009:613). It is therefore important to standardise a kinase assay for *Pf*GSK-3 to allow IC_{50} value comparison in future studies.

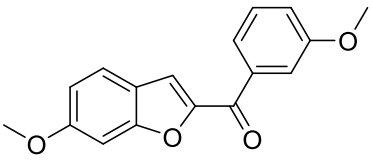




	19	**20**
***Pf*GSK-3 IC_{50}**	0.48 µM[a] (0.24 µM)[b]	0.72 µM[b]
***Hs*GSK-3β IC_{50}**	3.3 µM[a] (9.08 µM)[b]	40.2 µM[b]
SI[d]	7 (38)	56







	5p	**5m**	**5r**
***Pf*GSK-3 IC_{50}**	0.00049 µM[c]	0.00168 µM[c]	0.127 µM[c]
***Hs*GSK-3β IC_{50}**	0.155 µM[c]	0.294 µM[c]	70.997 µM[c]
SI[d]	316	175	559

Figure 5.1: Chemical structures and biological data of the most active 4-phenylthieno[2,3-*b*]pyridines (**19** and **20**) and benzofurans (**5p**, **5m** and **5r**). [a] IC_{50} values determined using a radiometric assay (Fugel *et al.*, 2013:272); [b] IC_{50} values determined using the Kinase Glo® Plus assay (Masch *et al.*, 2019:8); [c] IC_{50} values determined using the ADP-Glo™ Kinase assay (present study); [d] Selectivity index (SI): the selectivity for *Pf*GSK-3 is given as the ratio of (*Hs*GSK-3β IC_{50})/(*Pf*GSK-3 IC_{50}).

This study aimed to gain insight into the structural modifications to ring A and B of the benzofuran scaffold necessary for potent and selective inhibition of *Pf*GSK-3. Evaluation of the structure-activity relationships (objective 6) indicated that C6-OCH_3 substitution on ring A promotes selectivity towards *Pf*GSK-3. The effect of ring B substitution on activity, in decreasing order, was: C4'-CN > C4'-F > C3'-OCH_3 > C3,4'-diCl. The structure-activity data reported herein provide valuable insight for further optimisation of the benzofuran scaffold.

Overall, the benzofurans proved to be highly potent and selective *Pf*GSK-3 inhibitors. As *Pf*GSK-3 inhibitors are currently limited to the 4-phenylthieno[2,3-*b*]pyridine class, the benzofurans are promising new hit

compounds that can be explored in future medicinal chemistry efforts. Since *Pf*GSK-3 is essential to the erythrocytic stage of the *Plasmodium* parasite, the benzofurans have potential as curative antimalarial drugs. These compounds also differ from existing antimalarial drugs in terms of chemical scaffold and mechanism of action, which has potential in combating multidrug-resistant malaria. The results from this study were published in *Bioorganic Chemistry* (Moolman *et al.*, 2021).

5.3. Future prospects

The results from this study warrant further exploration of the benzofuran class of inhibitors, including:

- Homology modelling – as the crystal structure of *Pf*GSK-3 is not yet available, homology modelling can be used to generate three-dimensional structure information for this enzyme using a homologous protein as a template. *Hs*GSK-3 is ideal for this purpose as it shares a high degree of sequence similarity with *Pf*GSK-3. Previous studies have generated homology models for *Pf*GSK-3, using the available *Hs*GSK-3β crystal structure as a template (Droucheau *et al.*, 2004:183; Kruggel & Lemcke, 2009b:328). The homology model can then be used to study the interaction of the benzofuran derivatives with the *Pf*GSK-3 active site. This will provide insight into the interactions required for selectivity which can be used for further optimisation of the benzofuran scaffold.
- Screening against blood stage *P. falciparum* cultures (drug-sensitive and -resistant strains) to determine whether these compounds demonstrate the desired antimalarial activity *in vitro*, and how their activity compares to that of existing antimalarial drugs. Discrepancies between the enzymatic and whole-cell activities may indicate off-target activity or permeability issues.
- Assessing the *in vitro* ADME properties. This includes determining the lipophilicity, solubility, hepatic and plasma stability, permeability, and cytotoxicity of the benzofuran derivatives. Compounds demonstrating good *in vitro* ADME profiles are more likely to have better efficacy *in vivo*. The ADME profiles can be used to identify promising lead compounds for evaluation *in vivo*, or indicate which compounds require further optimisation.
- Screening against human kinase panels. Kinase inhibitor promiscuity is always a concern due to the highly conserved nature of this target group. As ATP-competitive inhibitors, the benzofuran derivatives, in particular, are more likely to be promiscuous. Simultaneous inhibition of *Pf*GSK-3 and other plasmodial kinases could be beneficial as this would limit the risk of the parasite developing drug resistance. However, inhibition of host kinases could result in toxicity and unwanted side-effects, thus it is important to screen potential plasmodial kinase inhibitors against human kinases.
- Determining the *in vivo* efficacy in a malaria mouse model. Benzofuran derivatives demonstrating favourable *in vitro* activity, ADME properties and selectivity, should be evaluated for their ability to reduce parasitaemia *in vivo*.

5.4. References

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Annexure A: Author guidelines for *Molecules*

Instructions for Authors

Manuscript Submission Overview

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- *Reviews:* These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA **guidelines.**

Submission Process

Manuscripts for *Molecules* should be submitted online at **susy.mdpi.com**. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the **criteria to qualify for authorship**) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the **submission website**. Once you have registered, **click here to go to the submission form for *Molecules***. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

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Authors must use the **Microsoft Word template** or **LaTeX template** to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office **molecules@mdpi.com**. Accepted file formats are:

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A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

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Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
  - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords
  - **Research manuscript sections**: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).
  - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.
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  A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing content, it should represent the topic of the article in an attention-grabbing way.

  The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, EPS, SVG, PSD or AI. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

  The minimum required size for the GA is 560 × 1100 pixels (height × width). When submitting larger images, please make sure to keep to the same ratio.

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- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on **Deposition of Sequences and of Expression Data**.
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- A concise summary of the diffraction parameters for a single structure should be included in the main body of the article. For example, the following popular format can be followed: "Crystal Data for C42H63P (M = 598.89 g/mol): monoclinic, space group P21/c (no. 14), a = 33.5426(3) Å, b = 10.06131(11) Å, c = 46.2696(5) Å, β = 93.3735(9)°, V = 15588.1(3) Å3, Z = 16, T = 99.9(2) K, μ(CuKα) = 0.790 mm-1, Dcalc = 1.021 g/cm3, 98894 reflections measured (6.7° ≤ 2Θ ≤ 155.122°), 32038 unique (Rint = 0.0381, Rsigma = 0.0401) which were used in all calculations. The final R1 was 0.0562 (I > 2σ(I)) and wR2 was 0.1452 (all data)."

  Only selected derived geometric data should be included that is directly relevant to describing the structure and its significant features. More extensive parameter tables can be included in the Optional Supplementary Materials. However, the inclusion of a suitable CIF file amongst the Supporting Materials is considered to be preferable.

Front Matter

These sections should appear in all manuscript types

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

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1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: **http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf**.

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- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

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Registration

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register or and to cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

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Annexure B: Author guidelines for *Bioorganic Chemistry*

BIOORGANIC CHEMISTRY

AUTHOR INFORMATION PACK

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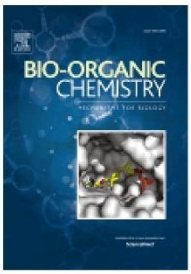

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DESCRIPTION

Bioorganic Chemistry publishes research that addresses biological questions at the molecular level, using organic chemistry and principles of physical organic chemistry. The scope of the journal covers a range of topics at the organic chemistry-biology interface, including: enzyme catalysis, biotransformation and enzyme inhibition; nucleic acids chemistry; medicinal chemistry; natural product chemistry, natural product synthesis and natural product biosynthesis; antimicrobial agents; lipid and peptide chemistry; biophysical chemistry; biological probes; bio-orthogonal chemistry and biomimetic chemistry.

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Annexure C: Paper II - Supplementary material

Exploration of benzofuran-based compounds as potent and selective *Plasmodium falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) inhibitors

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Supplementary material

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1. Chemistry

NMR: The proton (^{1}H) and carbon (C^{13}) NMR spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz and 151 MHz, respectively. Either deuterated chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO-*d6*) was used as solvent and tetramethylsilane (TMS) as internal reference for NMR analysis. All chemical shifts (δ) are reported as parts per million (ppm) in relation to the solvent signal ($CDCl_3$ residual peak at 7.26 ppm and DMSO-*d6* residual peak at 2.50 ppm for ^{1}H NMR). Spin multiplicities are reported as follows: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), q (quartet) and m (multiplet). Coupling constants (*J*) are reported in Hz.

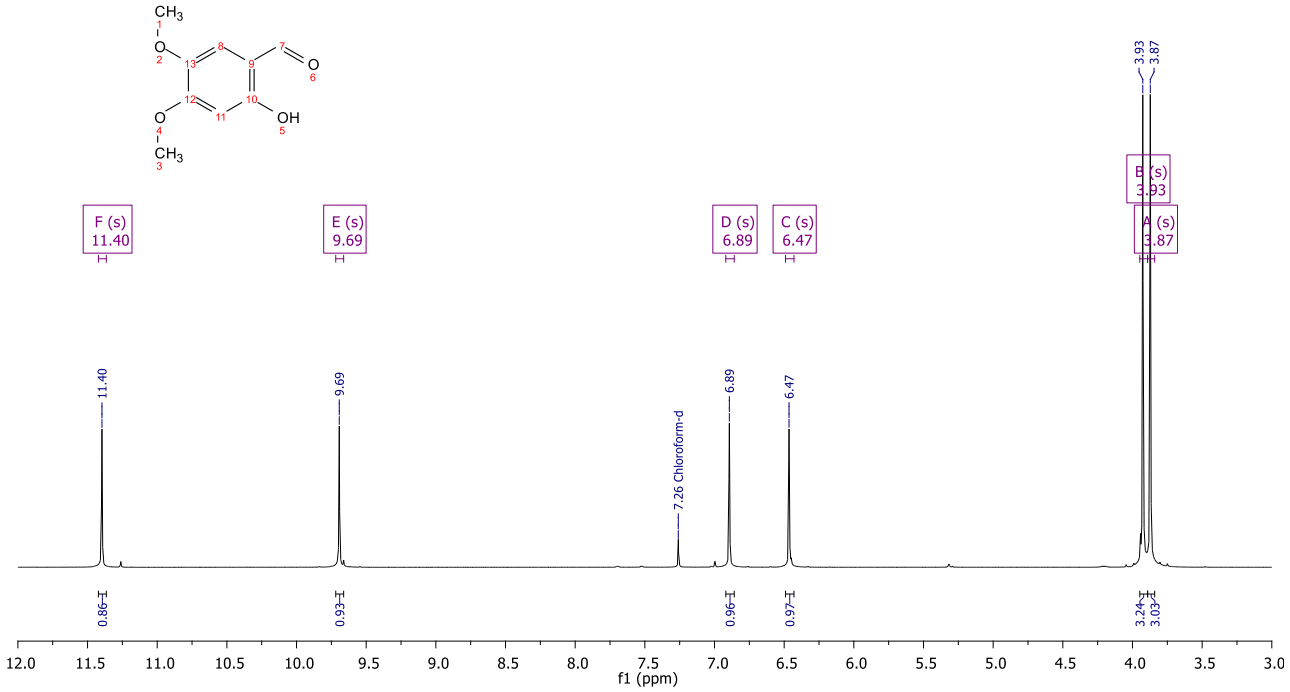
HRMS: High-resolution mass spectra were recorded on a Bruker micrOTOF-Q II mass spectrometer using atmospheric pressure chemical ionisation (APCI) in positive mode.

HPLC: High-performance liquid chromatography analyses were done using an Agilent 1100 HPLC system with a diode array detector (DAD). 10 µl of sample was injected onto a Venusil XBP C18 column (4.6 x 150 mm, 5 µM). The initial mobile phase consisted of 30% acetonitrile and 70% Milli-Q water with a flow rate of 1 ml/min. During the 15 min run, the composition of the mobile phase was changed by linearly increasing the acetonitrile concentration to 85%. The column was re-equilibrated for 5 min between runs. DAD wavelengths (nm) are indicated on each chromatogram.

1.1. NMR, HRMS and HPLC data for compounds 3a, 4a and 5a-v

2-Hydroxy-4,5-dimethoxybenzaldehyde (**3a**)

^{1}H NMR



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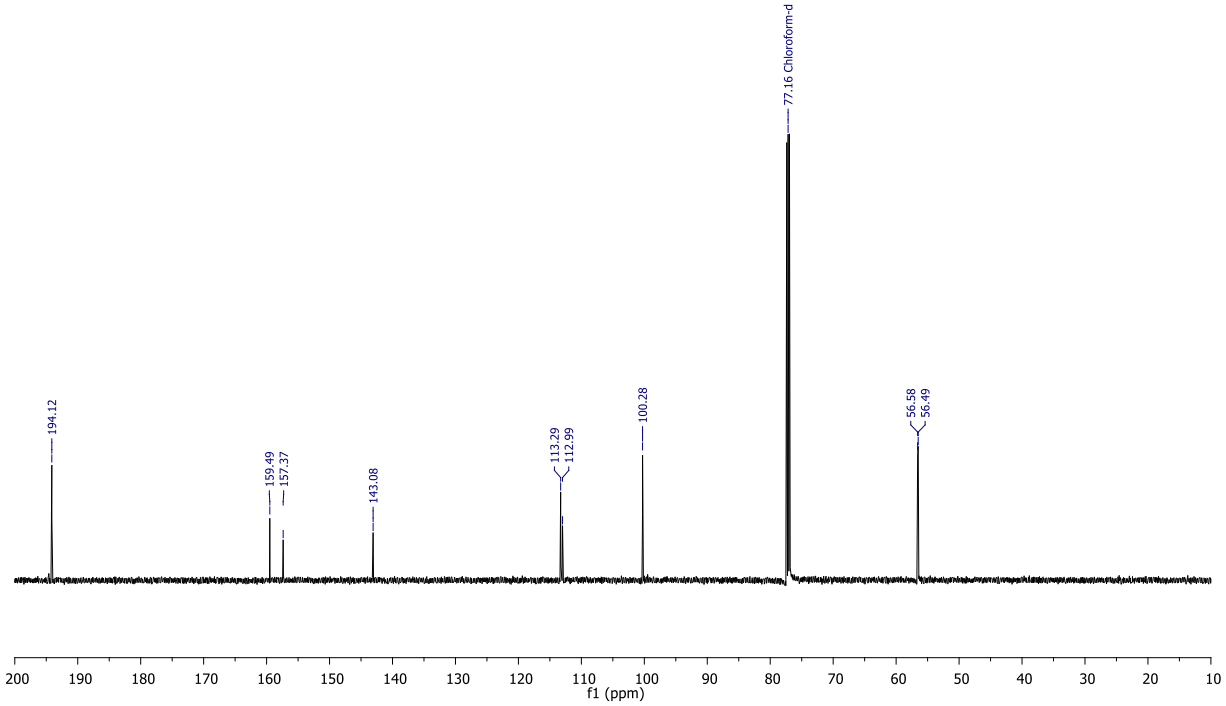

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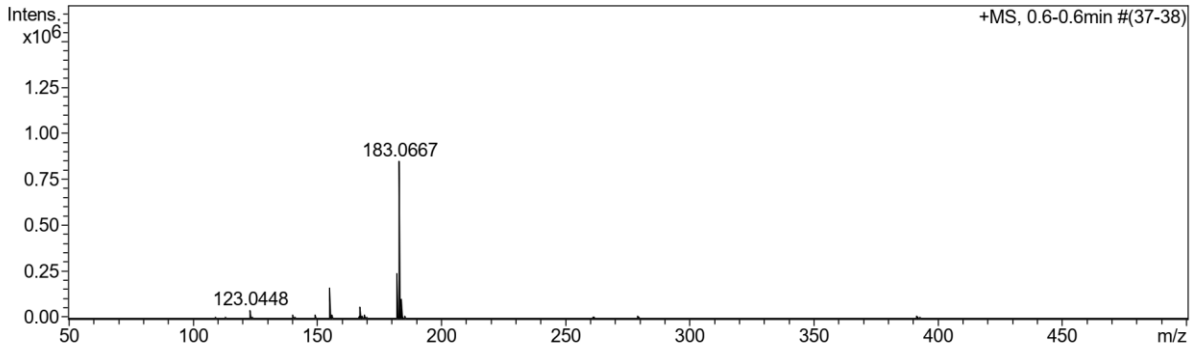
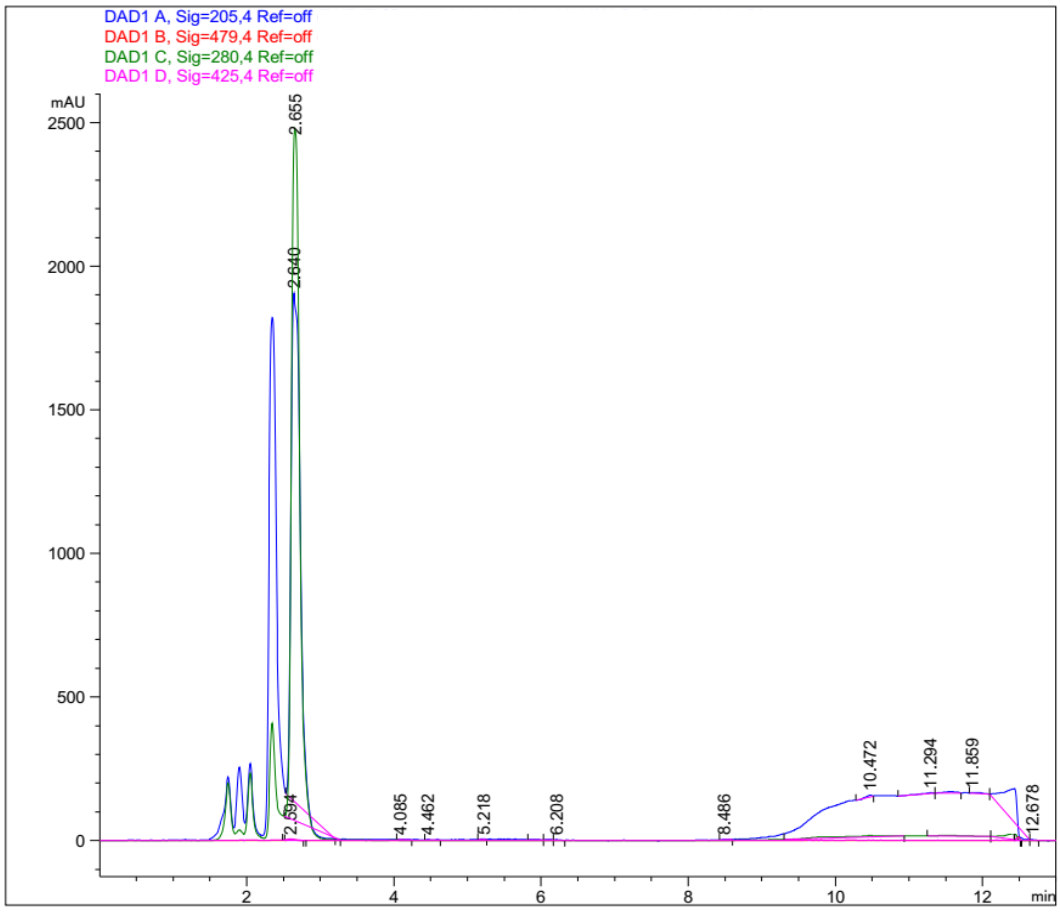


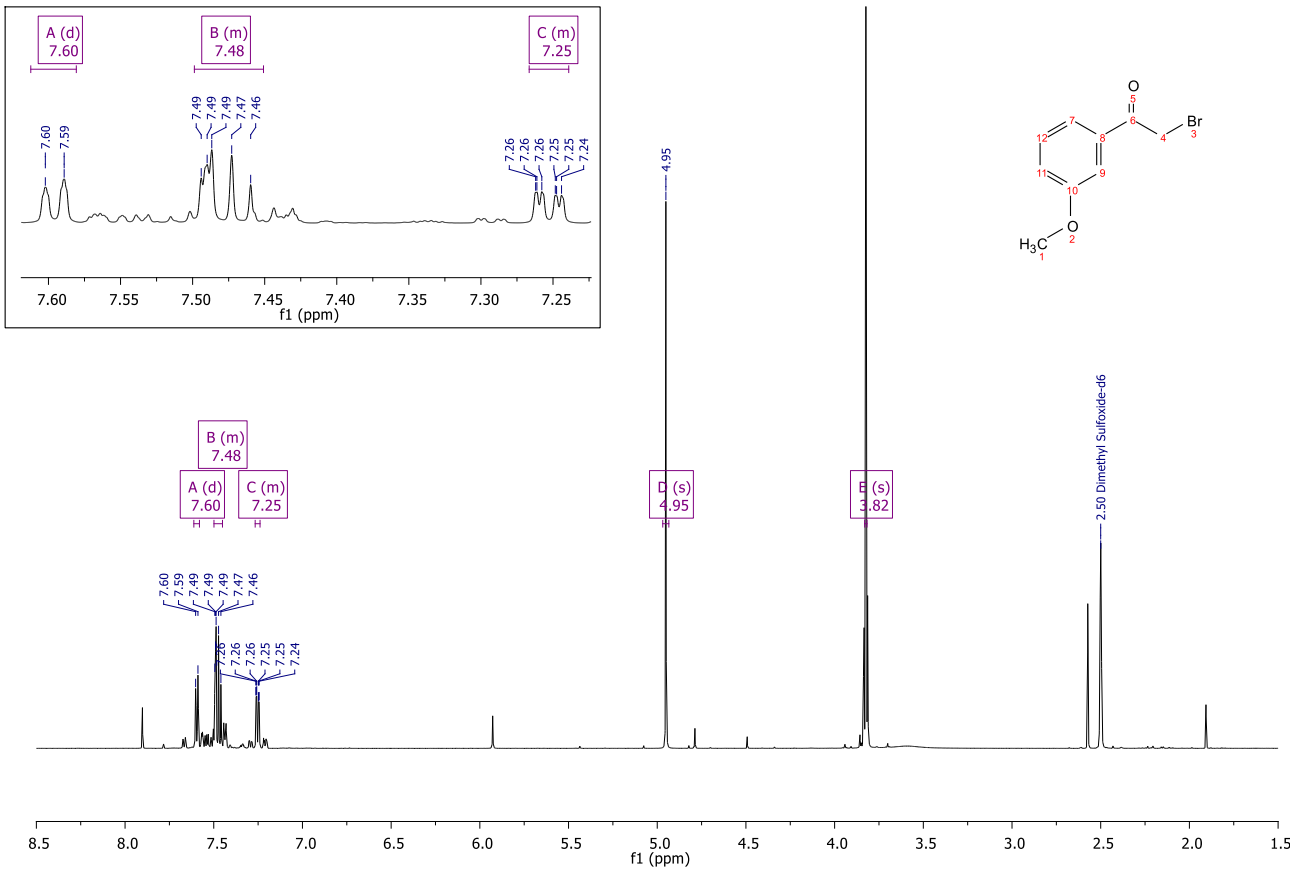
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
183.0667	1	C 9 H 11 O 4	100.00	183.0652	-1.5	-8.2	13.7	4.5	even	ok

HPLC

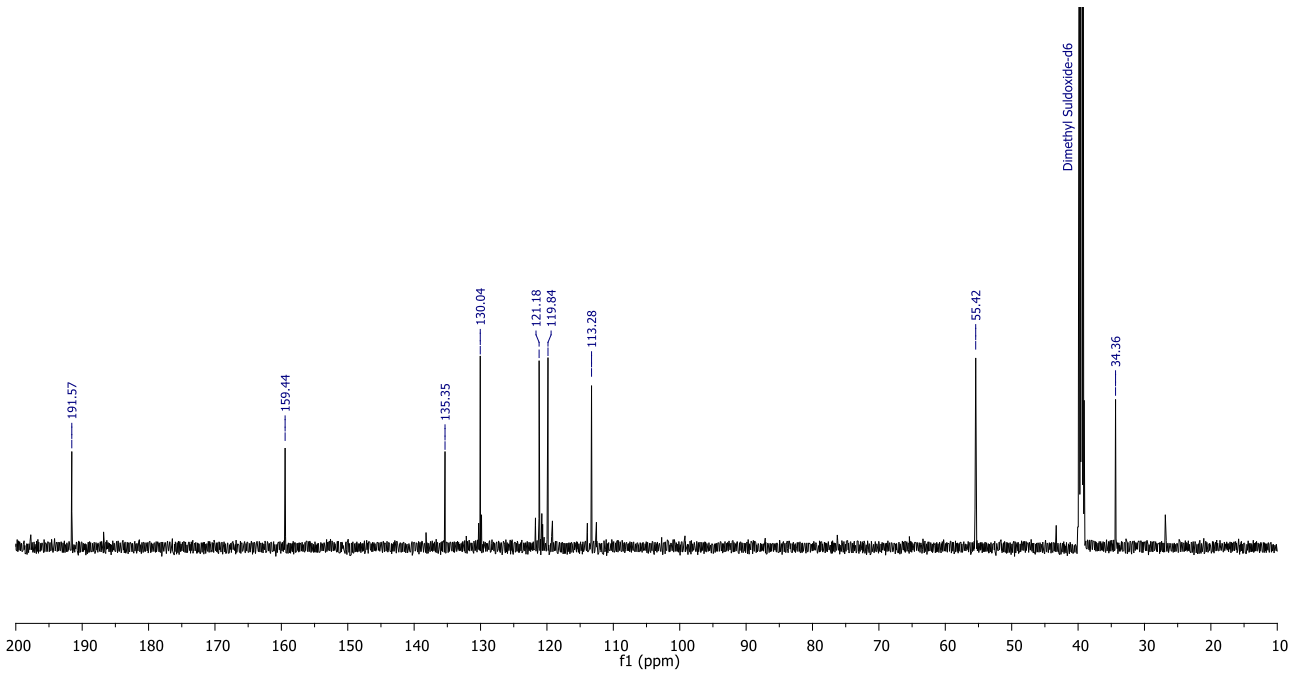


2-Bromo-3'-methoxyacetophenone (**4a**)

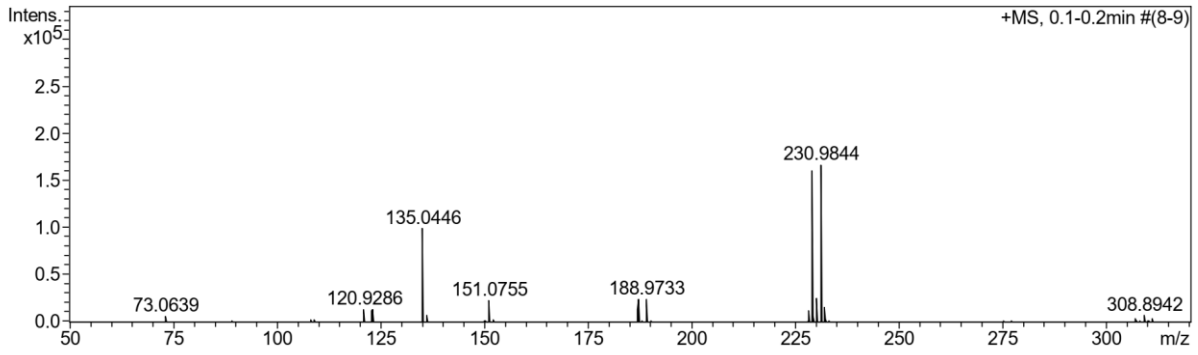
^{1}H NMR



^{13}C NMR

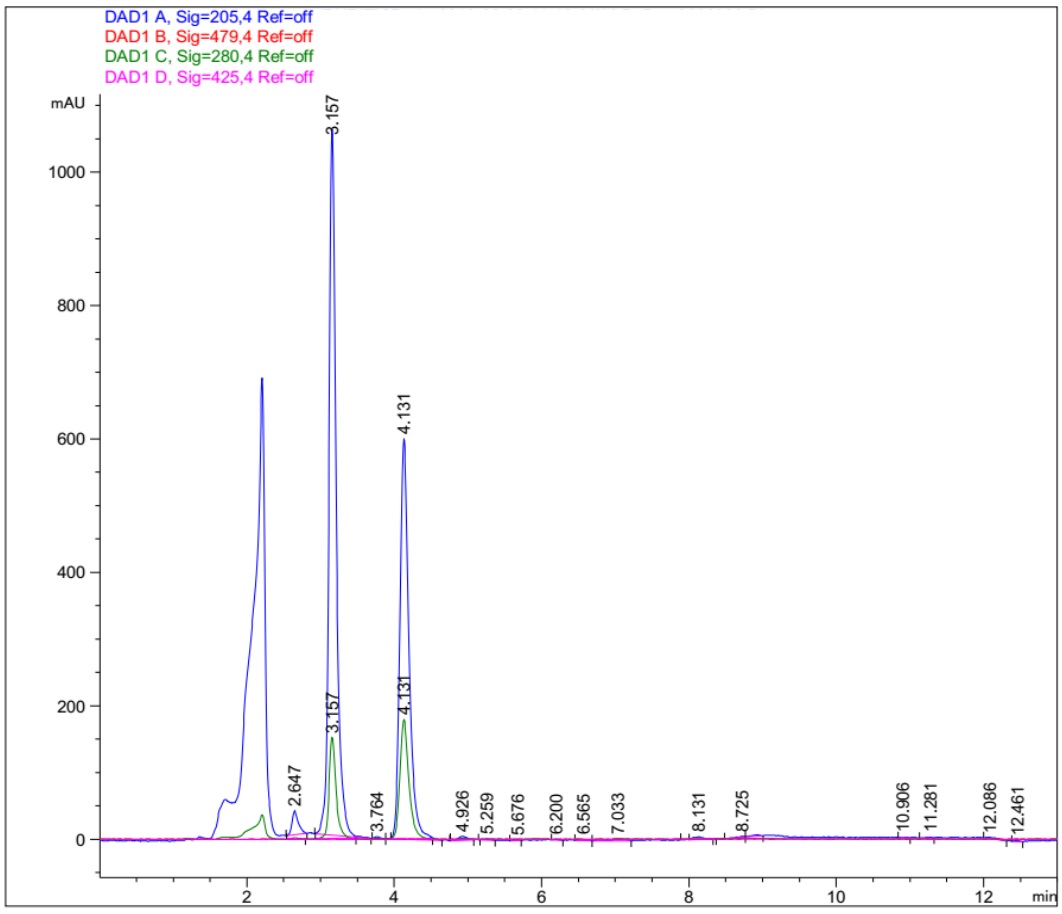


HRMS



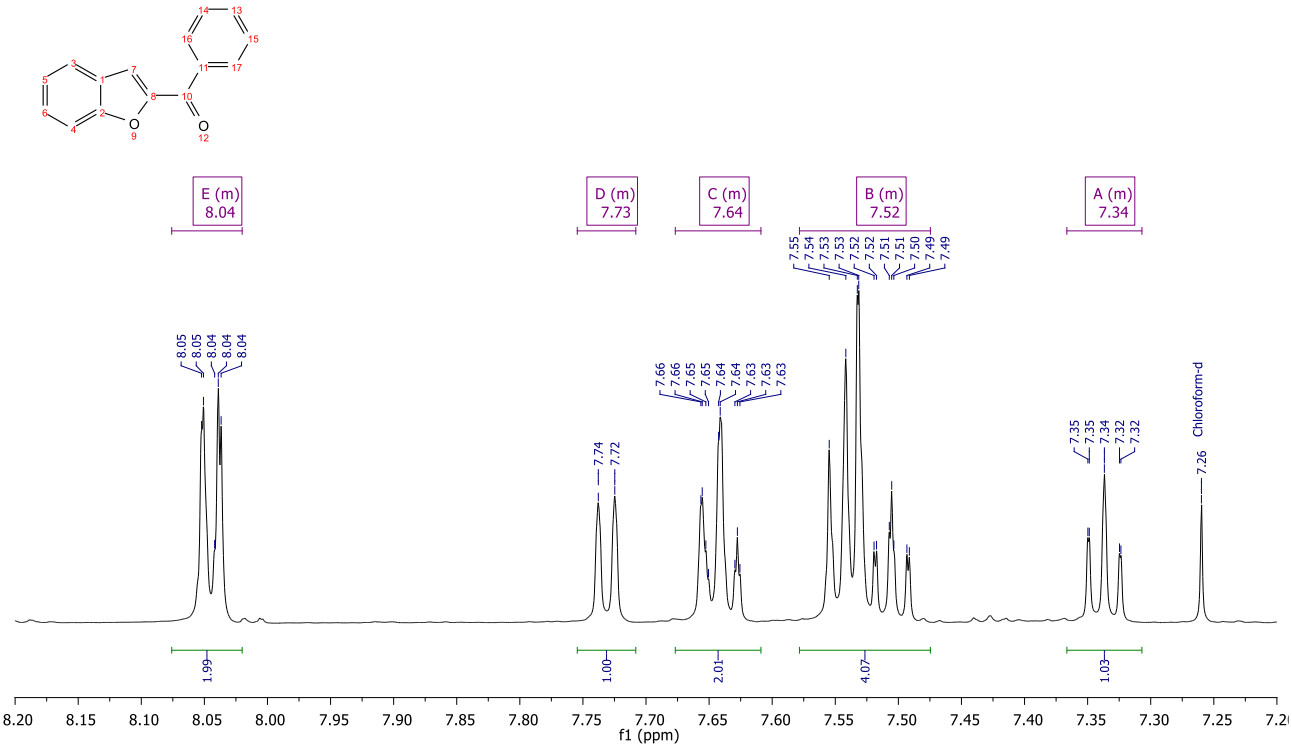
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
135.0446	1	C 8 H 7 O 2	100.00	135.0441	-0.6	-4.2	4.0	5.5	ok	even
228.9862	1	C 9 H 10 Br O 2	100.00	228.9859	-0.4	-1.6	30.9	4.5	ok	even

HPLC

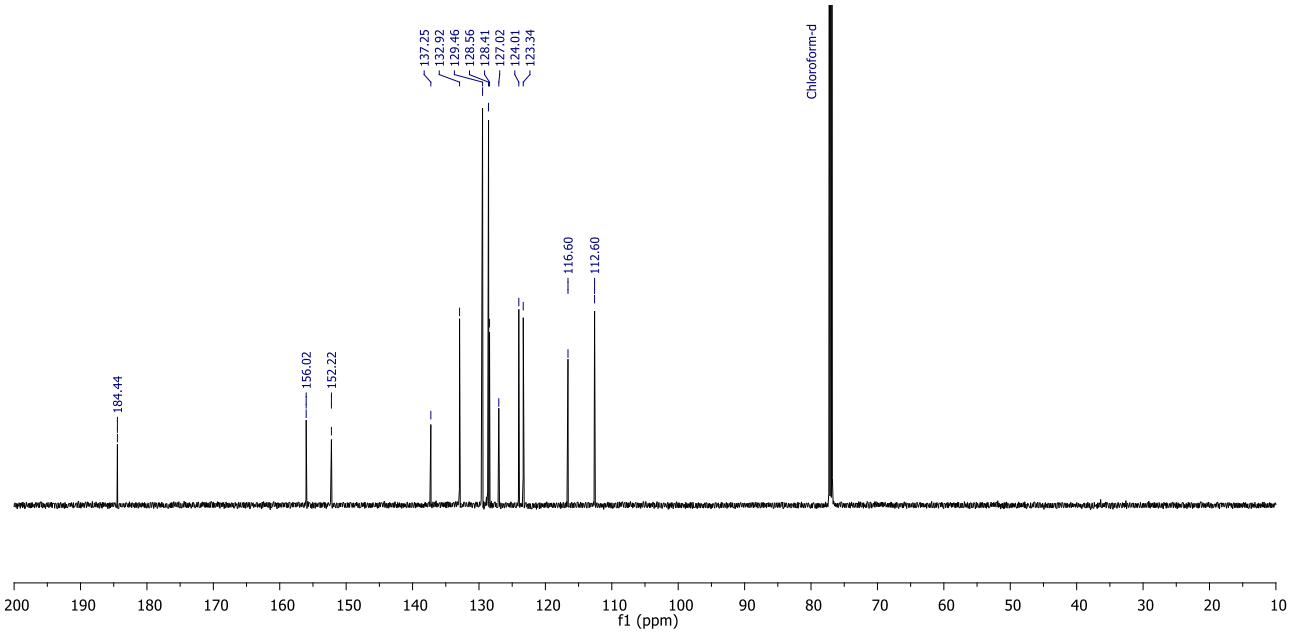


1-Benzofuran-2-yl(phenyl)methanone (**5a**)

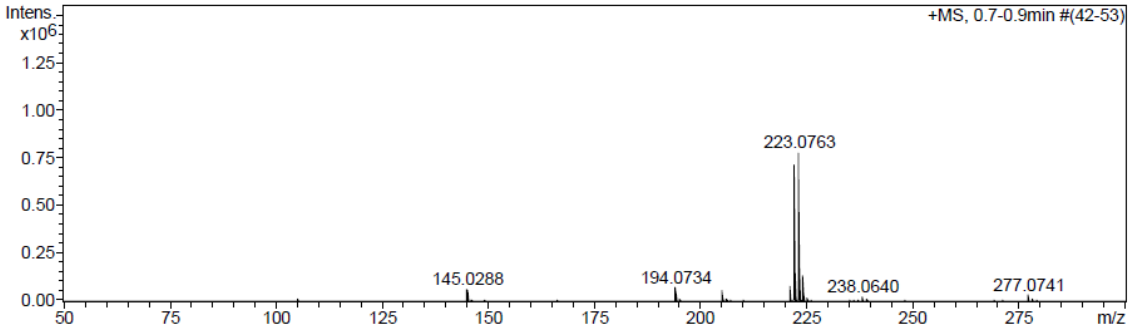
^{1}H NMR



^{13}C NMR

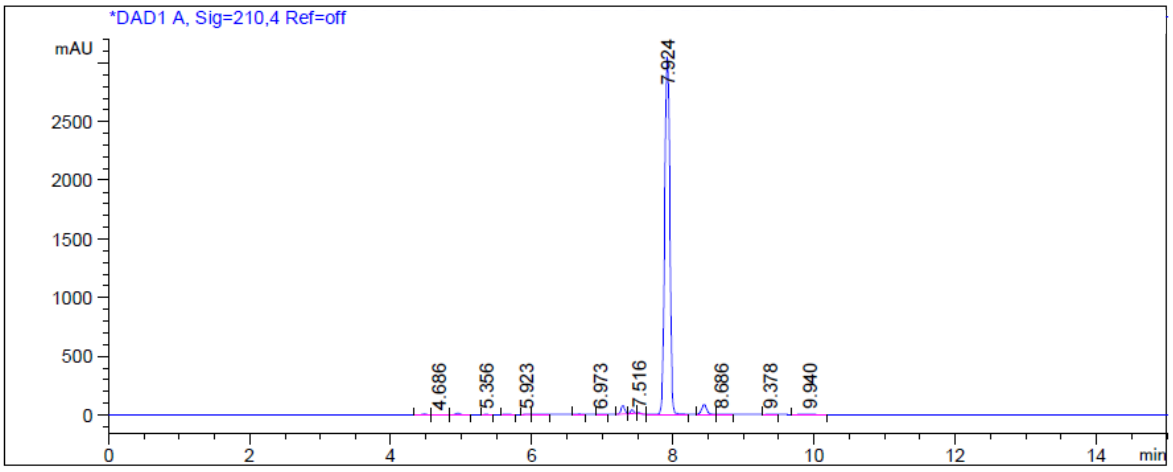


HRMS



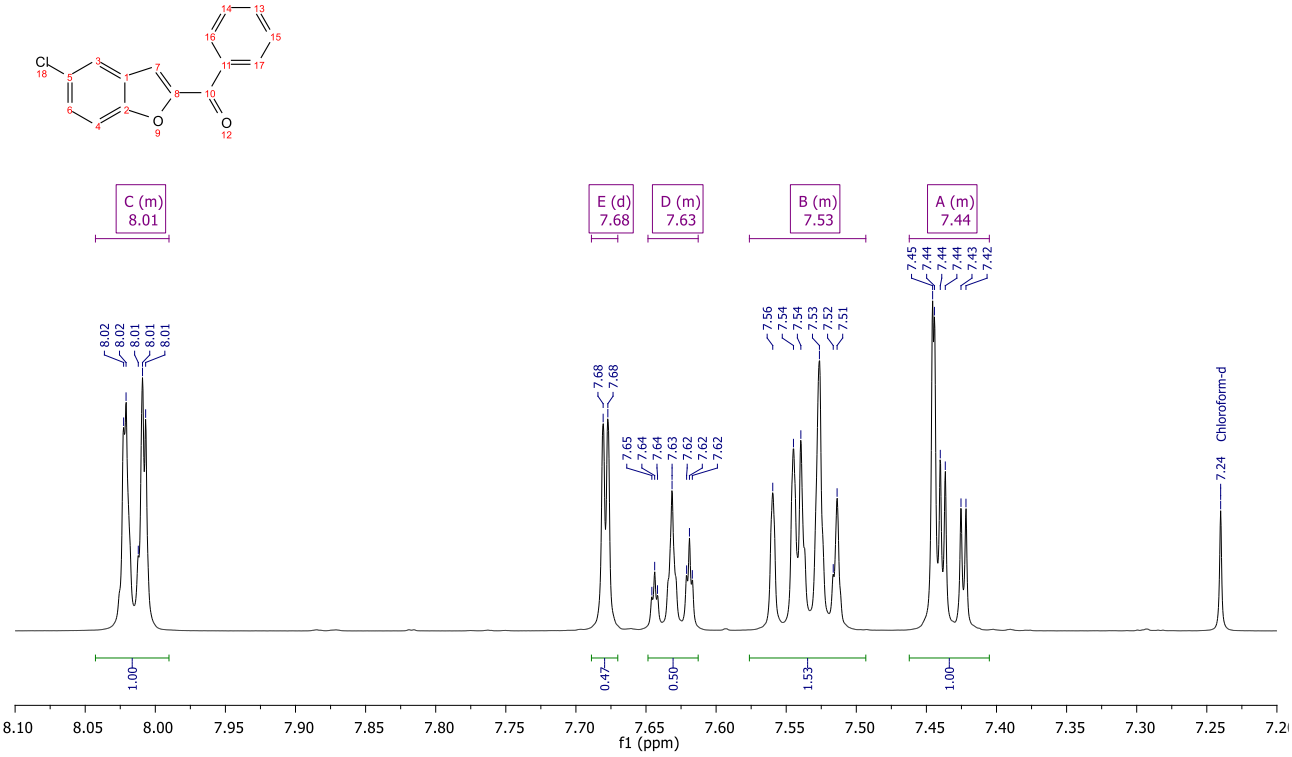
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
223.0763	1	C 15 H 11 O 2	100.00	223.0754	-1.0	-4.3	5.9	10.5	even	ok

HPLC

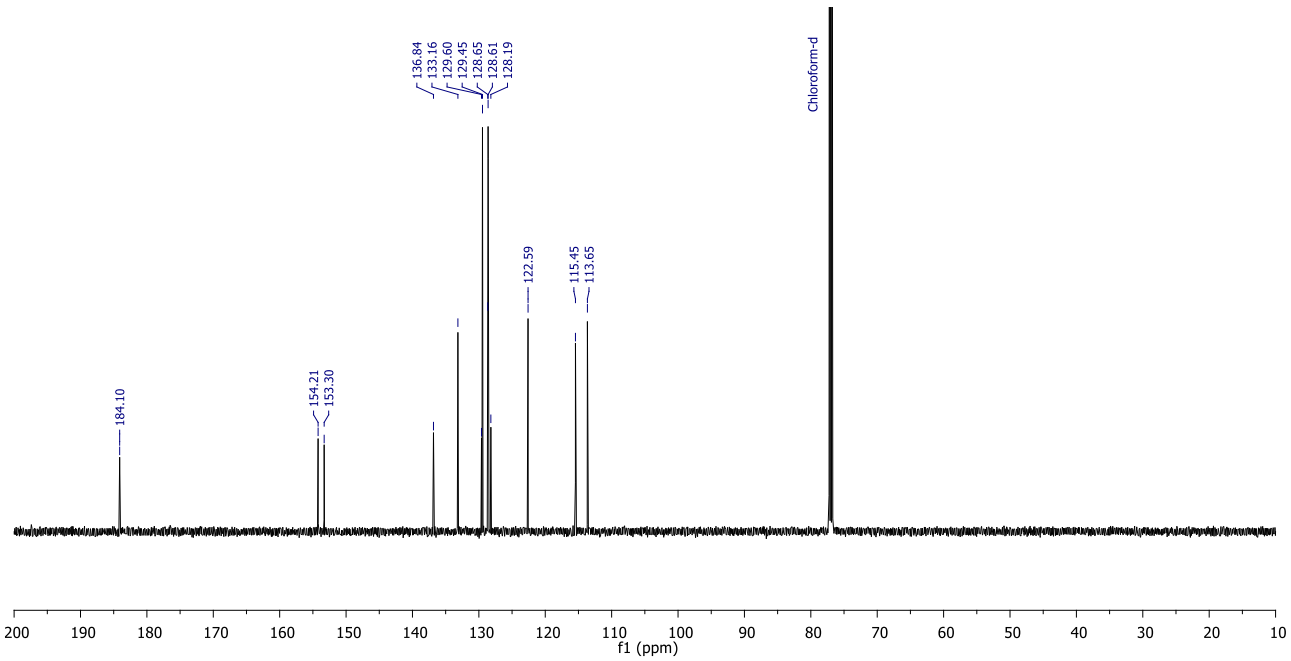


(5-chloro-1-benzofuran-2-yl)(phenyl)methanone (**5b**)

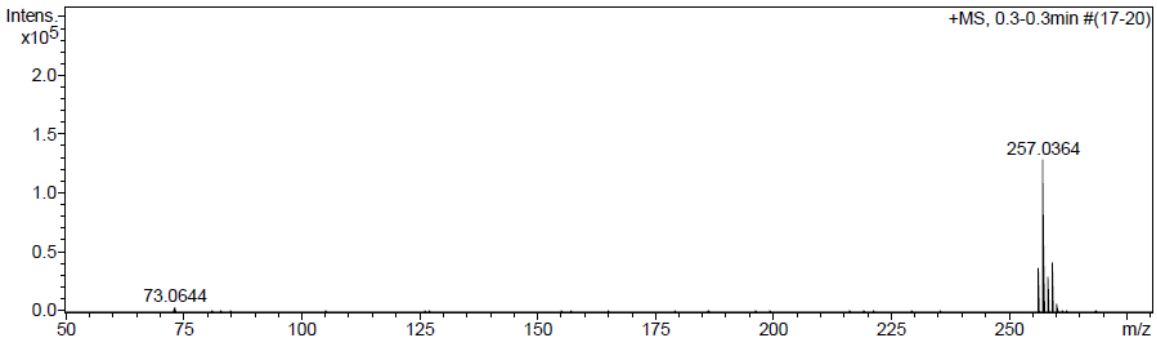
^{1}H NMR



^{13}C NMR

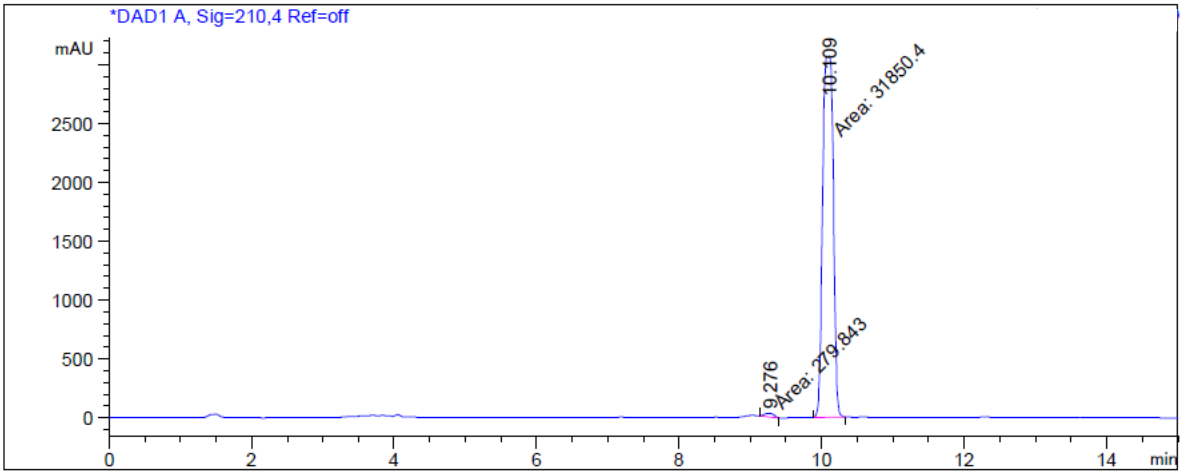


HRMS



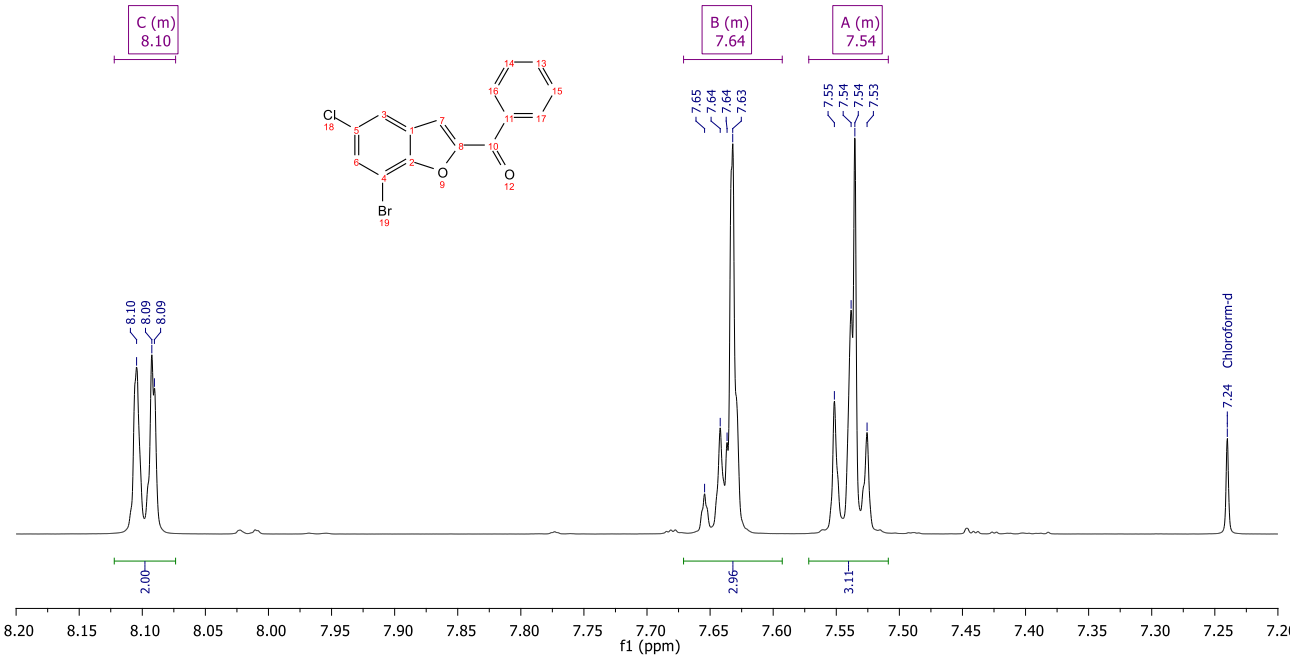
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
257.0364	1	C 15 H 10 Cl O 2	100.00	257.0364	-0.0	-0.1	30.0	10.5	ok	even
	2	C 21 H 5	0.03	257.0386	2.2	8.4	172.0	19.5	ok	even
	3	C 16 H 5 N 2 O 2	0.03	257.0346	-1.9	-7.2	177.1	15.5	ok	even
	4	C 15 H 5 N 4 O	0.00	257.0458	9.4	36.5	180.1	15.5	ok	even
	5	C 14 H 3 N 5 O	0.01	257.0332	-3.2	-12.4	181.6	16.0	ok	odd

HPLC

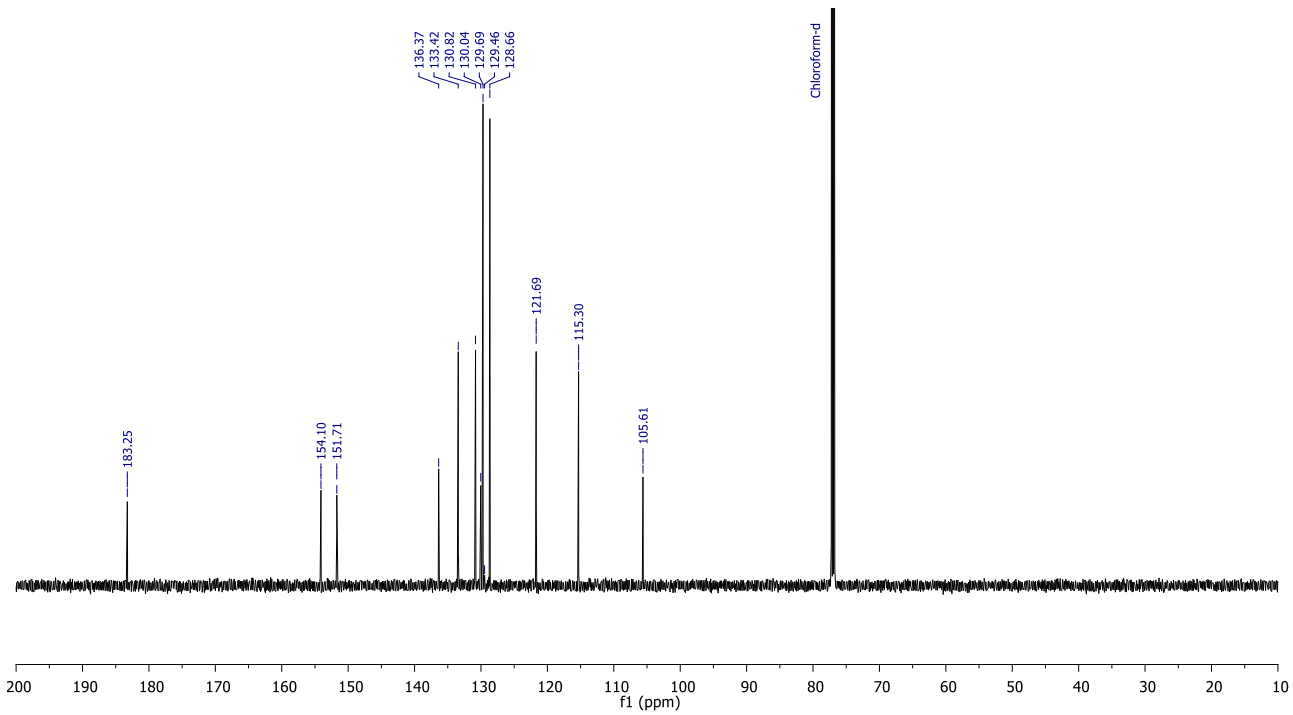


(7-bromo-5-chloro-1-benzofuran-2-yl)(phenyl)methanone (**5c**)

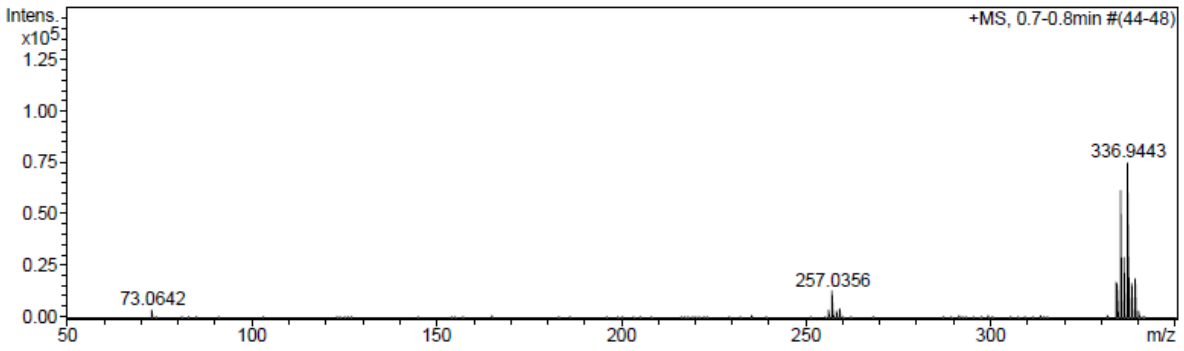
^{1}H NMR



^{13}C NMR

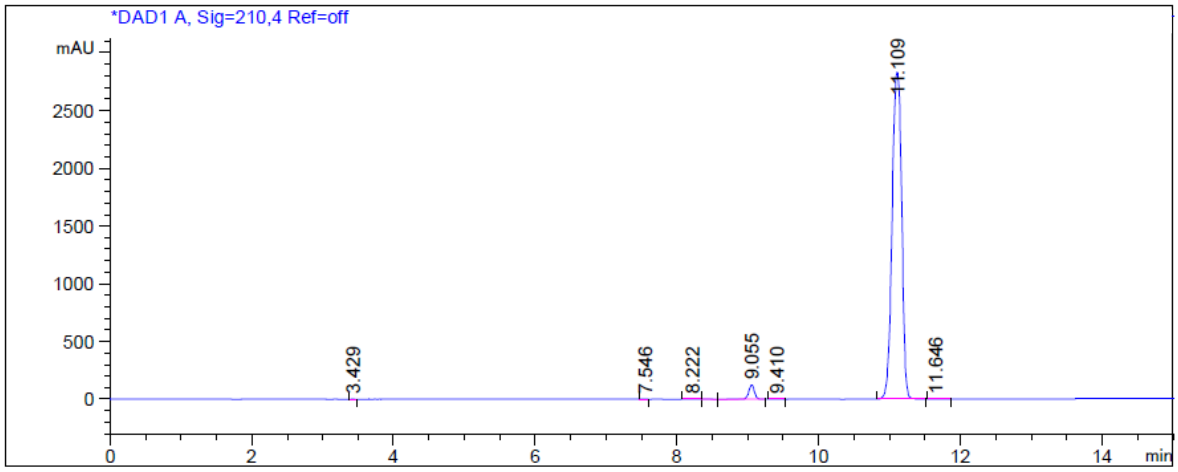


HRMS



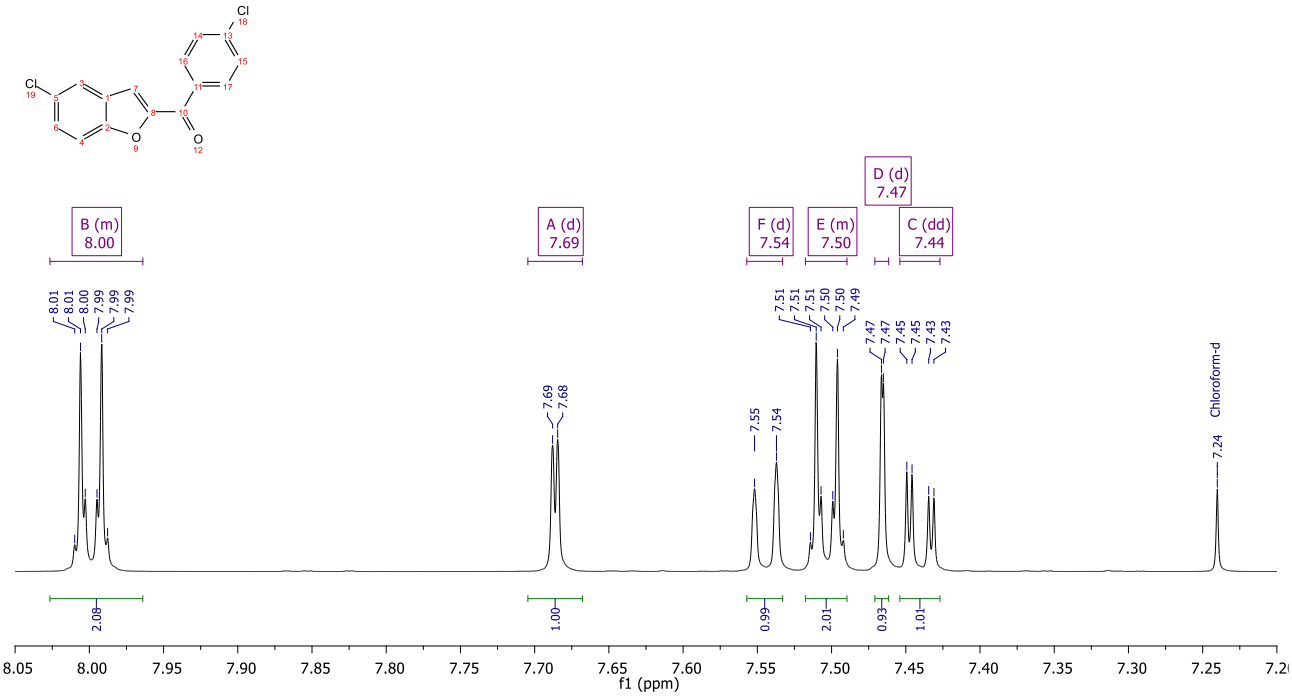
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
334.9465	1	C 15 H 9 Br Cl O 2	100.00	334.9469	0.4	1.2	104.1	10.5	ok	even
	2	C 13 H 7 Br Cl N 3 O	41.01	334.9456	-0.9	-2.8	116.2	11.0	ok	odd
	3	C 11 H 5 Br Cl N 6	13.03	334.9442	-2.3	-6.8	120.3	11.5	ok	even
	4	C 7 H 7 Br Cl N 7 O 2	0.03	334.9528	6.3	18.7	136.9	7.0	ok	odd
	5	C 6 H 5 Br Cl N 8 O 2	0.03	334.9402	-6.3	-18.8	140.0	7.5	ok	even
	6	C 5 H 5 Br Cl N 10 O	0.21	334.9514	4.9	14.7	141.4	7.5	ok	even
	7	C 4 H 3 Br Cl N 11 O	0.00	334.9388	-7.6	-22.8	144.6	8.0	ok	odd
	8	C 3 H 3 Br Cl N 13	0.81	334.9501	3.6	10.7	146.1	8.0	ok	odd
	9	C 2 H Br Cl N 14	0.00	334.9375	-9.0	-26.8	149.3	8.5	ok	even
	10	C 21 H 4 Br	1.66	334.9491	2.6	7.7	150.1	19.5	ok	even
	11	C 20 H 2 Br N	0.00	334.9365	-10.0	-29.8	152.1	20.0	ok	odd
	12	C 16 H 4 Br N 2 O 2	1.60	334.9451	-1.4	-4.3	163.8	15.5	ok	even
	13	C 15 H 4 Br N 4 O	0.00	334.9563	9.8	29.3	166.3	15.5	ok	even
	14	C 14 H 2 Br N 5 O	0.39	334.9437	-2.8	-8.3	168.9	16.0	ok	odd
	15	C 13 H 2 Br N 7	0.00	334.9550	8.5	25.3	170.2	16.0	ok	odd
	16	C 12 Br N 8	0.07	334.9424	-4.1	-12.3	172.9	16.5	ok	even
	17	C 6 Br N 12 O	0.16	334.9496	3.1	9.2	176.8	12.5	ok	even

HPLC

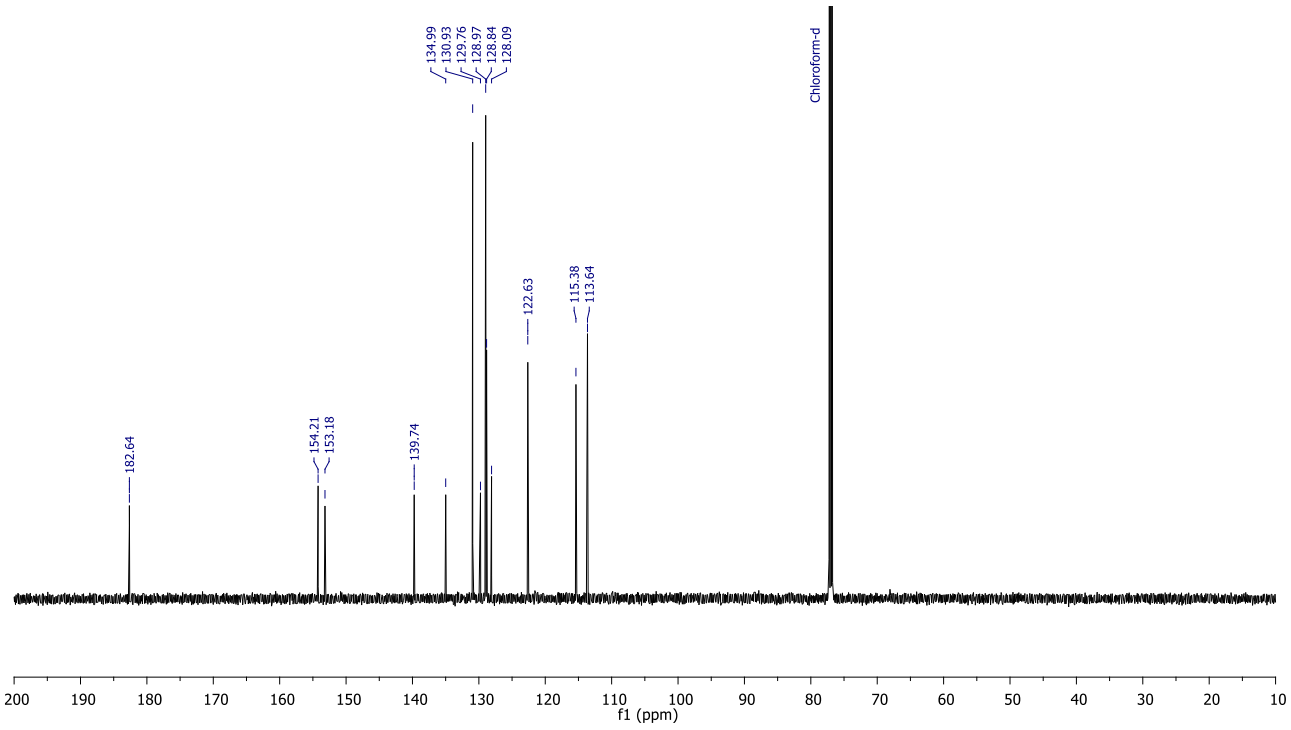


(5-chloro-1-benzofuran-2-yl)(4-chlorophenyl)methanone (**5d**)

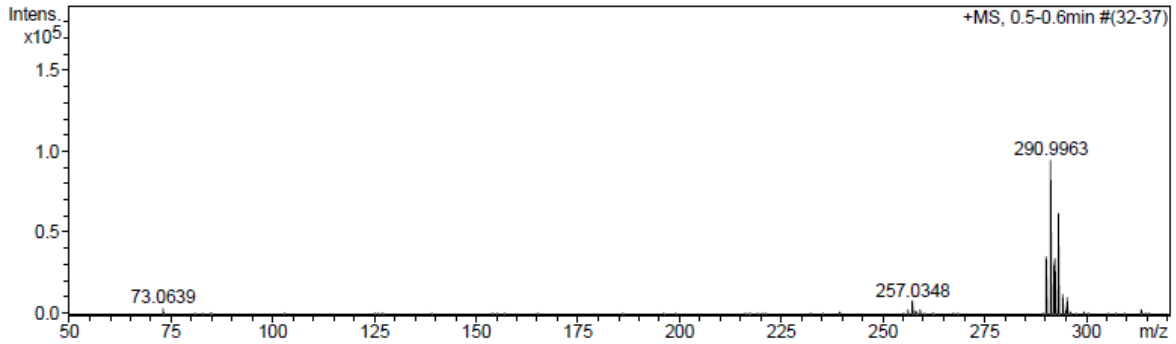
^{1}H NMR



^{13}C NMR

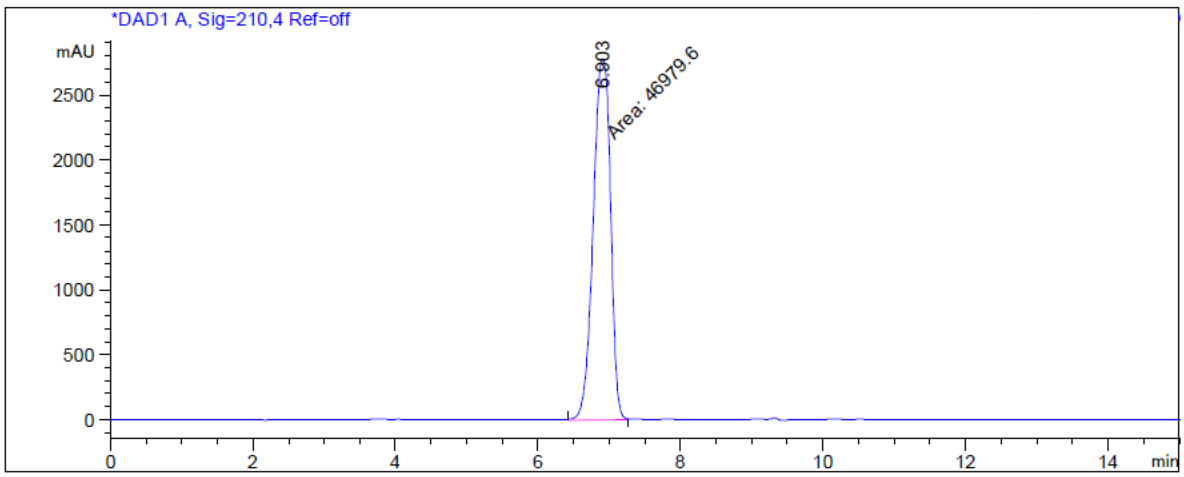


HRMS



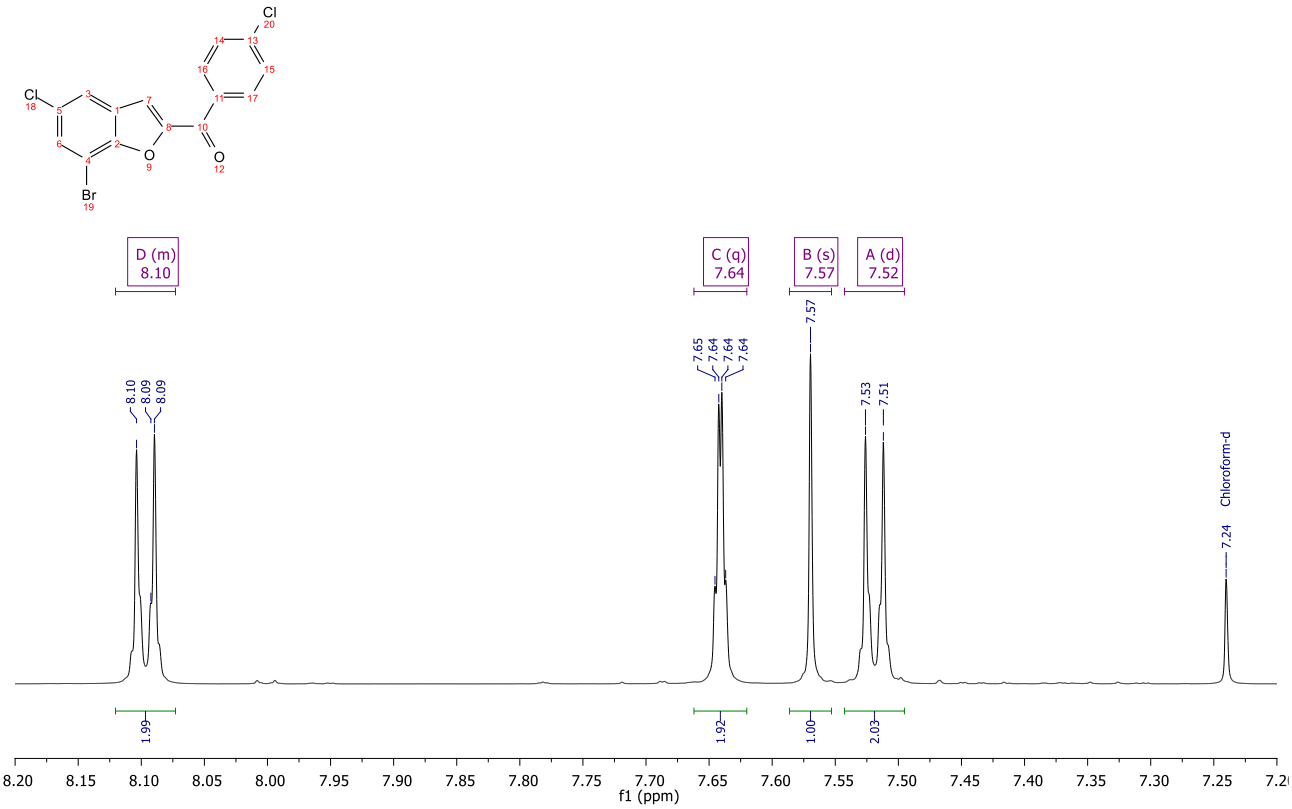
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
290.9963	1	C 15 H 9 Cl 2 O 2	76.51	290.9974	1.1	3.9	81.9	10.5	ok	even
	2	C 13 H 7 Cl 2 N 3 O	100.00	290.9961	-0.2	-0.8	86.6	11.0	ok	odd
	3	C 11 H 5 Cl 2 N 6	38.80	290.9947	-1.6	-5.4	91.3	11.5	ok	even
	4	C 7 H 7 Cl 2 N 7 O 2	0.02	291.0033	7.0	24.0	109.3	7.0	ok	odd
	5	C 6 H 5 Cl 2 N 8 O 2	0.17	290.9907	-5.6	-19.2	112.5	7.5	ok	even
	6	C 5 H 5 Cl 2 N 10 O	0.14	291.0019	5.6	19.4	114.2	7.5	ok	even
	7	C 4 H 3 Cl 2 N 11 O	0.01	290.9894	-6.9	-23.8	117.5	8.0	ok	odd
	8	C 3 H 3 Cl 2 N 13	0.71	291.0006	4.3	14.8	119.1	8.0	ok	odd
	9	C 2 H Cl 2 N 14	0.00	290.9880	-8.3	-28.4	122.4	8.5	ok	even
	10	C 21 H 4 Cl	0.19	290.9996	3.3	11.4	158.9	19.5	ok	even
	11	C 20 H 2 Cl N	0.00	290.9870	-9.3	-31.8	160.9	20.0	ok	odd
	12	C 16 H 4 Cl N 2 O 2	0.59	290.9956	-0.7	-2.4	171.0	15.5	ok	even
	13	C 14 H 2 Cl N 5 O	0.06	290.9942	-2.0	-7.0	190.2	16.0	ok	odd
	14	C 13 H 2 Cl N 7	0.00	291.0055	9.2	31.6	191.5	16.0	ok	odd
	15	C 12 Cl N 8	0.01	290.9929	-3.4	-11.7	194.1	16.5	ok	even
	16	C 8 H 2 Cl N 9 O 2	0.00	291.0014	5.2	17.7	208.1	12.0	ok	odd
	17	C 7 Cl N 10 O 2	0.00	290.9889	-7.4	-25.5	210.5	12.5	ok	even
	18	C 6 Cl N 12 O	0.00	291.0001	3.8	13.1	213.2	12.5	ok	even

HPLC

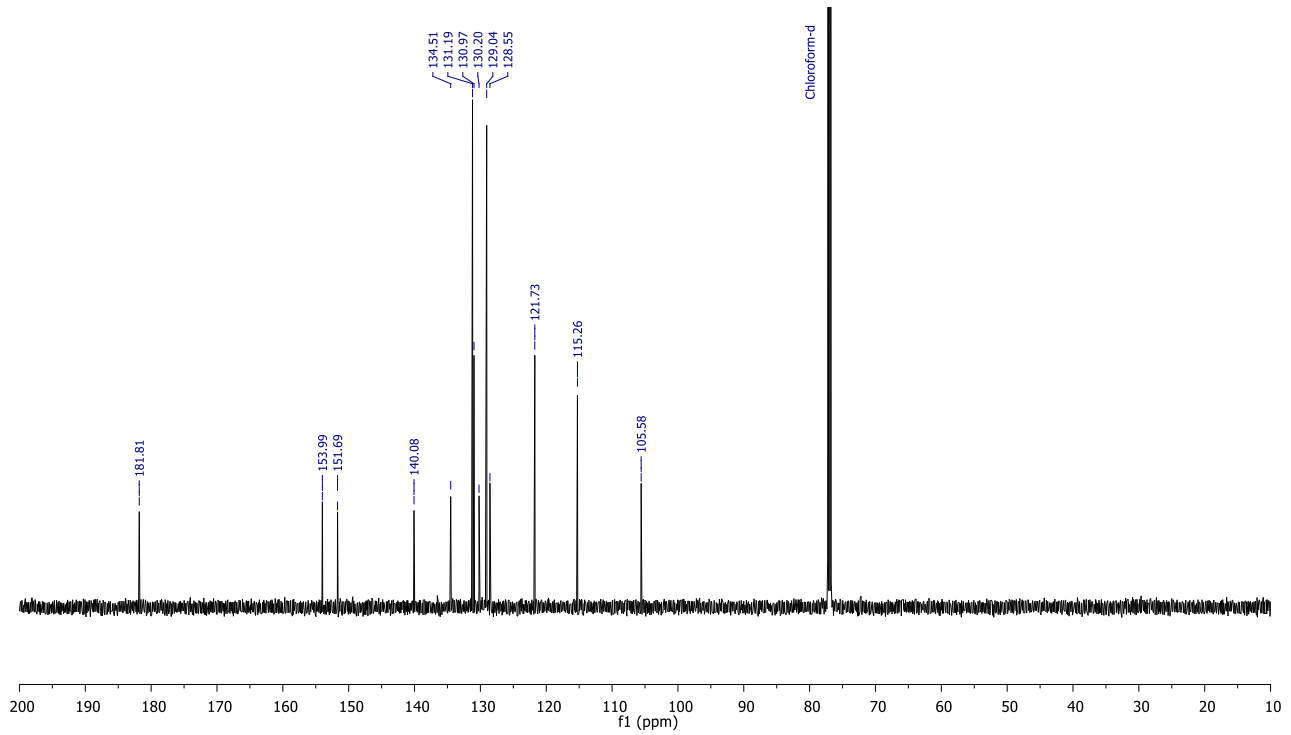


(7-bromo-5-chloro-1-benzofuran-2-yl)(4-chlorophenyl)methanone (**5e**)

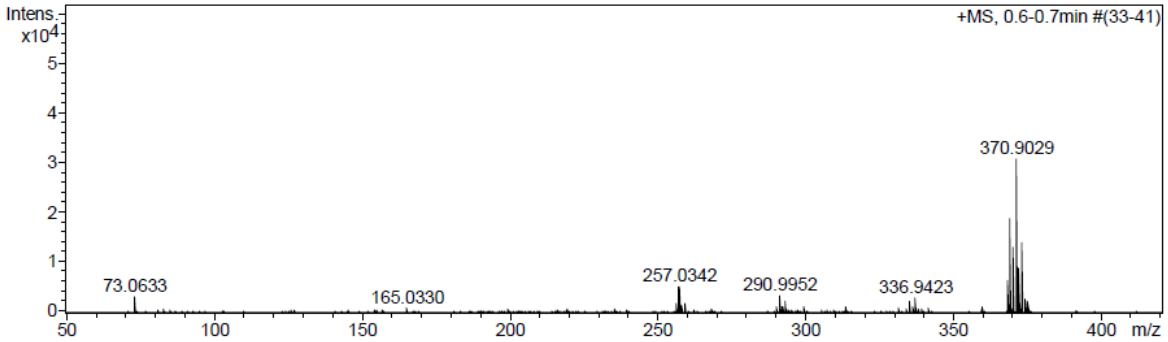
^{1}H NMR



^{13}C NMR

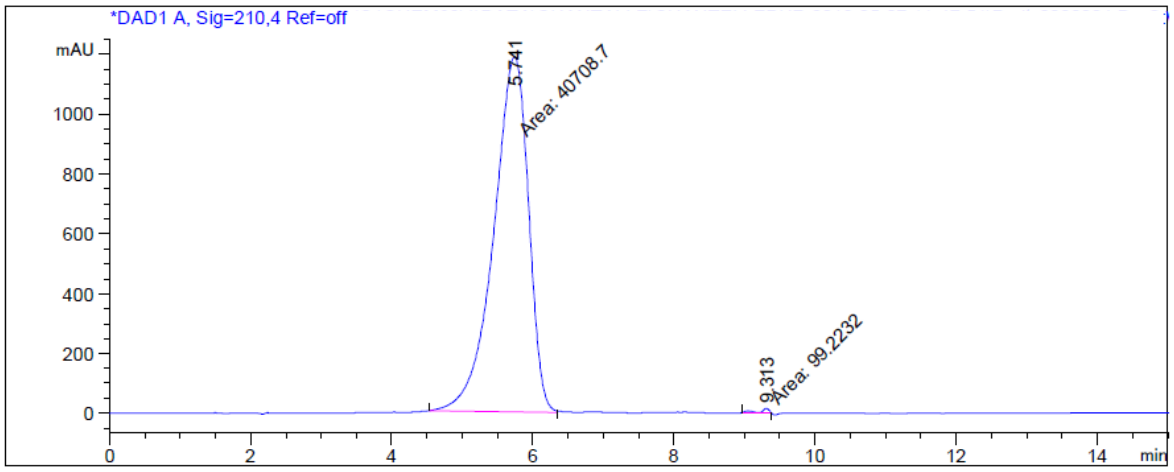


HRMS



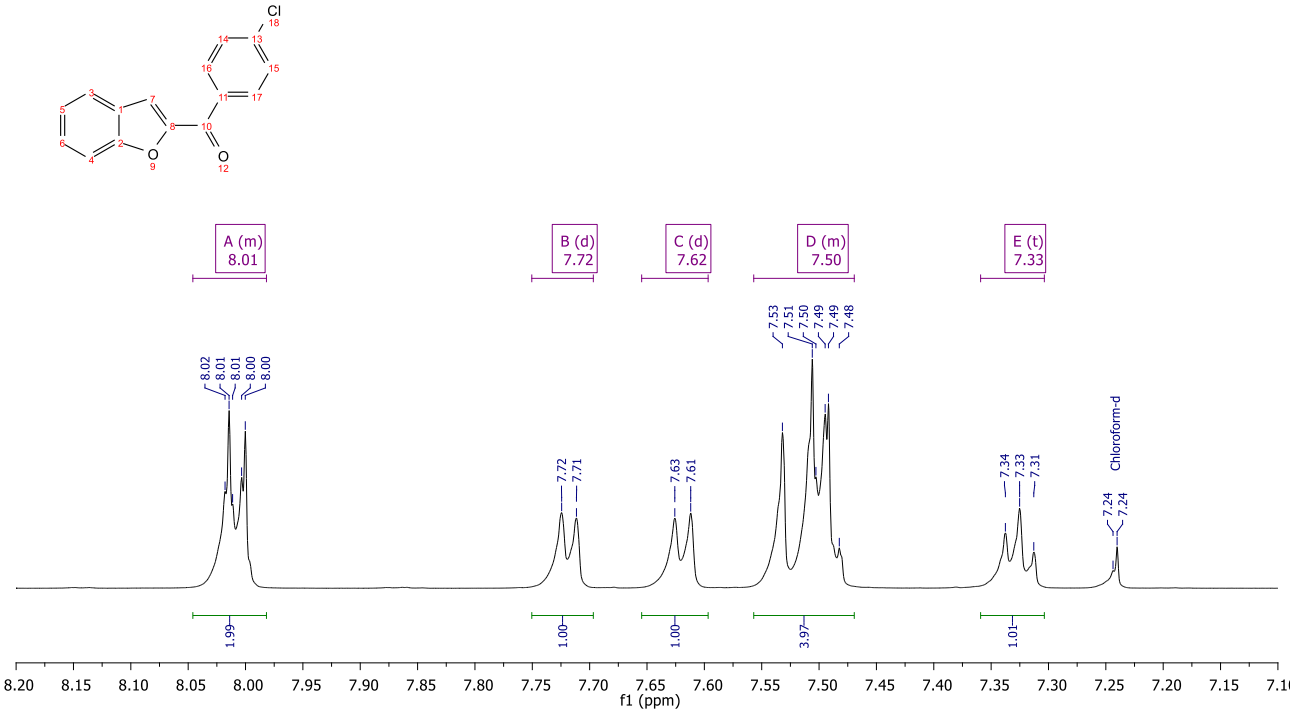
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
368.9052	1	C 15 H 8 Br Cl 2 O 2	100.00	368.9079	2.7	7.4	121.0	10.5	ok	even
	2	C 21 H 3 Br Cl	3.45	368.9101	4.9	13.4	135.6	19.5	ok	even
369.8980	1	C 18 H 5 Br Cl 2	100.00	369.8946	-3.4	-9.2	438.6	15.0	ok	odd
	2	C 15 H 9 Br Cl 2 O 2	0.00	369.9157	17.7	47.9	445.9	10.0	ok	odd
	3	C 21 H 4 Br Cl	0.00	369.9179	19.9	53.8	446.8	19.0	ok	odd
	4	C 20 Br Cl O	0.00	369.8816	-16.5	-44.5	455.6	20.0	ok	odd
370.9029	1	C 20 H Br Cl O	0.00	370.8894	-13.5	-36.3	246.2	19.5	ok	even
	2	C 23 Br O	0.00	370.9127	9.8	26.5	255.6	23.5	ok	even
	3	C 18 H 6 Br Cl 2	100.00	370.9024	-0.4	-1.1	285.4	14.5	ok	even

HPLC

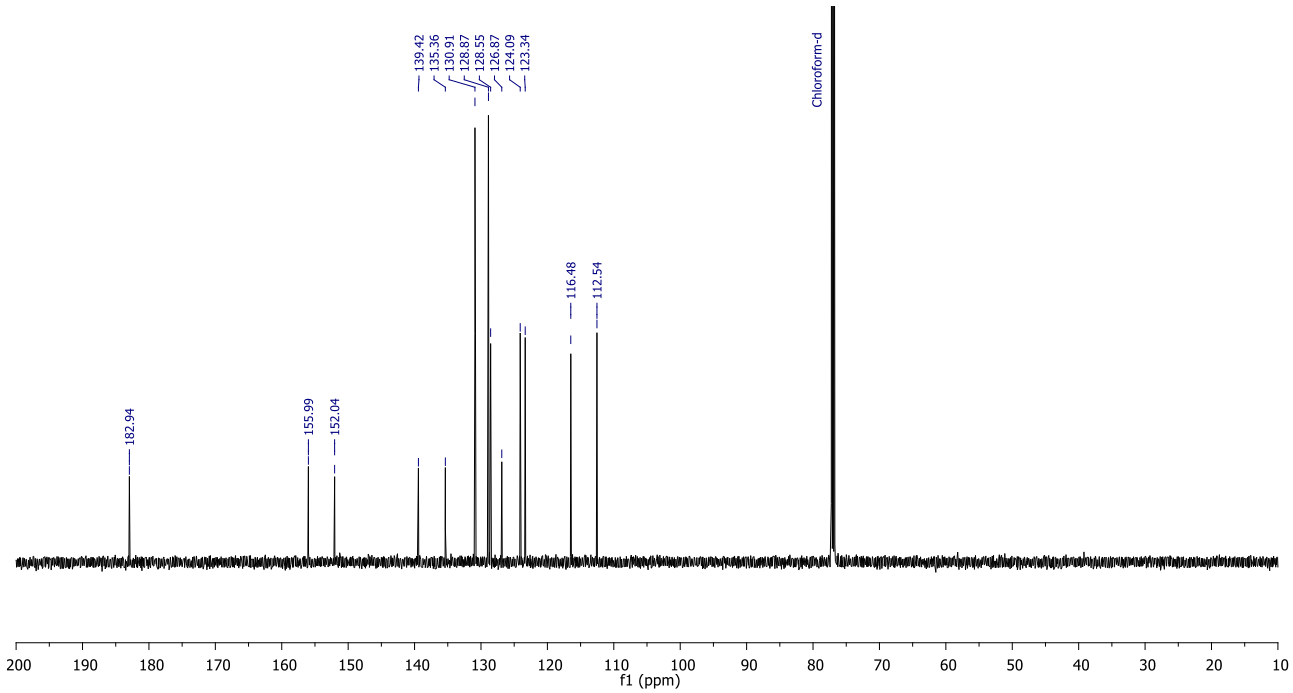


1-benzofuran-2-yl(4-chlorophenyl)methanone (**5f**)

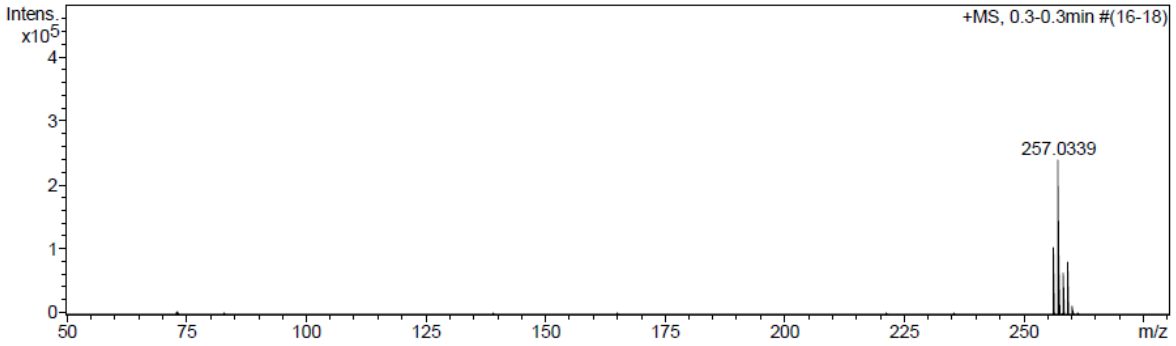
^{1}H NMR



^{13}C NMR

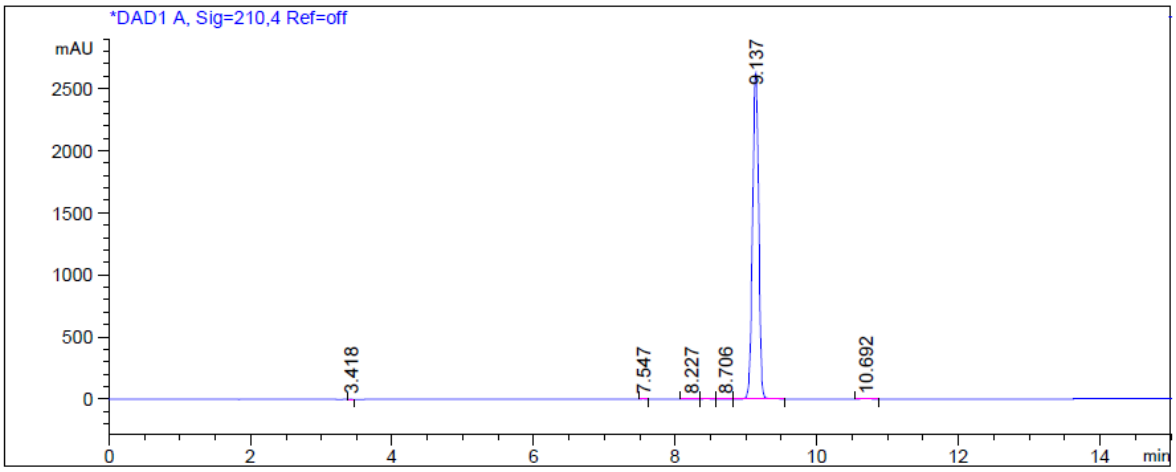


HRMS



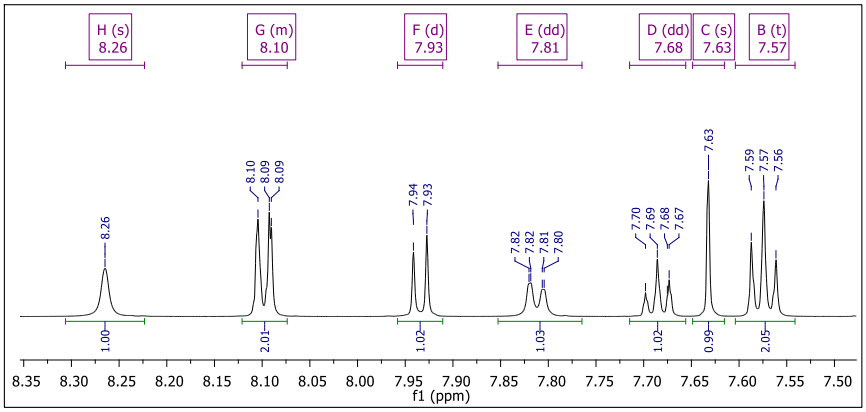
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
257.0339	1	C 15 H 10 Cl O 2	100.00	257.0364	2.5	9.8	45.7	10.5	ok	even
	2	C 17 H 7 N O 2	0.00	257.0471	13.3	51.6	45.9	15.0	ok	odd
	3	C 15 H 5 N 4 O	0.00	257.0458	11.9	46.4	51.1	15.5	ok	even
	4	C 21 H 5	0.01	257.0386	4.7	18.3	180.3	19.5	ok	even
	5	C 20 H 3 N	0.00	257.0260	-7.9	-30.6	181.7	20.0	ok	odd
	6	C 16 H 5 N 2 O 2	0.16	257.0346	0.7	2.6	188.3	15.5	ok	even
	7	C 15 H 3 N 3 O 2	0.00	257.0220	-11.9	-46.3	190.1	16.0	ok	odd

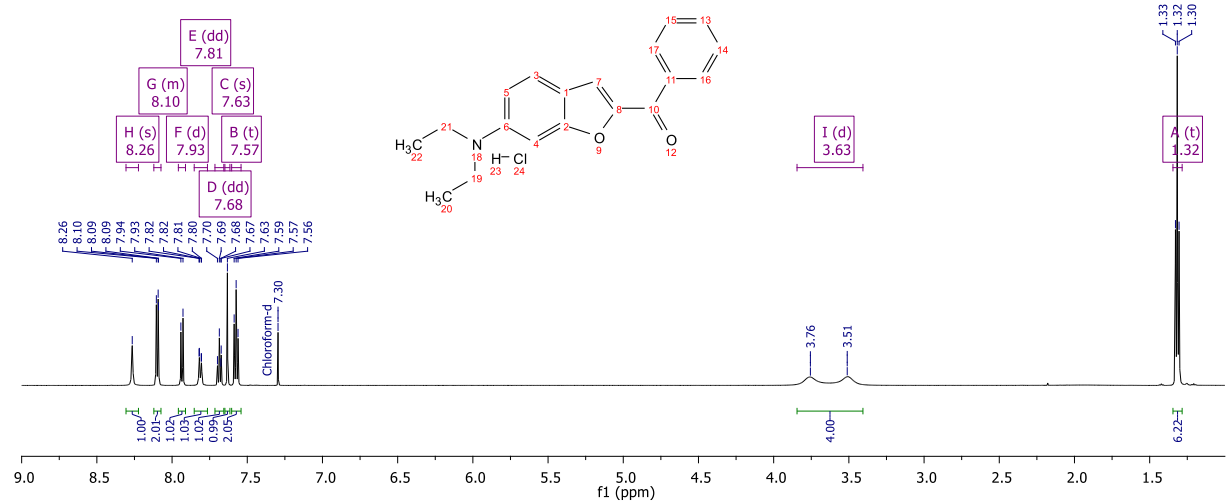
HPLC



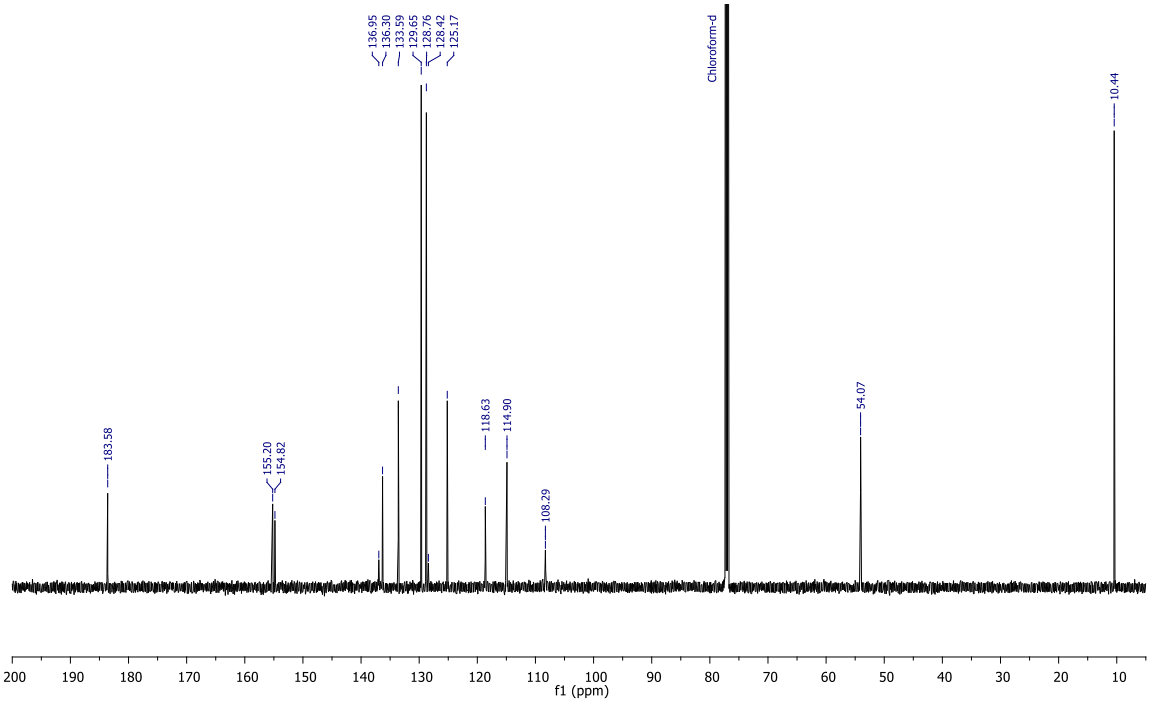
[6-(diethylamino)benzofuran-2-yl](phenyl)methanone hydrochloride (**5g**)

^{1}H NMR

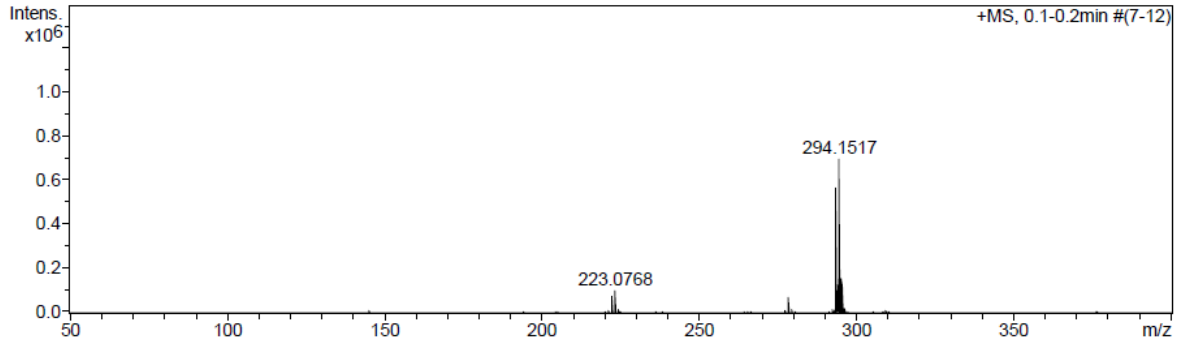




^{13}C NMR

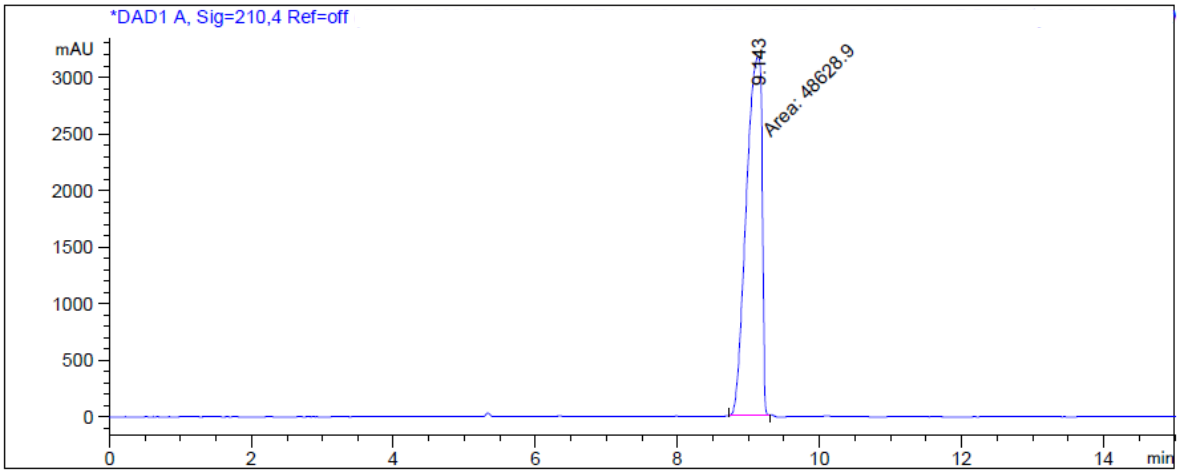


HRMS



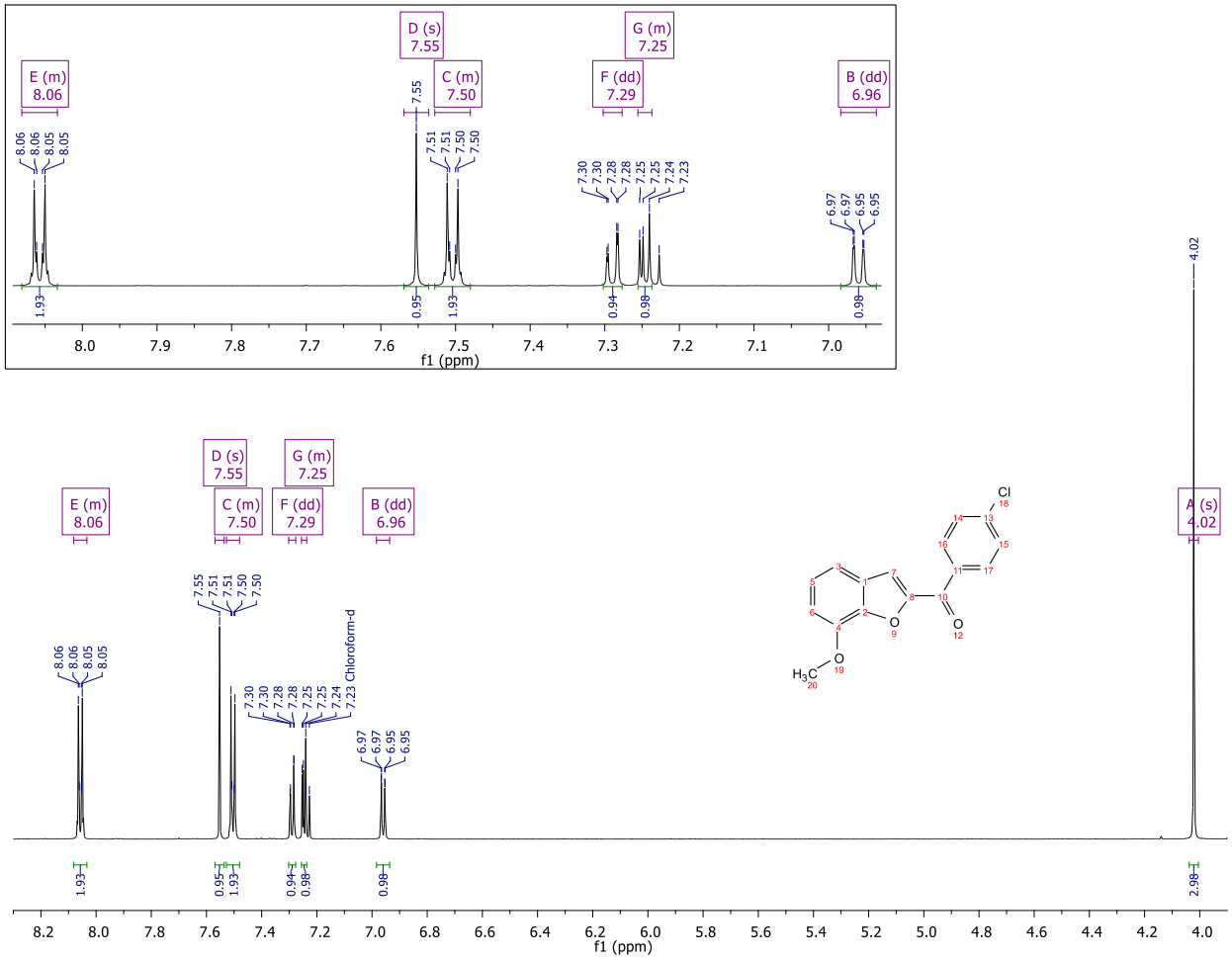
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
293.1443	1	C 20 H 21 O 2	0.01	293.1536	9.3	31.7	478.8	10.5	even	ok
	2	C 19 H 19 N O 2	100.00	293.1410	-3.3	-11.2	482.9	11.0	odd	ok
294.1517	1	C 20 H 22 O 2	0.00	294.1614	9.8	33.2	3.9	10.0	odd	ok
	2	C 19 H 20 N O 2	100.00	294.1489	-2.8	-9.5	8.0	10.5	even	ok

HPLC

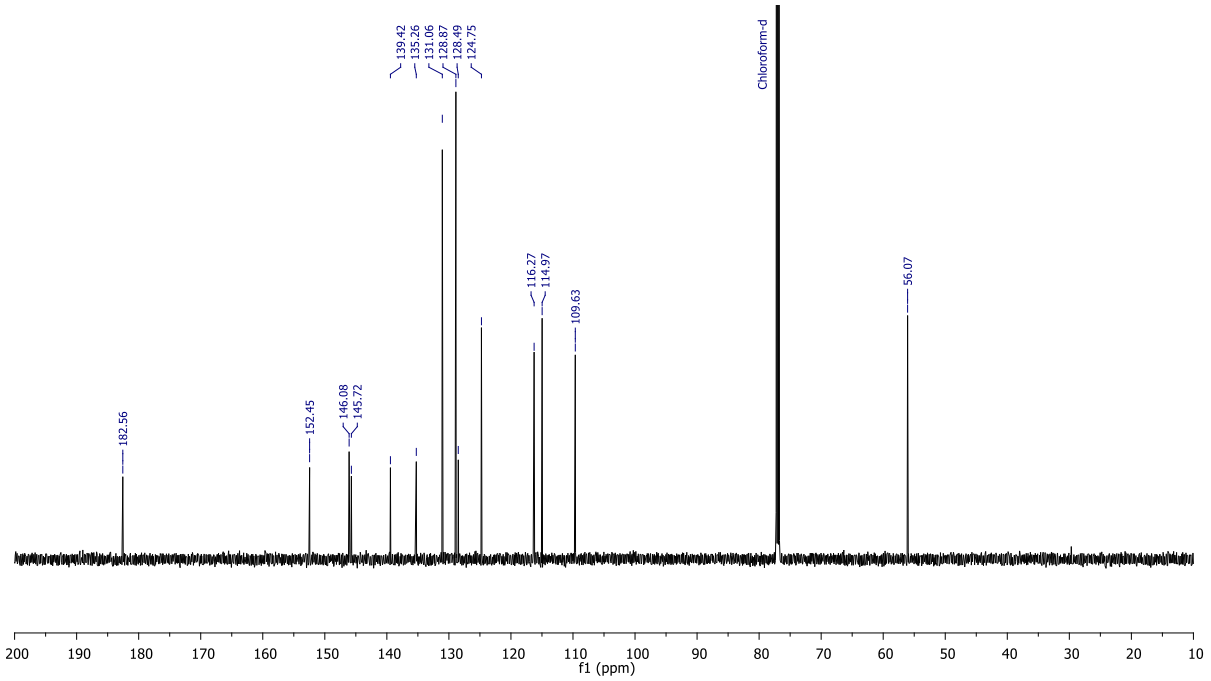


(4-chlorophenyl)(7-methoxy-1-benzofuran-2-yl)methanone (**5h**)

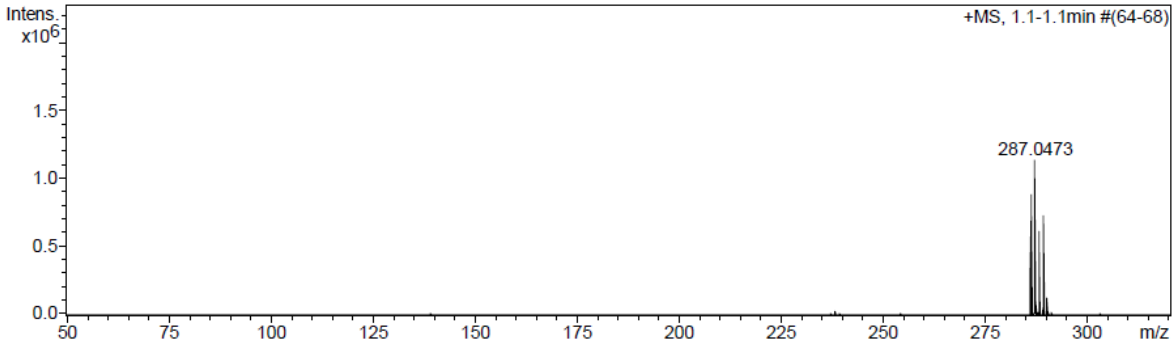
^{1}H NMR



^{13}C NMR

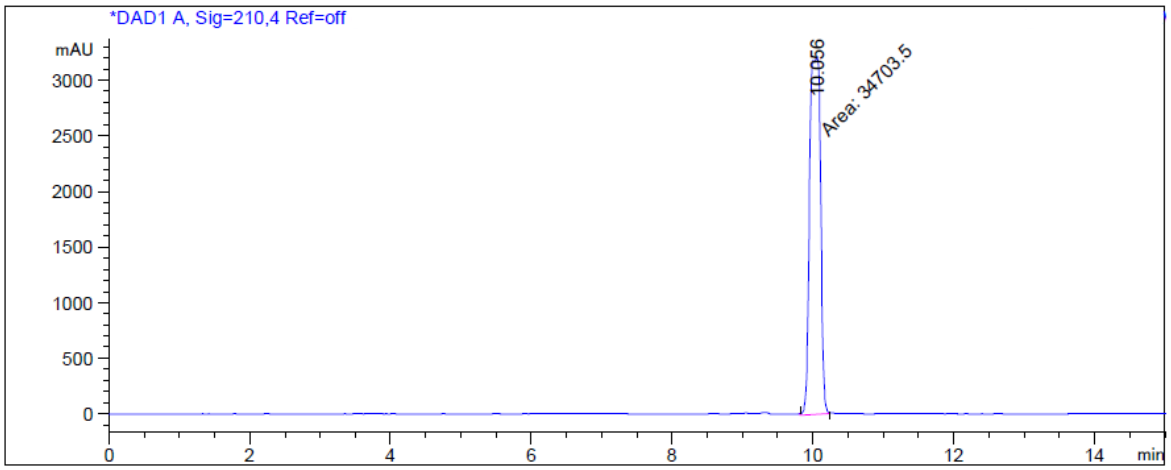


HRMS



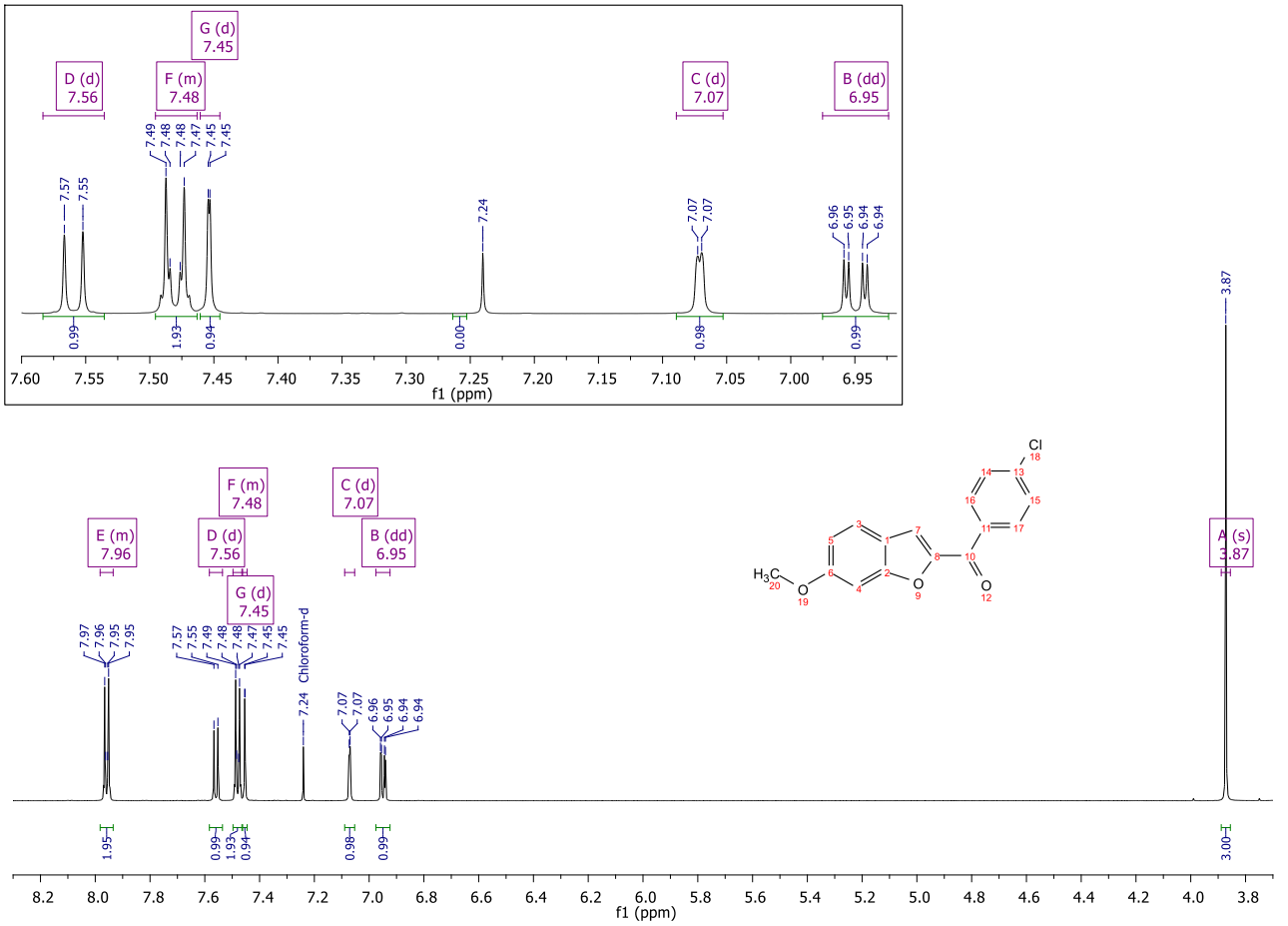
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
287.0473	1	C 19 H 10 Cl N	83.58	287.0496	2.4	8.2	201.2	15.0	ok	odd
	2	C 16 H 12 Cl O 3	100.00	287.0469	-0.3	-1.1	214.0	10.5	ok	even
	3	C 22 H 7 O	0.00	287.0491	1.9	6.5	395.3	19.5	ok	even
	4	C 20 H 5 N 3	0.00	287.0478	0.5	1.9	399.3	20.0	ok	odd
	5	C 17 H 7 N 2 O 3	0.00	287.0451	-2.1	-7.5	409.8	15.5	ok	even
	6	C 16 H 7 N 4 O 2	0.00	287.0564	9.1	31.7	412.1	15.5	ok	even

HPLC

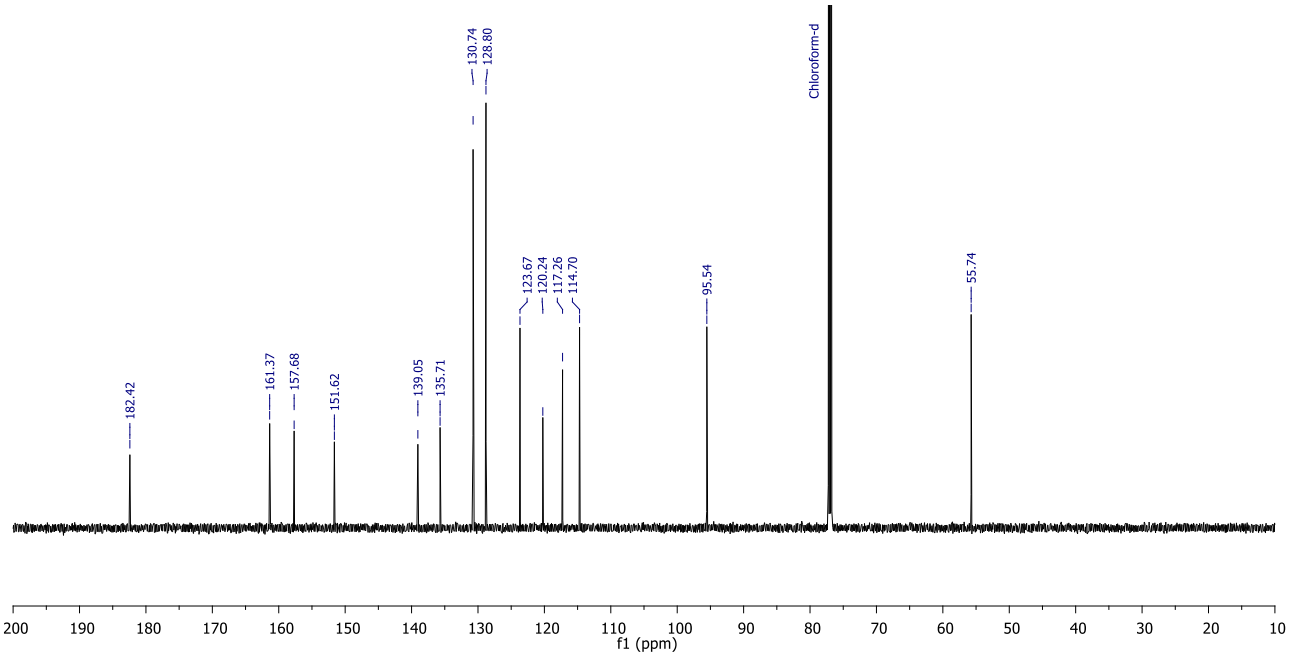


(4-chlorophenyl)(6-methoxy-1-benzofuran-2-yl)methanone (**5i**)

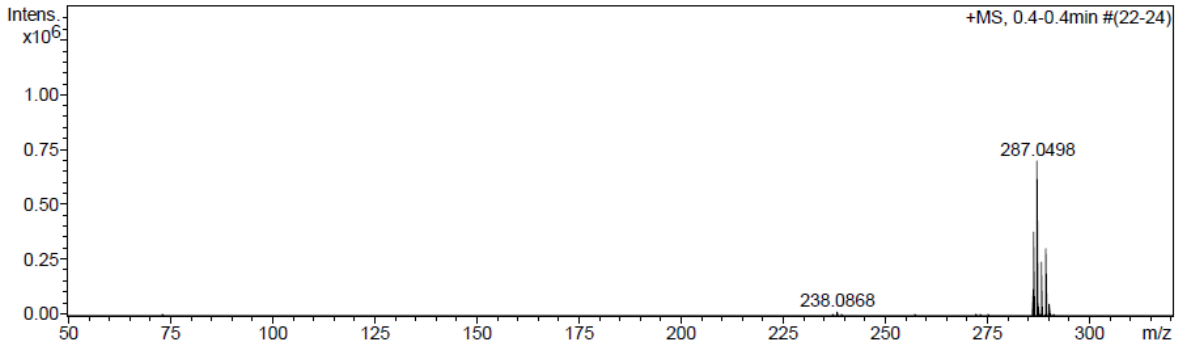
^{1}H NMR



^{13}C NMR

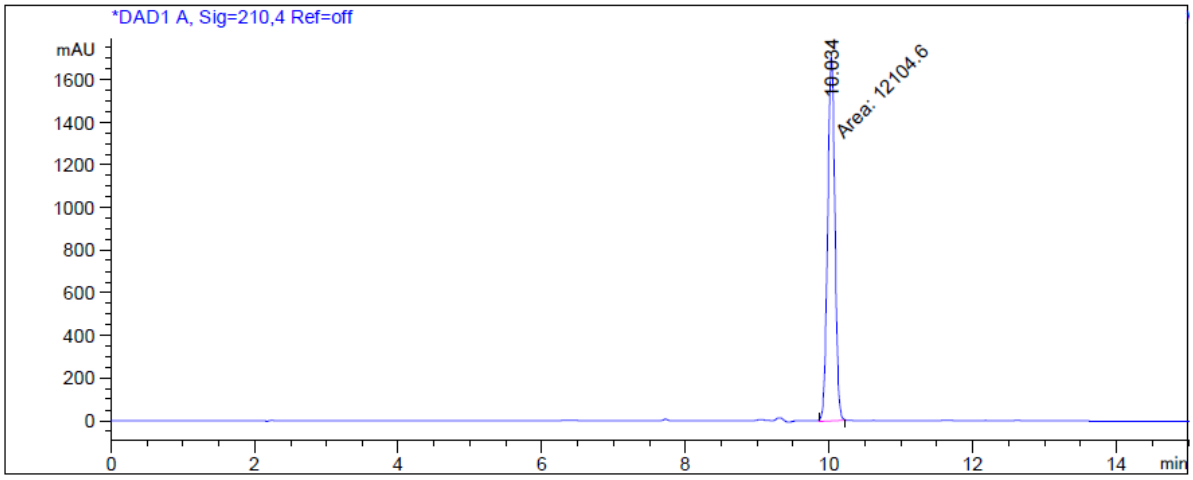


HRMS



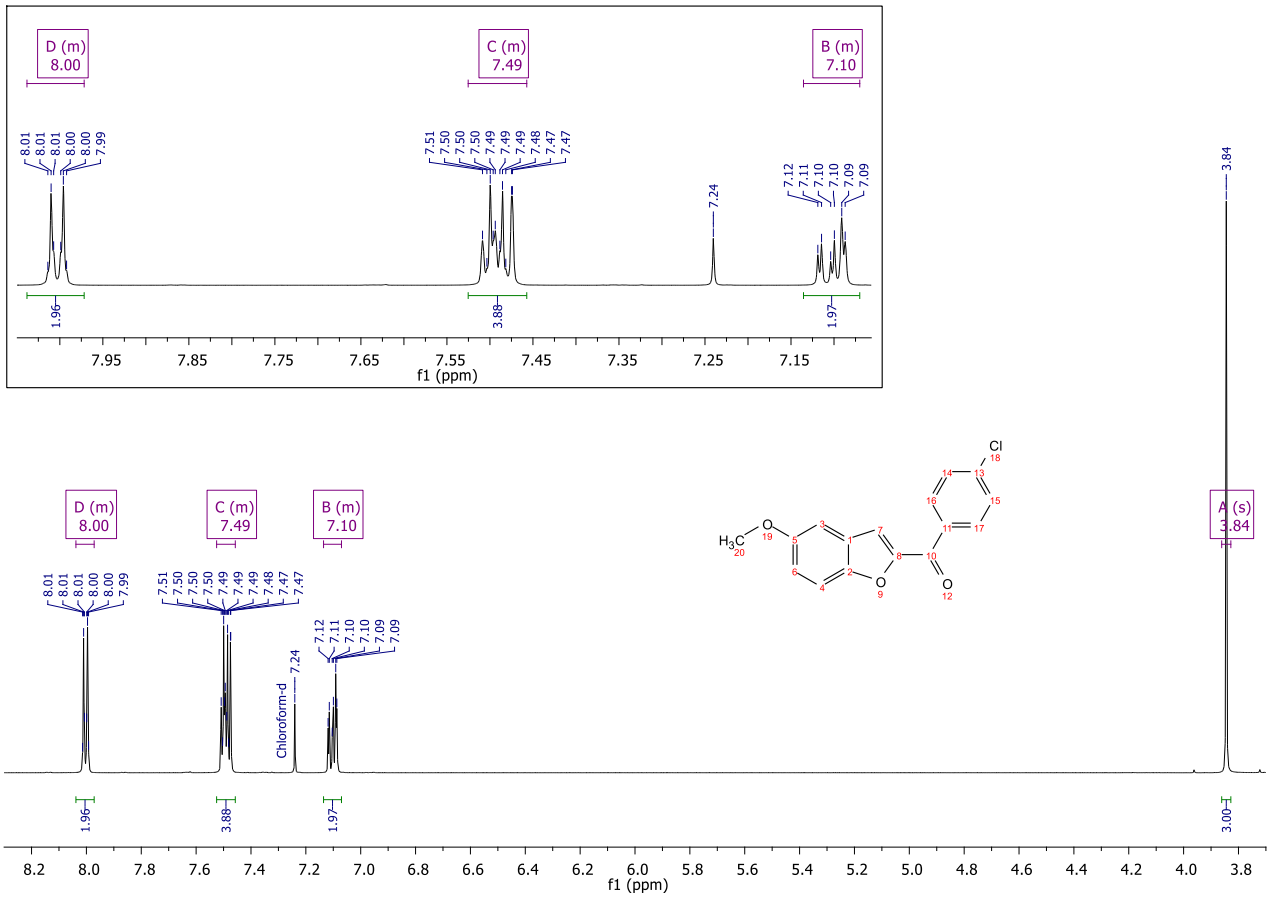
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
287.0498	1	C 19 H 10 Cl N	100.00	287.0496	-0.2	-0.7	73.8	15.0	ok	odd
	2	C 16 H 12 Cl O 3	9.05	287.0469	-2.9	-10.0	88.3	10.5	ok	even
	3	C 22 H 7 O	0.00	287.0491	-0.7	-2.4	240.5	19.5	ok	even
	4	C 20 H 5 N 3	0.00	287.0478	-2.0	-7.1	243.5	20.0	ok	odd
	5	C 18 H 9 N O 3	0.00	287.0577	7.9	27.4	248.8	15.0	ok	odd
	6	C 17 H 7 N 2 O 3	0.00	287.0451	-4.7	-16.4	250.9	15.5	ok	even
	7	C 16 H 7 N 4 O 2	0.00	287.0564	6.5	22.7	253.1	15.5	ok	even

HPLC

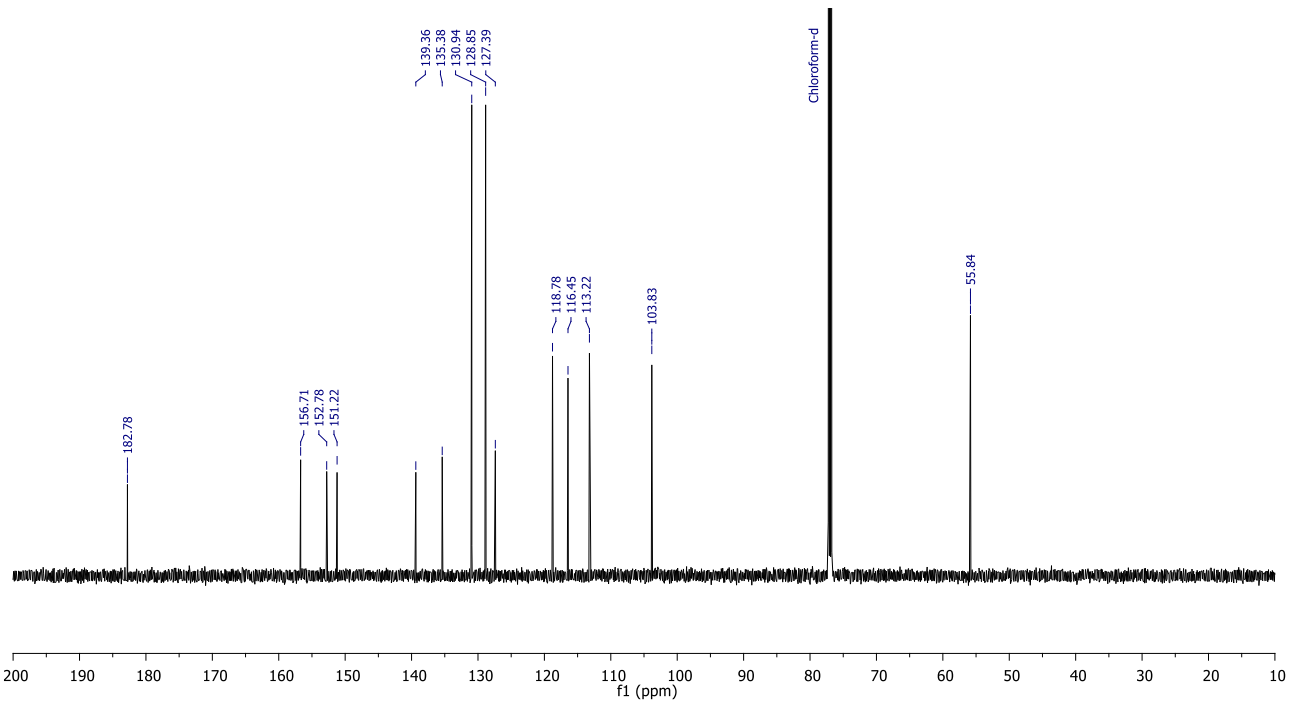


(4-chlorophenyl)(5-methoxy-1-benzofuran-2-yl)methanone (**5j**)

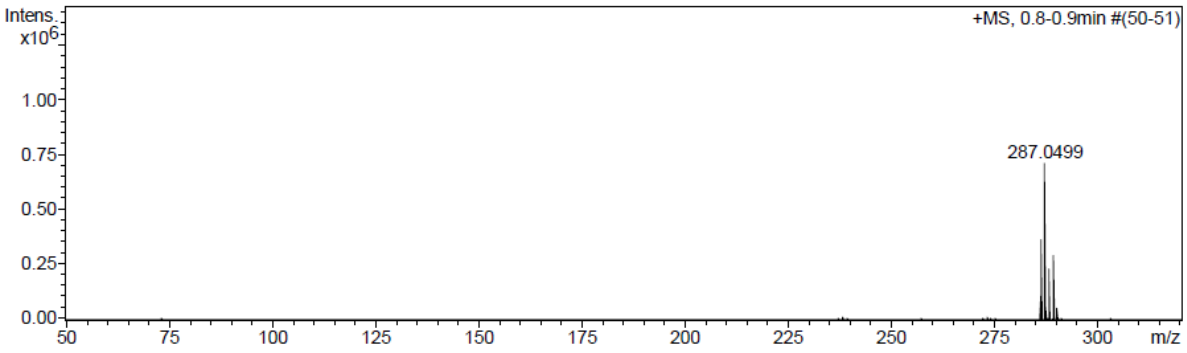
^{1}H NMR



^{13}C NMR

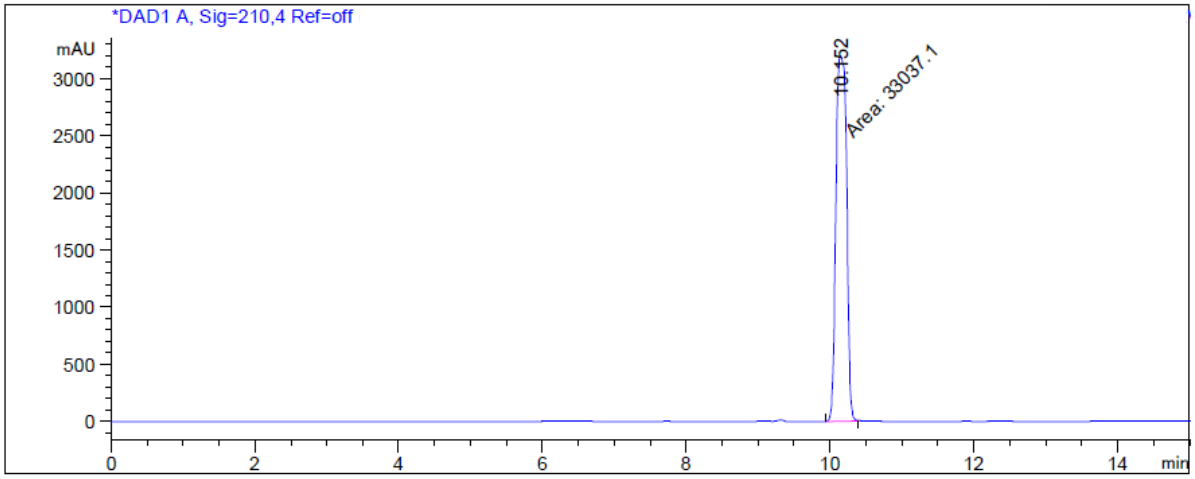


HRMS



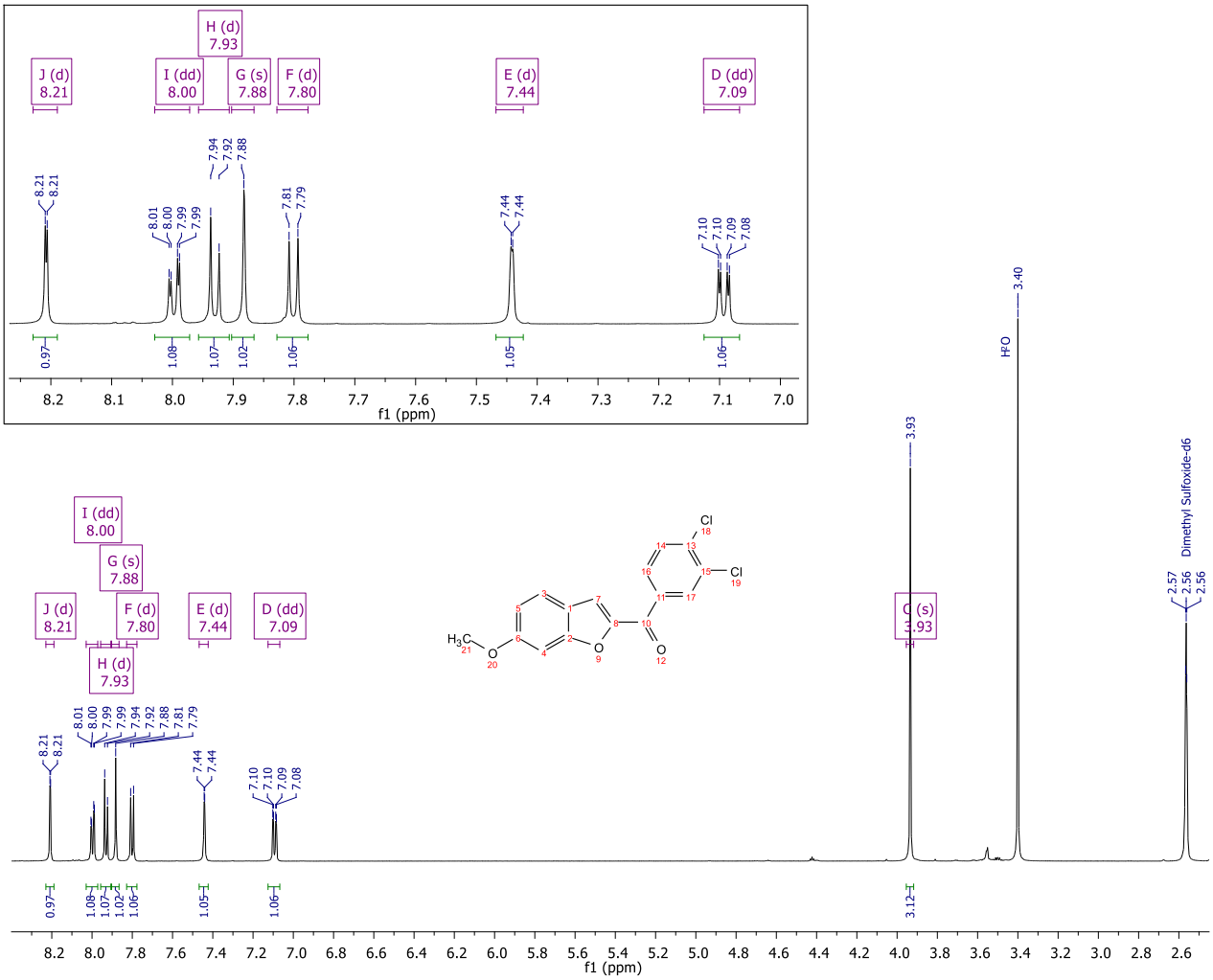
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
287.0499	1	C 19 H 10 Cl N	100.00	287.0496	-0.2	-0.8	61.6	15.0	ok	odd
	2	C 16 H 12 Cl O 3	9.40	287.0469	-2.9	-10.1	76.1	10.5	ok	even
	3	C 22 H 7 O	0.00	287.0491	-0.7	-2.5	228.8	19.5	ok	even
	4	C 20 H 5 N 3	0.00	287.0478	-2.1	-7.2	231.5	20.0	ok	odd
	5	C 18 H 9 N O 3	0.00	287.0577	7.8	27.3	236.1	15.0	ok	odd
	6	C 17 H 7 N 2 O 3	0.00	287.0451	-4.7	-16.5	238.2	15.5	ok	even
	7	C 16 H 7 N 4 O 2	0.00	287.0564	6.5	22.6	240.3	15.5	ok	even

HPLC

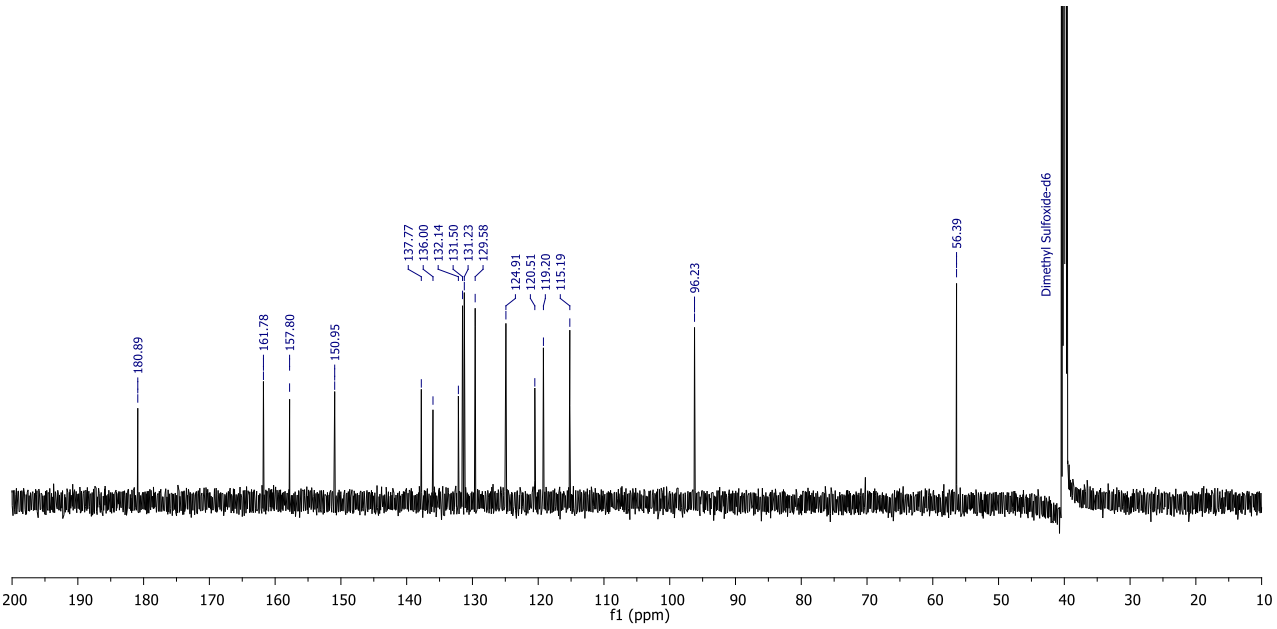


(3,4-dichlorophenyl)(6-methoxy-1-benzofuran-2-yl)methanone (**5k**)

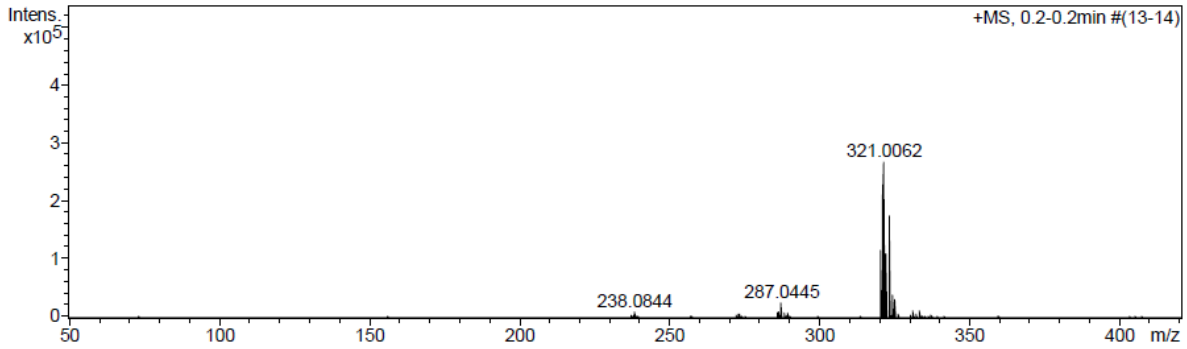
^{1}H NMR



^{13}C NMR

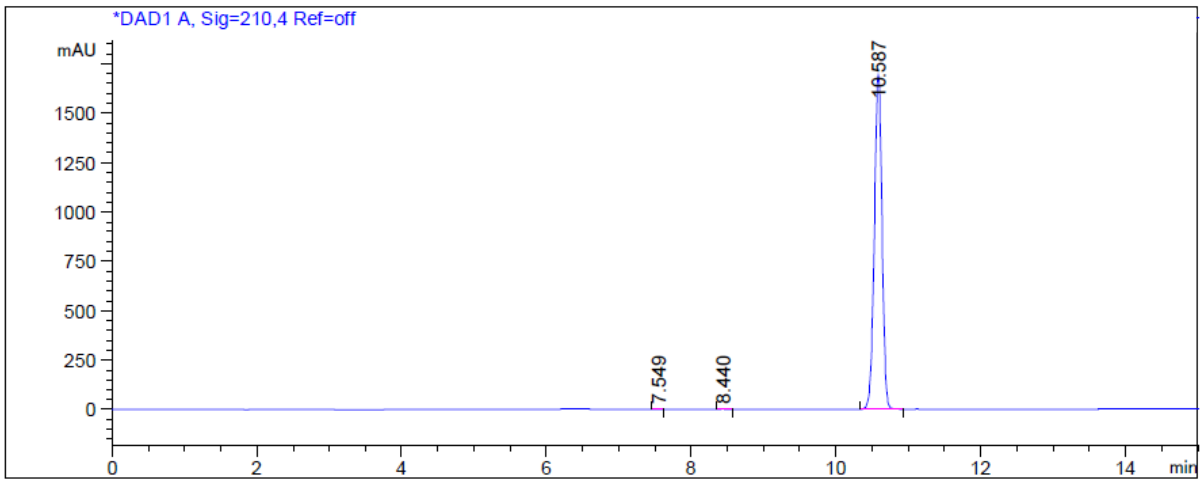


HRMS



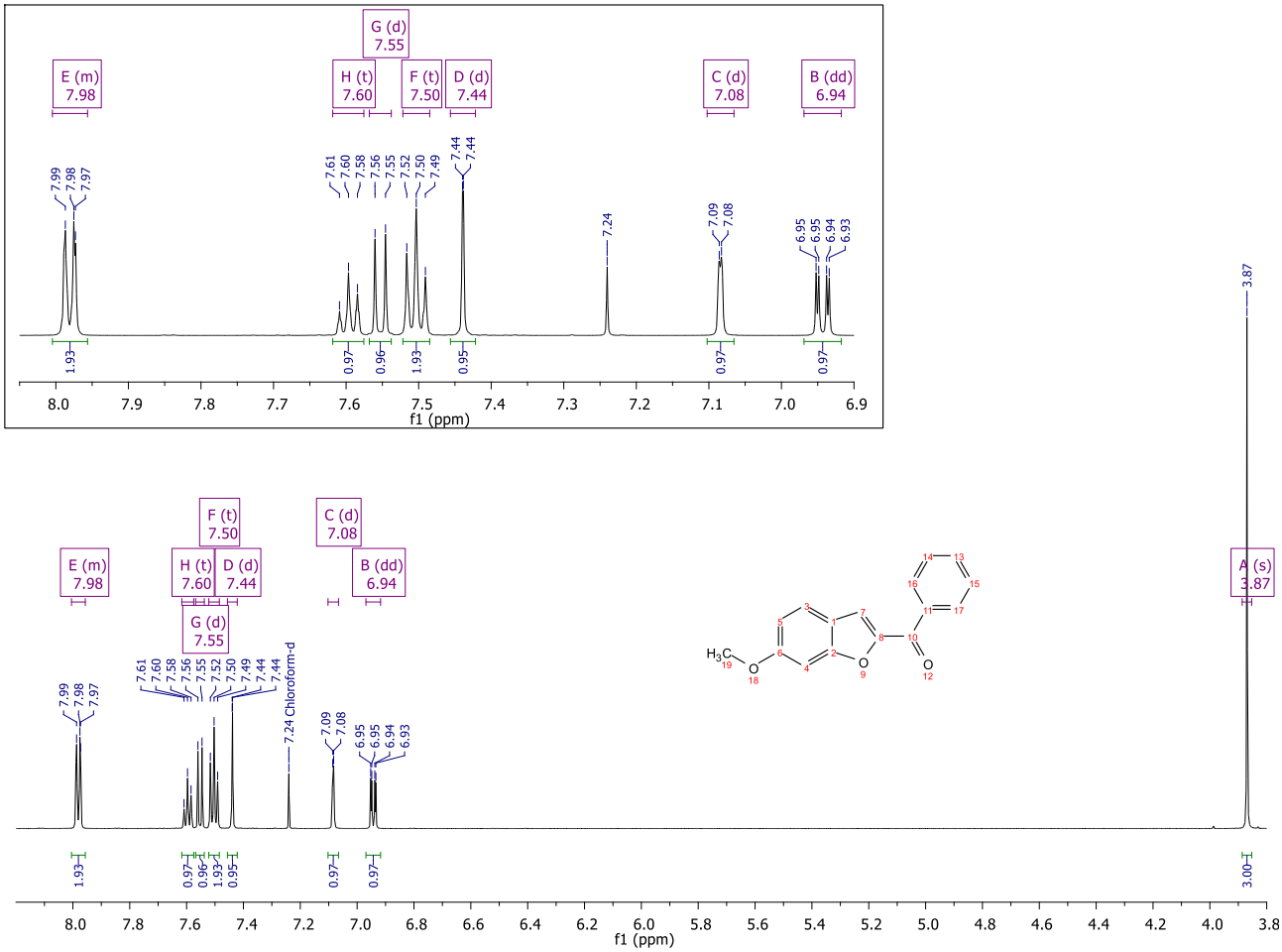
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
321.0062	1	C 19 H 9 Cl 2 N	13.16	321.0107	4.5	14.0	80.1	15.0	ok	odd
	2	C 18 H 7 Cl 2 N 2	0.03	320.9981	-8.1	-25.2	83.1	15.5	ok	even
	3	C 16 H 11 Cl 2 O 3	100.00	321.0080	1.8	5.7	95.6	10.5	ok	even
	4	C 22 H 6 Cl O	0.21	321.0102	4.0	12.5	165.1	19.5	ok	even
	5	C 21 H 4 Cl N O	0.00	320.9976	-8.6	-26.7	167.4	20.0	ok	odd
	6	C 20 H 4 Cl N 3	0.67	321.0088	2.7	8.3	168.5	20.0	ok	odd
	7	C 19 H 2 Cl N 4	0.00	320.9963	-9.9	-30.9	170.8	20.5	ok	even
	8	C 17 H 6 Cl N 2 O 3	1.82	321.0061	-0.0	-0.0	178.0	15.5	ok	even
	9	C 24 H O 2	0.00	320.9971	-9.0	-28.2	364.9	24.5	ok	even
	10	C 23 H N 2 O	0.00	321.0083	2.2	6.8	368.1	24.5	ok	even
	11	C 18 H N 4 O 3	0.00	321.0043	-1.8	-5.7	377.8	20.5	ok	even
	12	C 17 H N 6 O 2	0.00	321.0155	9.4	29.3	380.0	20.5	ok	even

HPLC

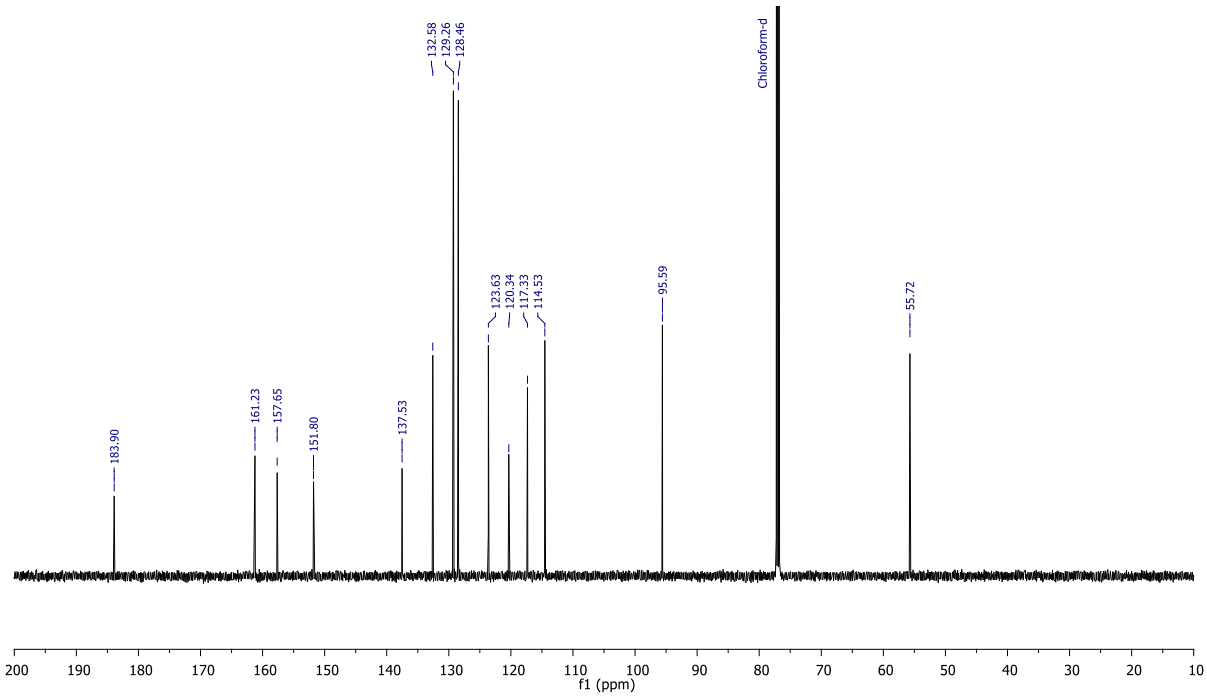


(6-methoxy-1-benzofuran-2-yl)(phenyl)methanone (**5l**)

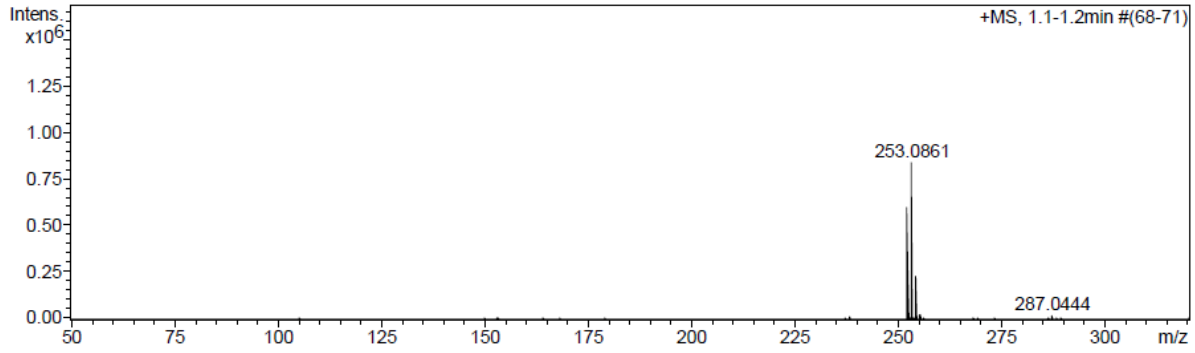
^{1}H NMR



^{13}C NMR

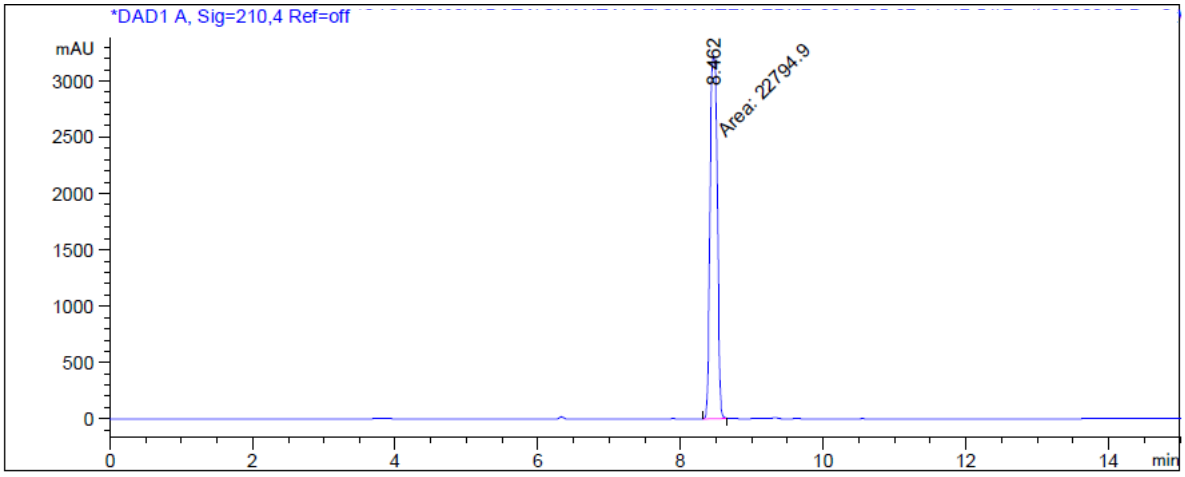


HRMS



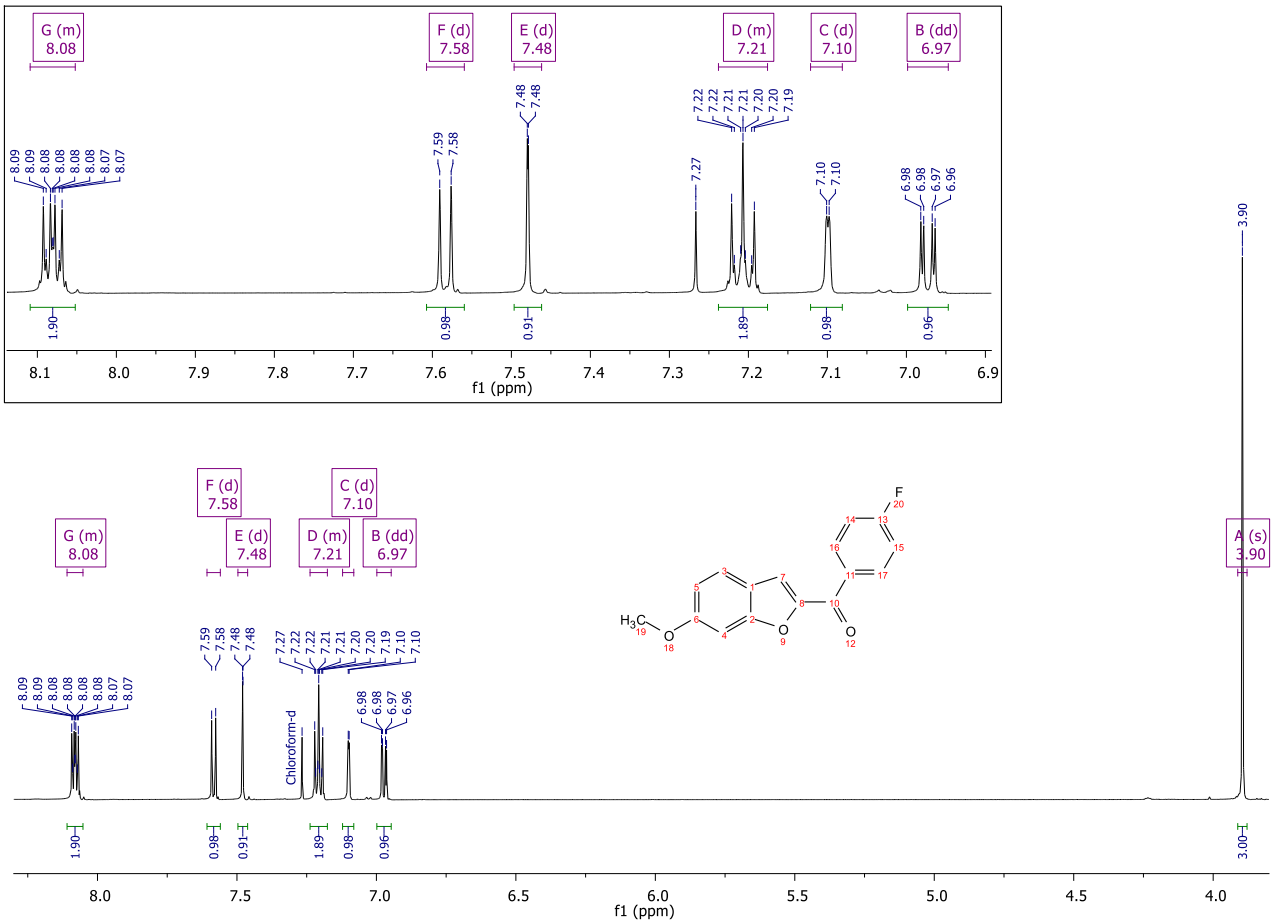
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
253.0861	1	C 19 H 11 N	39.87	253.0886	2.5	10.0	34.8	15.0	ok	odd
	2	C 16 H 13 O 3	100.00	253.0859	-0.1	-0.6	55.5	10.5	ok	even

HPLC

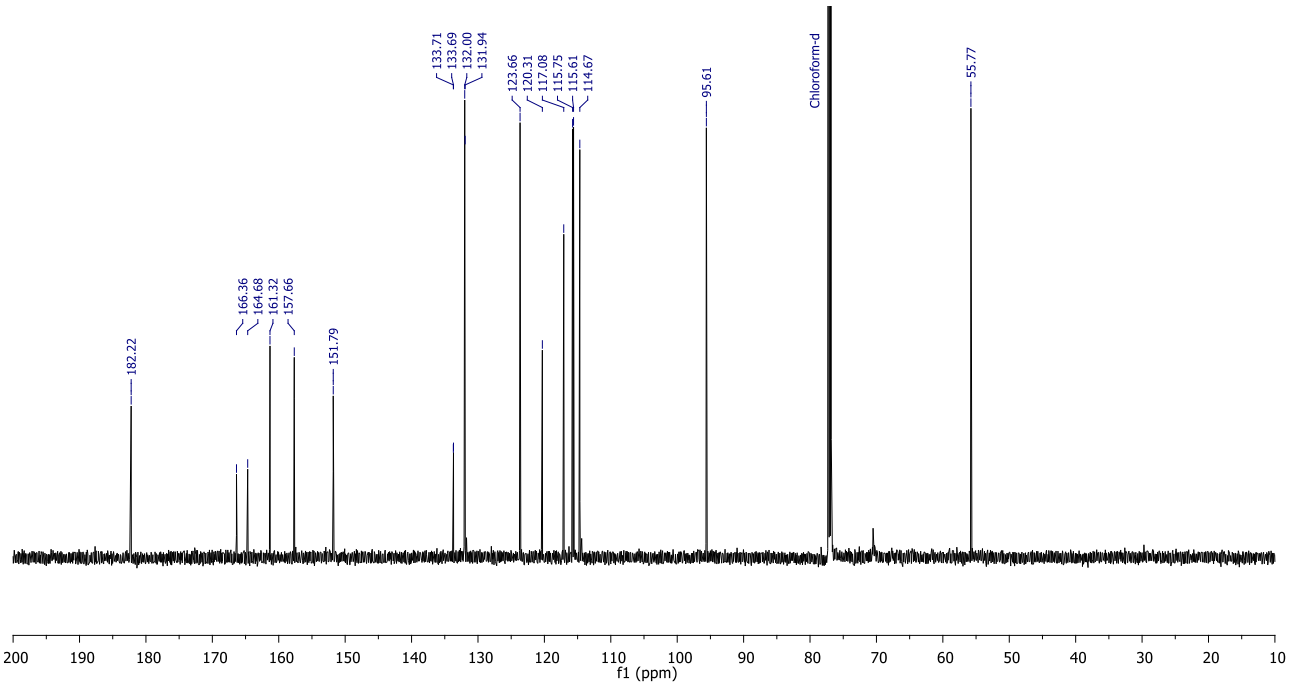


(4-fluorophenyl)(6-methoxy-1-benzofuran-2-yl)methanone (**5m**)

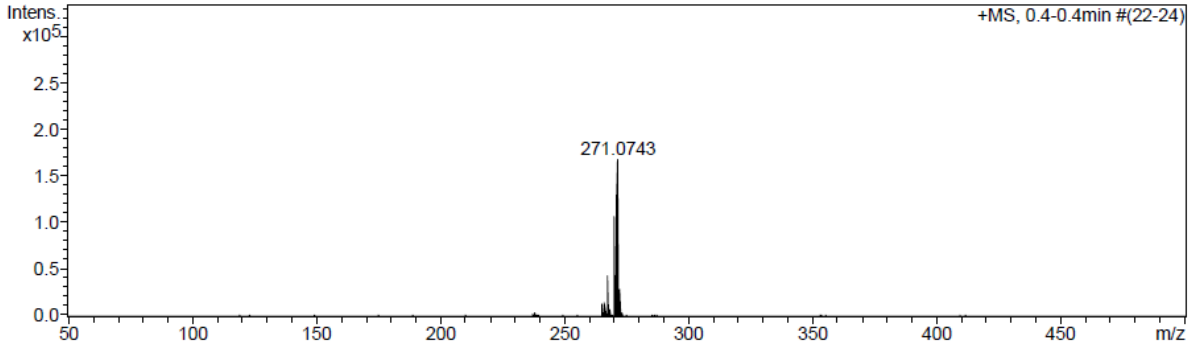
^{1}H NMR



^{13}C NMR

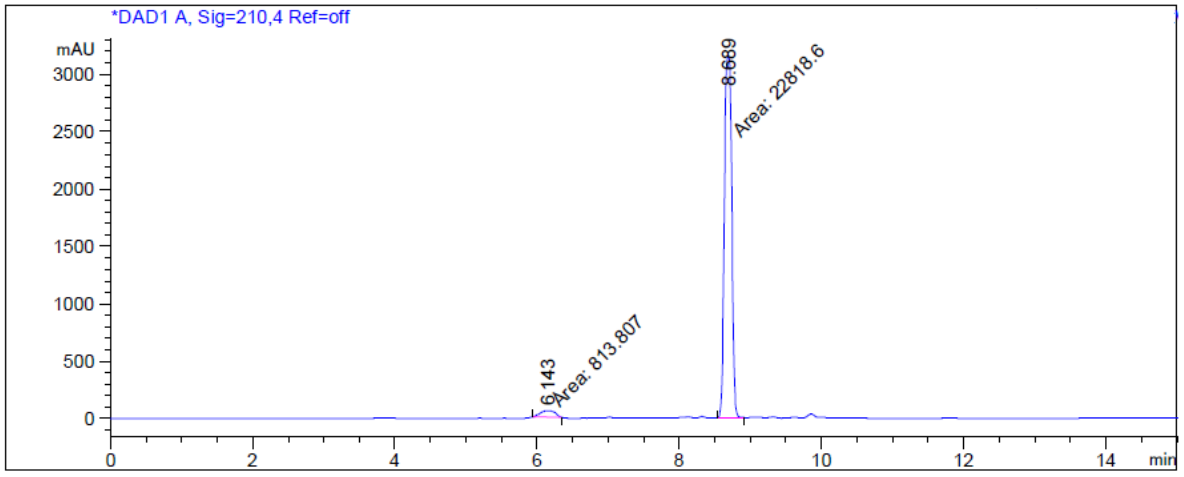


HRMS



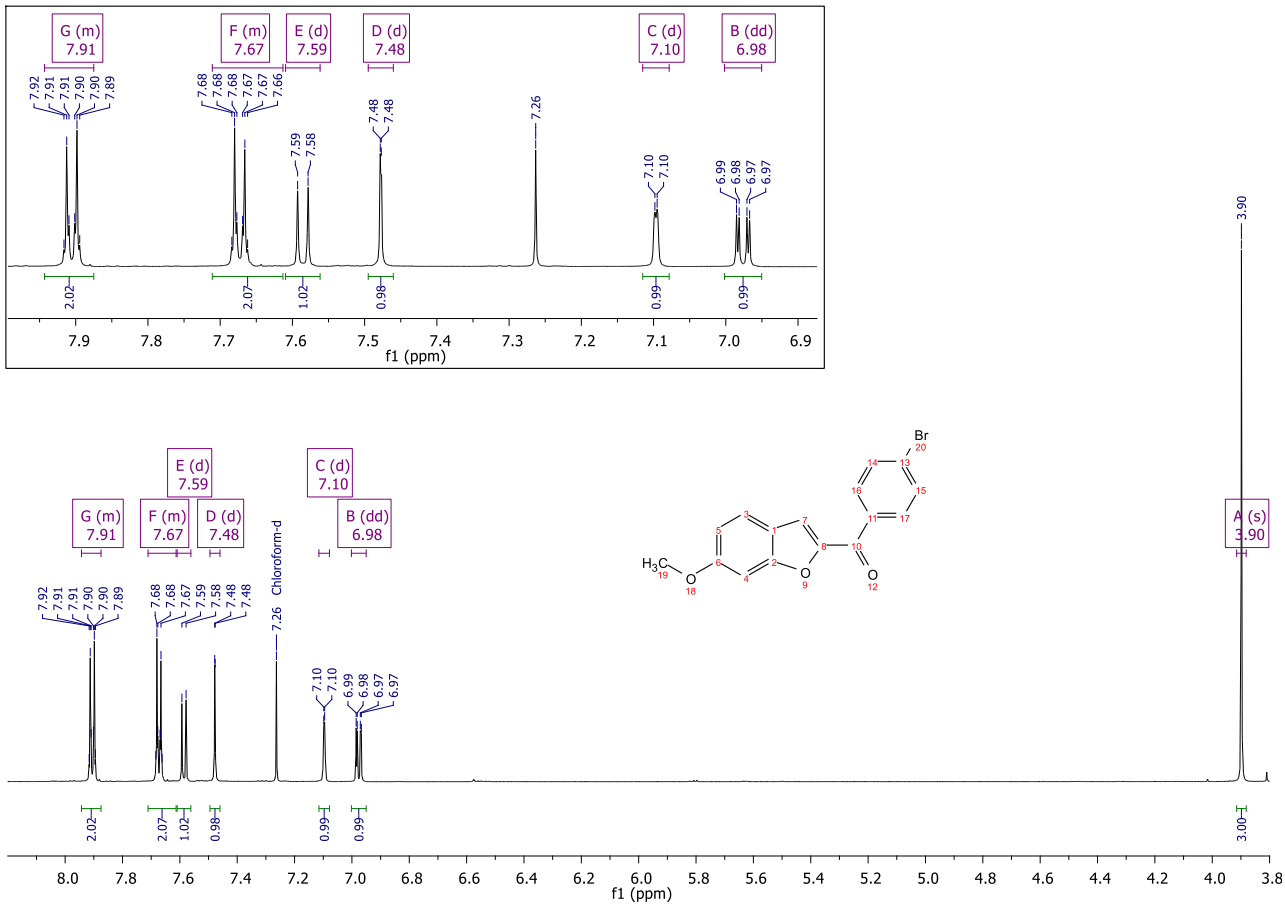
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
271.0743	1	C 16 H 12 F O 3	65.58	271.0765	2.2	8.0	2.2	10.5	even	ok
	2	C 19 H 11 O 2	100.00	271.0754	1.0	3.7	21.0	14.5	even	ok

HPLC

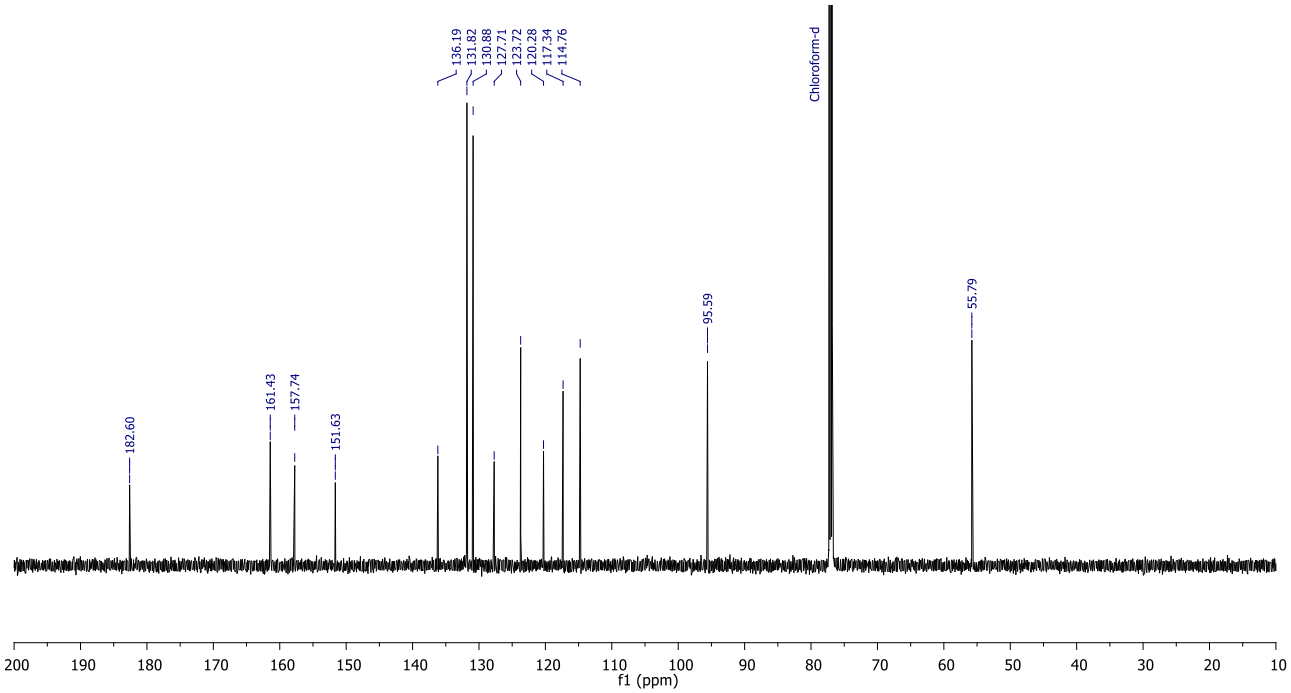


(4-bromophenyl)(6-methoxy-1-benzofuran-2-yl)methanone (**5n**)

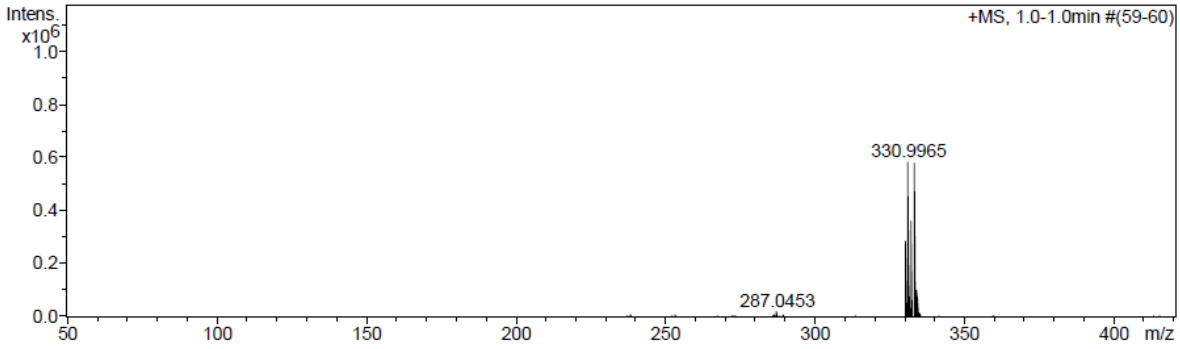
^{1}H NMR



^{13}C NMR

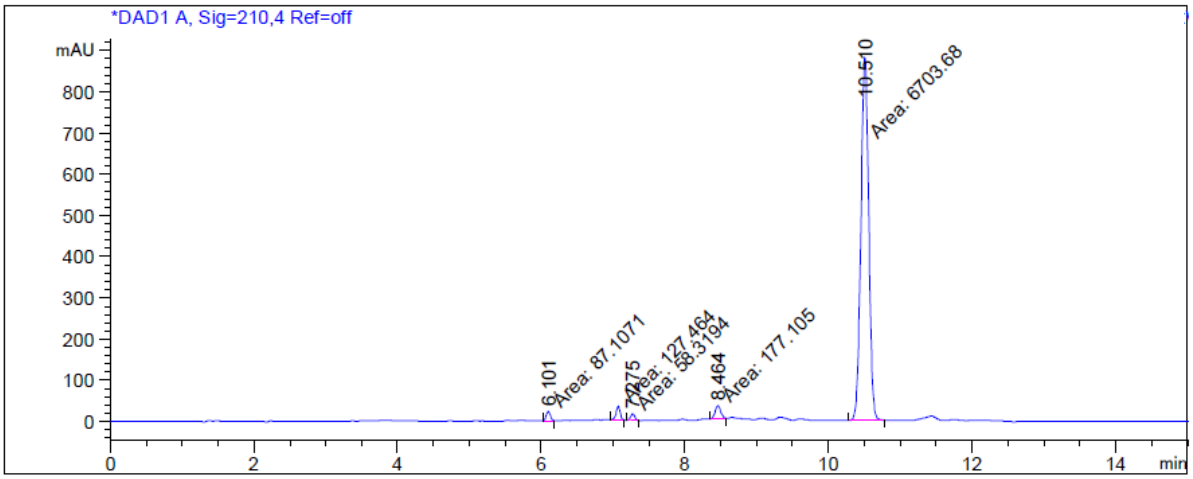


HRMS



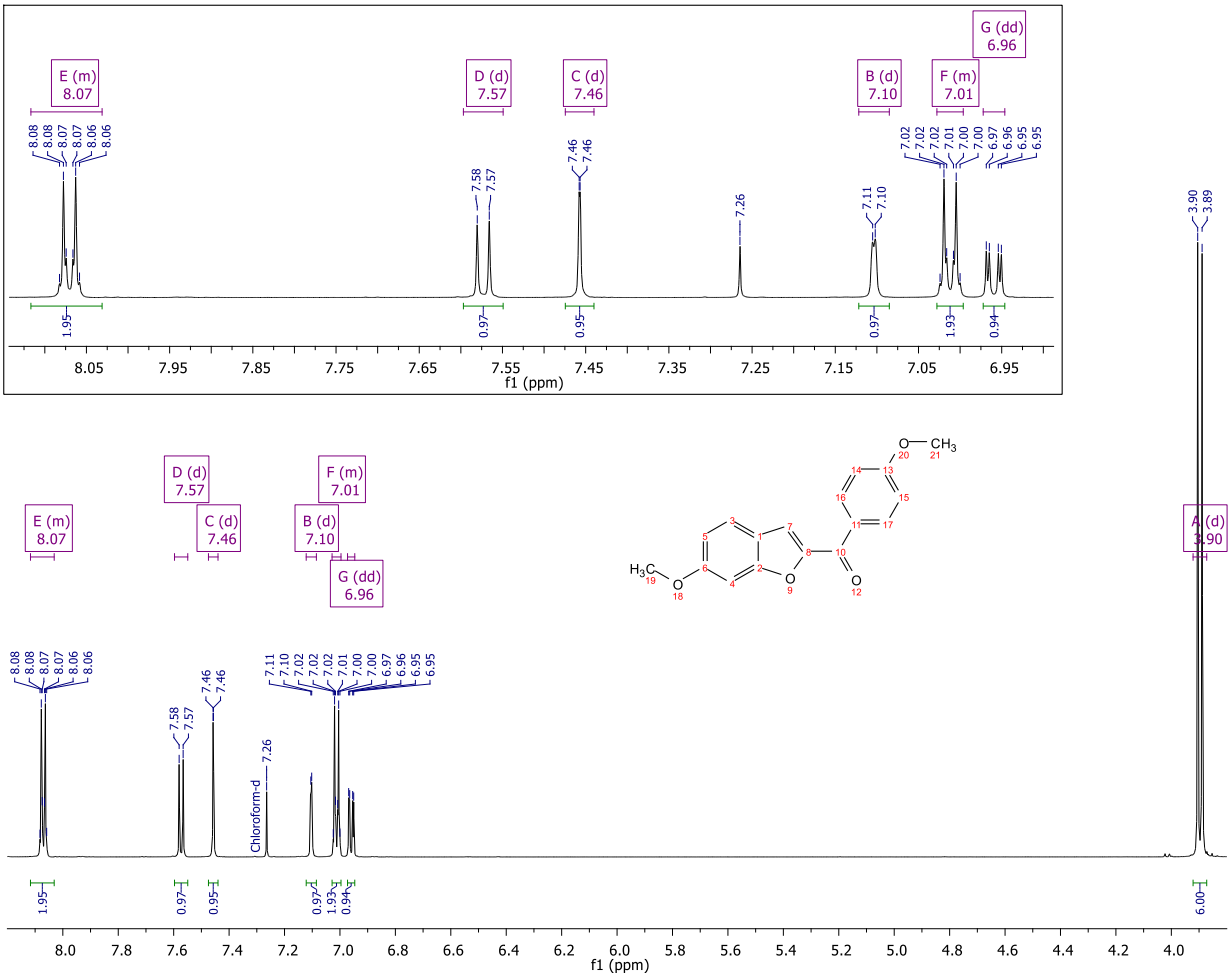
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
330.9965	1	C 19 H 10 Br N	67.65	330.9991	2.6	8.0	183.0	15.0	ok	odd
	2	C 18 H 8 Br N 2	0.00	330.9865	-9.9	-30.0	186.0	15.5	ok	even
	3	C 16 H 12 Br O 3	100.00	330.9964	-0.0	-0.1	198.8	10.5	ok	even
	4	C 25 H N O	0.00	331.0053	8.8	26.6	586.7	26.0	ok	odd
	5	C 20 H N 3 O 3	0.00	331.0012	4.8	14.4	599.2	22.0	ok	odd

HPLC

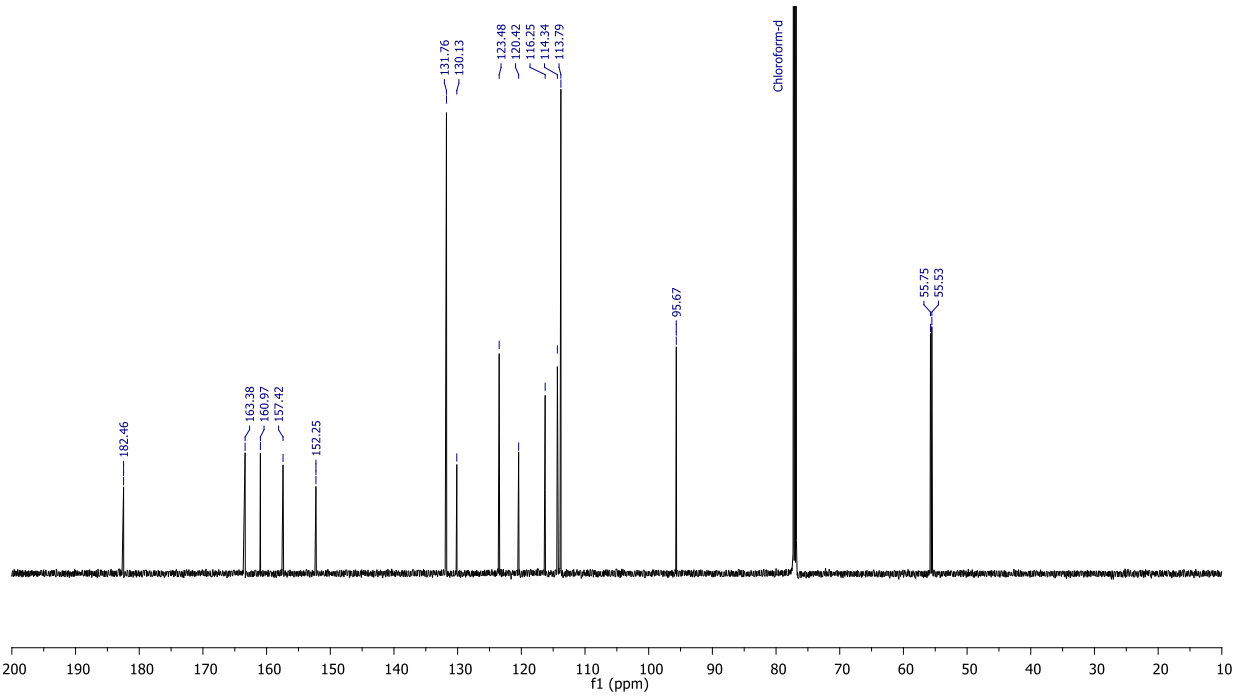


(6-methoxy-1-benzofuran-2-yl)(4-methoxyphenyl)methanone (**5o**)

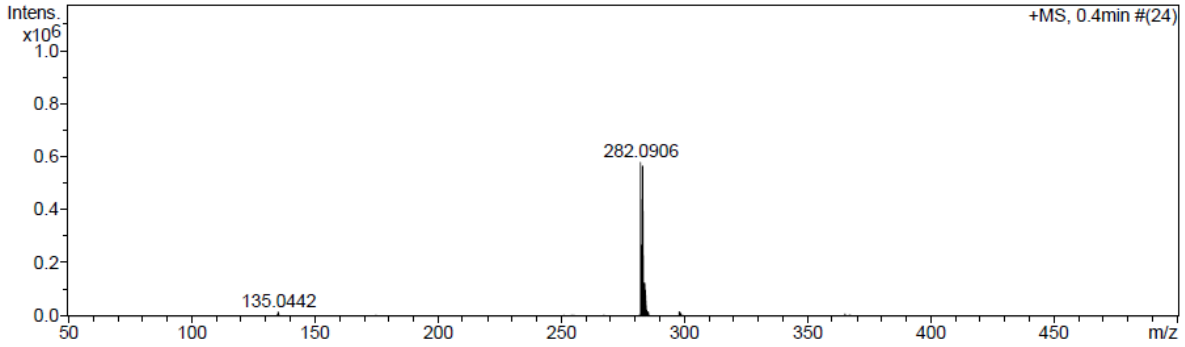
^{1}H NMR



^{13}C NMR

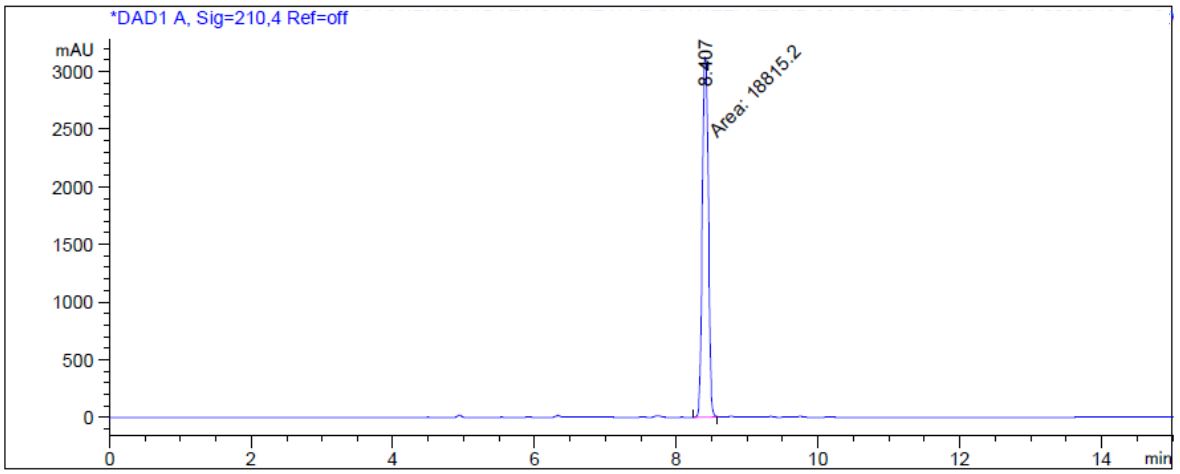


HRMS



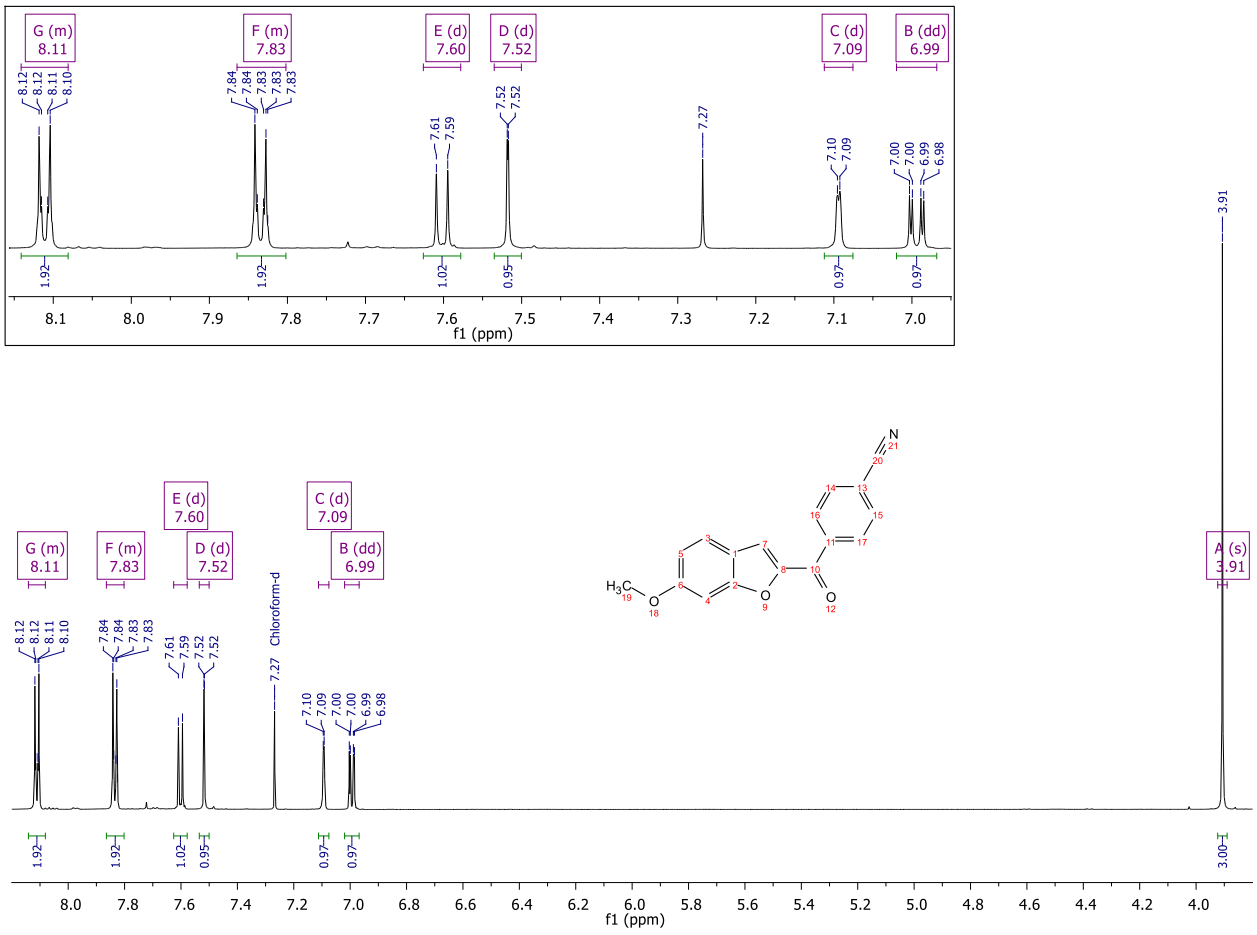
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
282.0906	1	C 17 H 14 O 4	100.00	282.0887	-1.9	-6.8	472.6	11.0	odd	ok
283.0974	1	C 17 H 15 O 4	100.00	283.0965	-0.9	-3.3	23.0	10.5	even	ok

HPLC

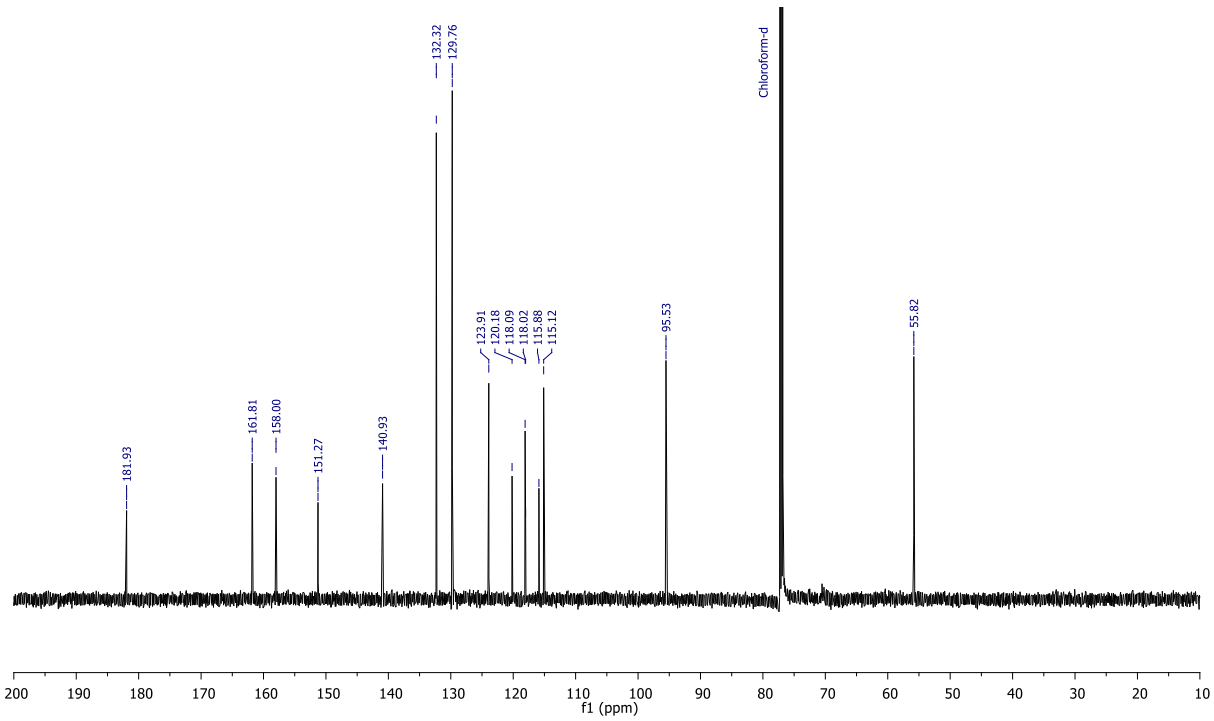


4-[(6-methoxy-1-benzofuran-2-yl)carbonyl]benzonitrile (**5p**)

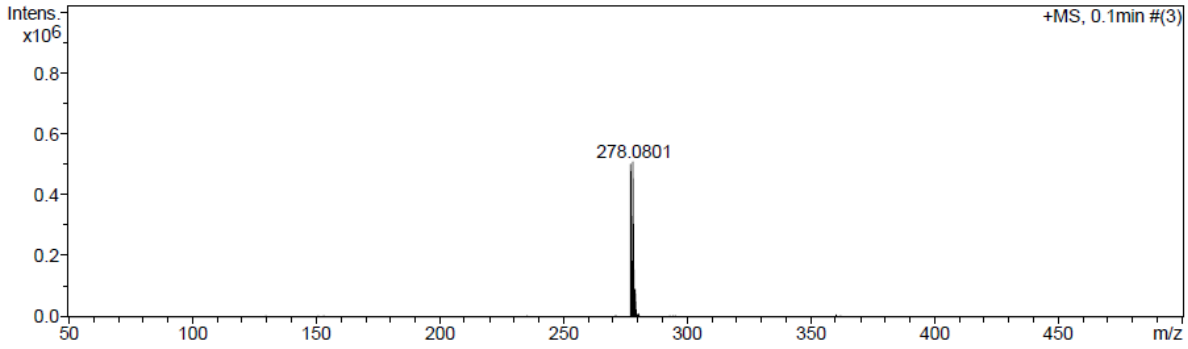
^{1}H NMR



^{13}C NMR

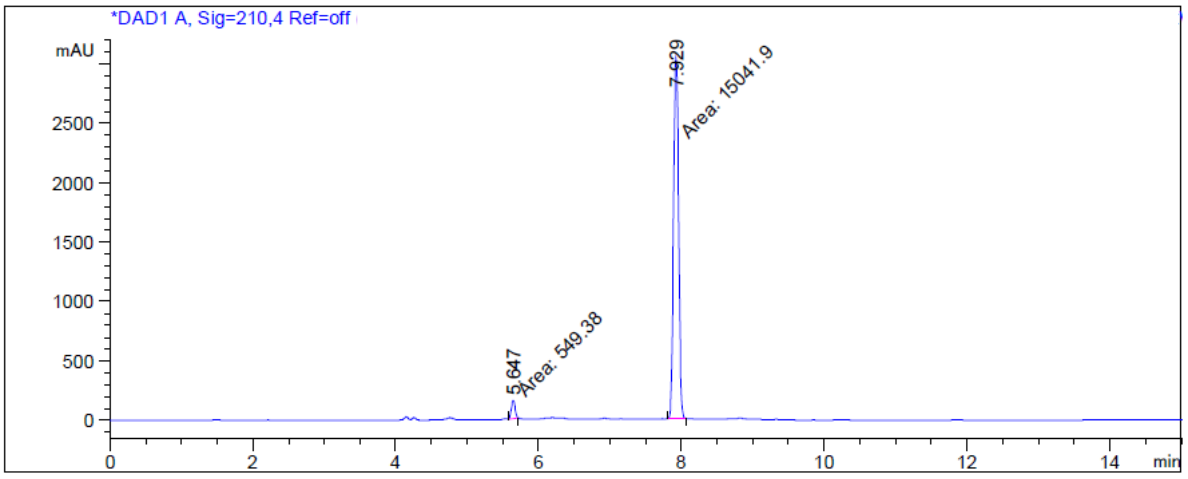


HRMS



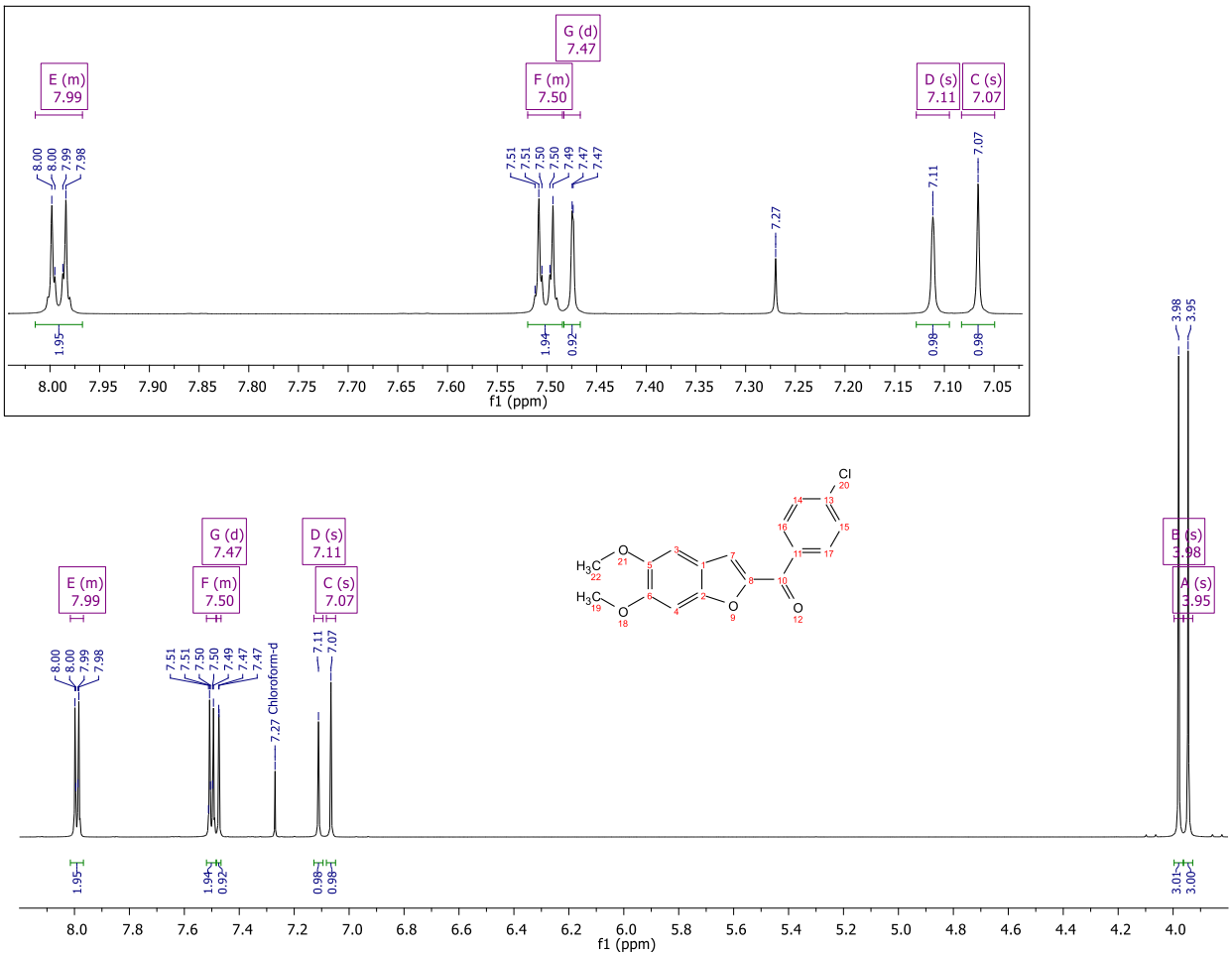
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
277.0733	1	C 17 H 11 N O 3	100.00	277.0733	-0.0	-0.0	477.9	13.0	odd	ok
278.0801	1	C 17 H 12 N O 3	100.00	278.0812	1.0	3.7	3.7	12.5	even	ok

HPLC

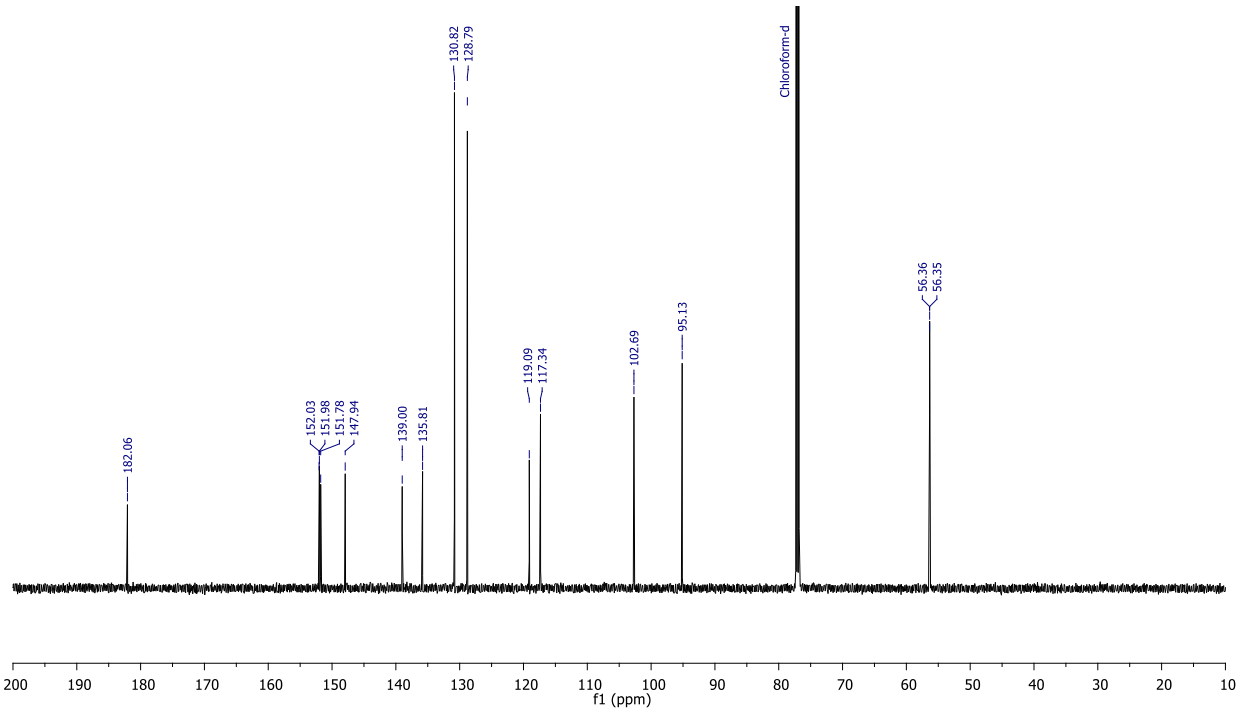


(4-chlorophenyl)(5,6-dimethoxy-1-benzofuran-2-yl)methanone (**5q**)

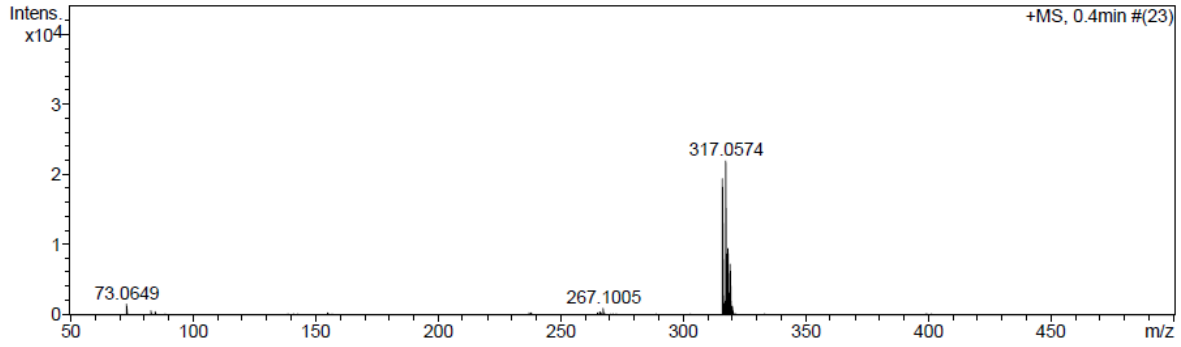
^{1}H NMR



^{13}C NMR

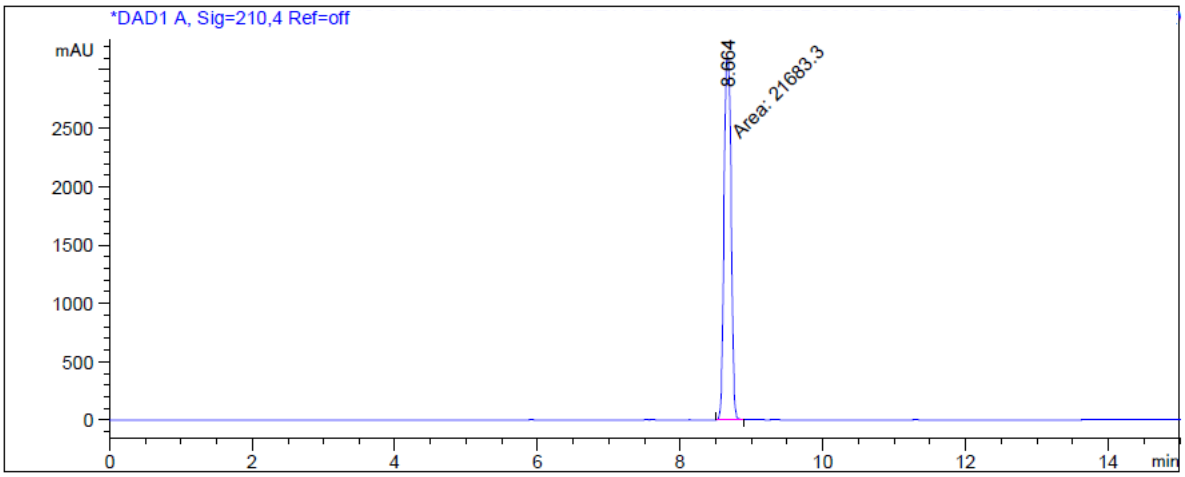


HRMS



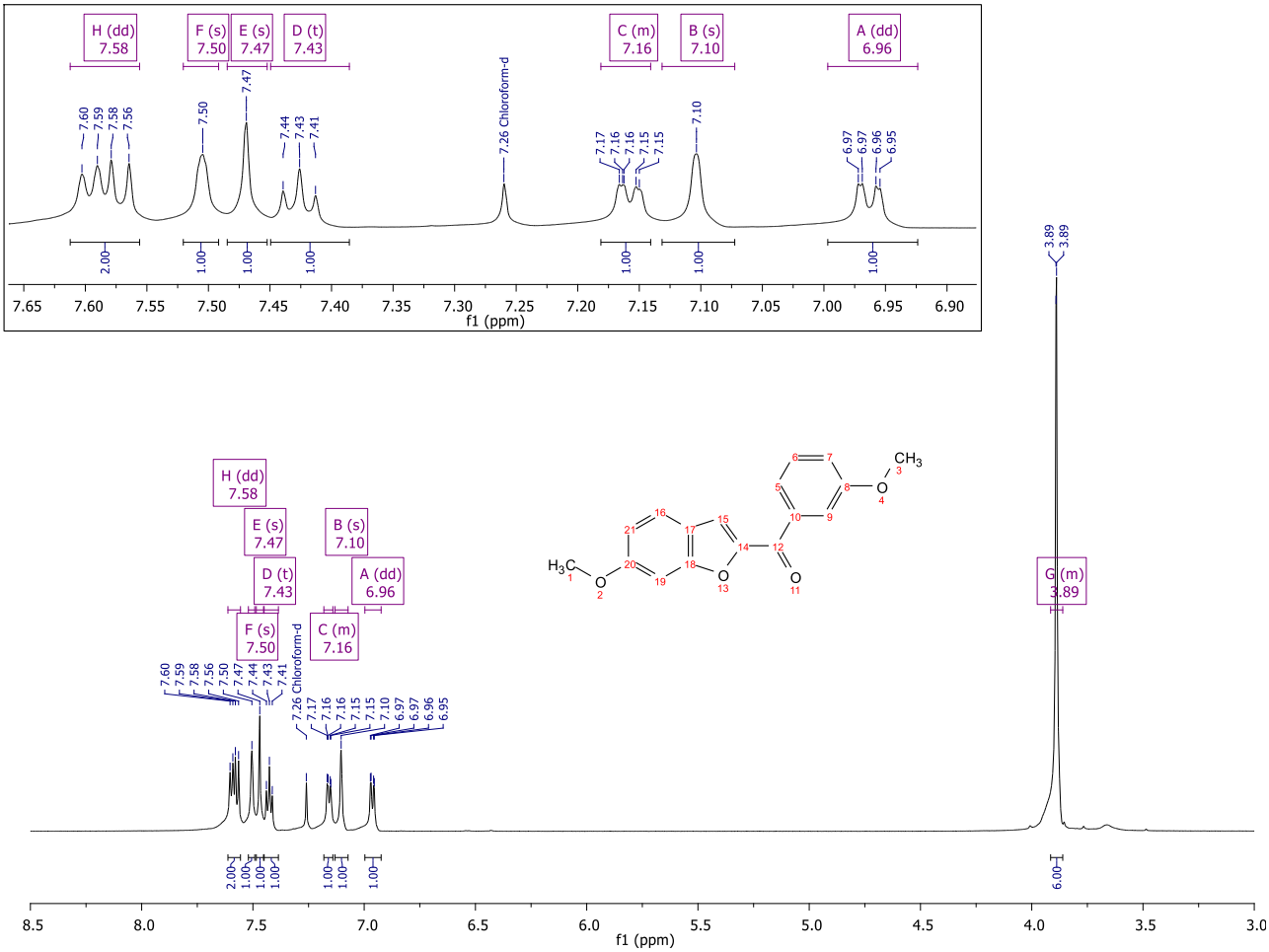
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
316.0508	1	C 17 H 13 Cl O 4	100.00	316.0497	-1.1	-3.5	391.3	11.0	odd	ok
	2	C 23 H 8 O 2	0.00	316.0519	1.1	3.4	495.2	20.0	odd	ok
317.0574	1	C 17 H 14 Cl O 4	100.00	317.0575	0.1	0.2	110.7	10.5	even	ok
	2	C 23 H 9 O 2	0.05	317.0597	2.3	7.1	203.5	19.5	even	ok

HPLC

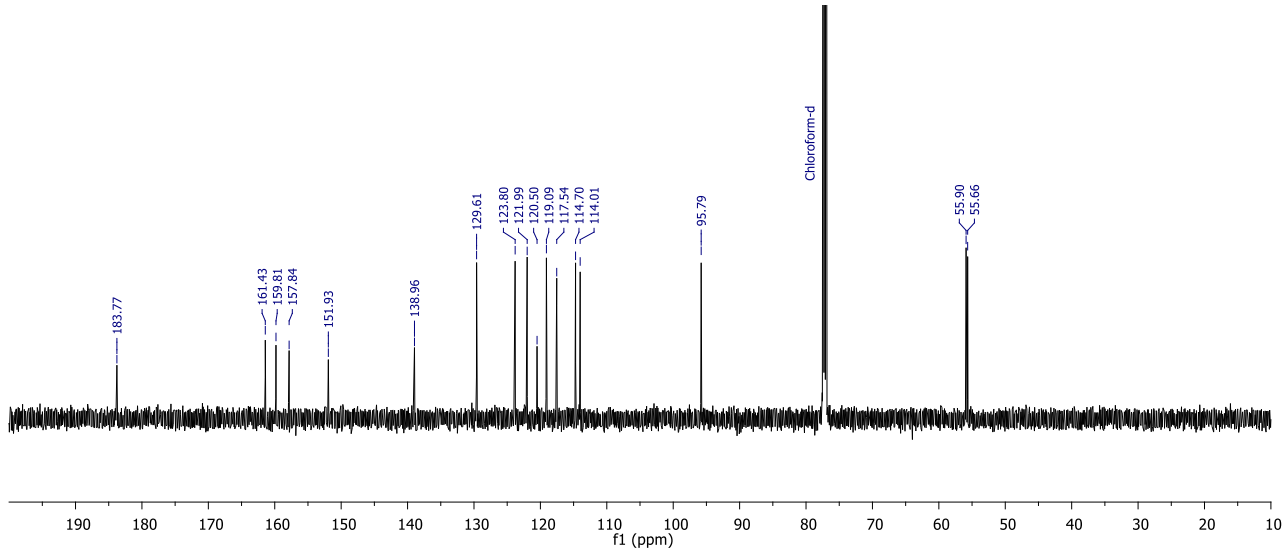


(6-Methoxybenzofuran-2-yl)(3-methoxyphenyl)methanone (**5r**)

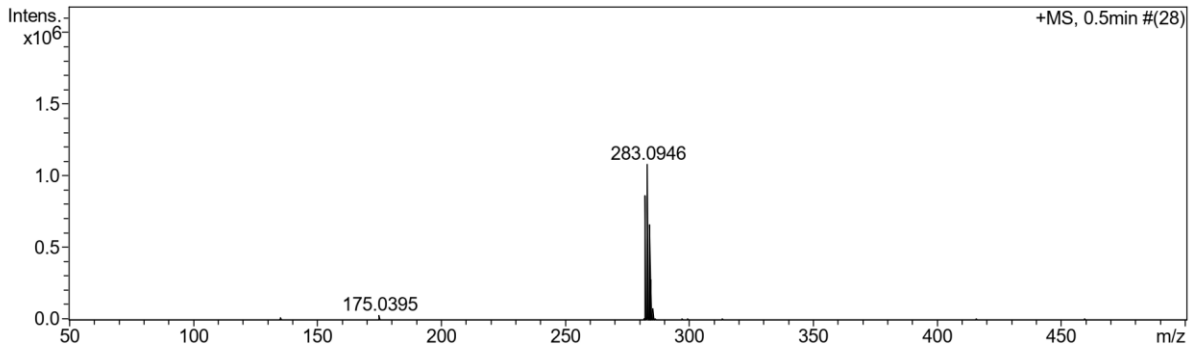
^{1}H NMR



^{13}C NMR



HRMS



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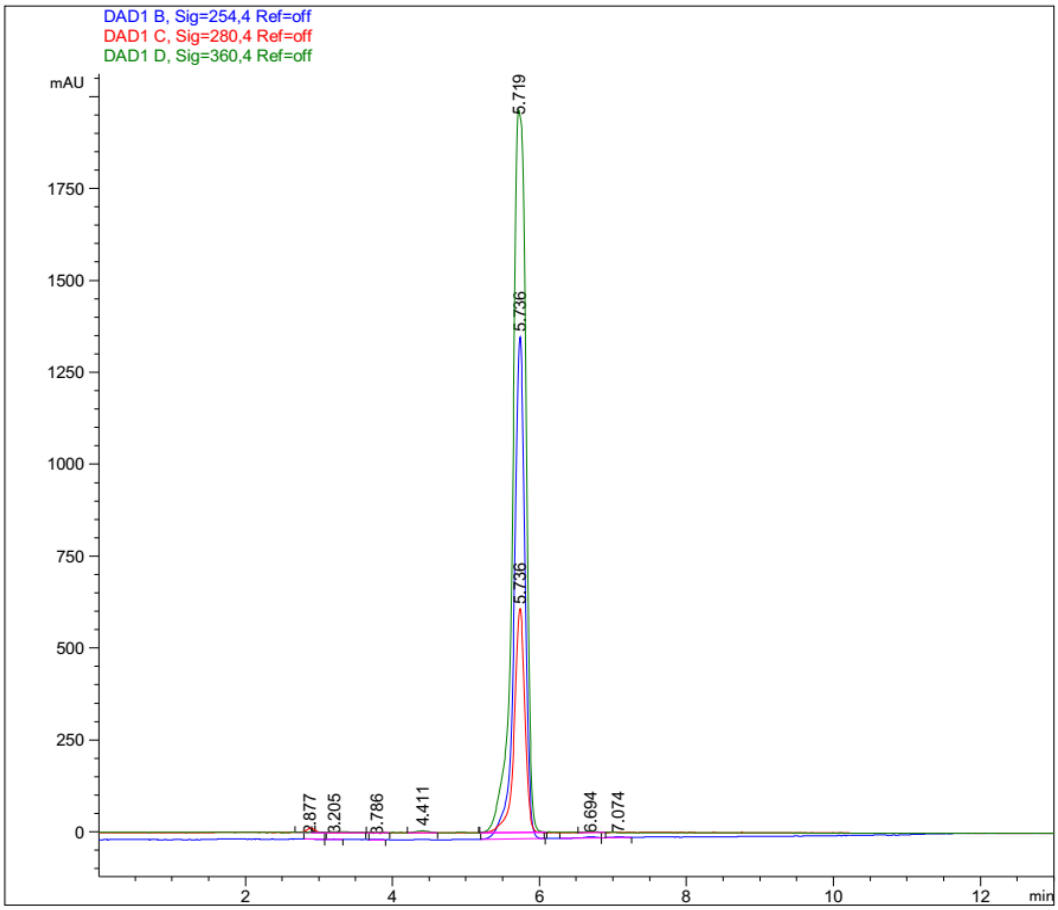
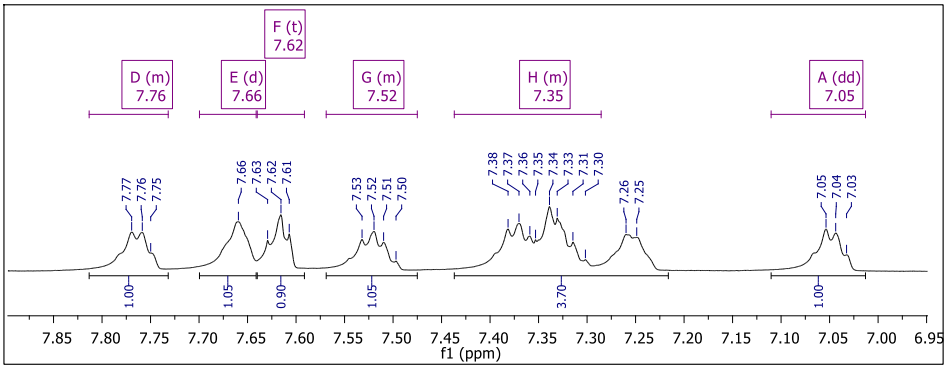
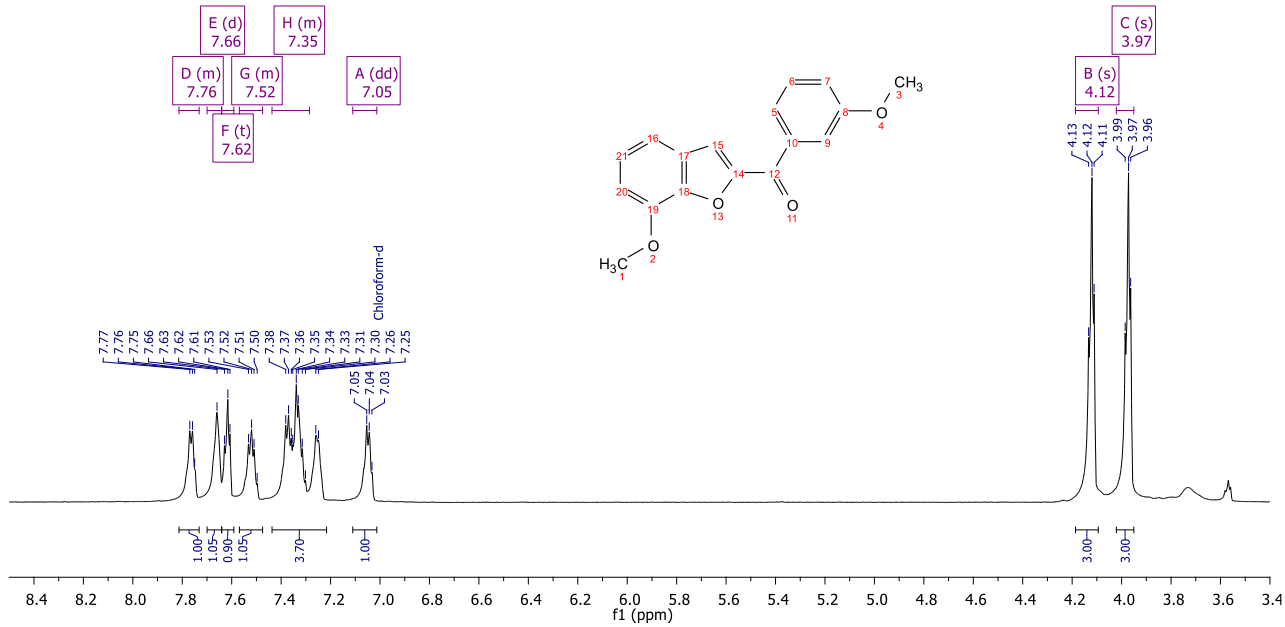
HPLC



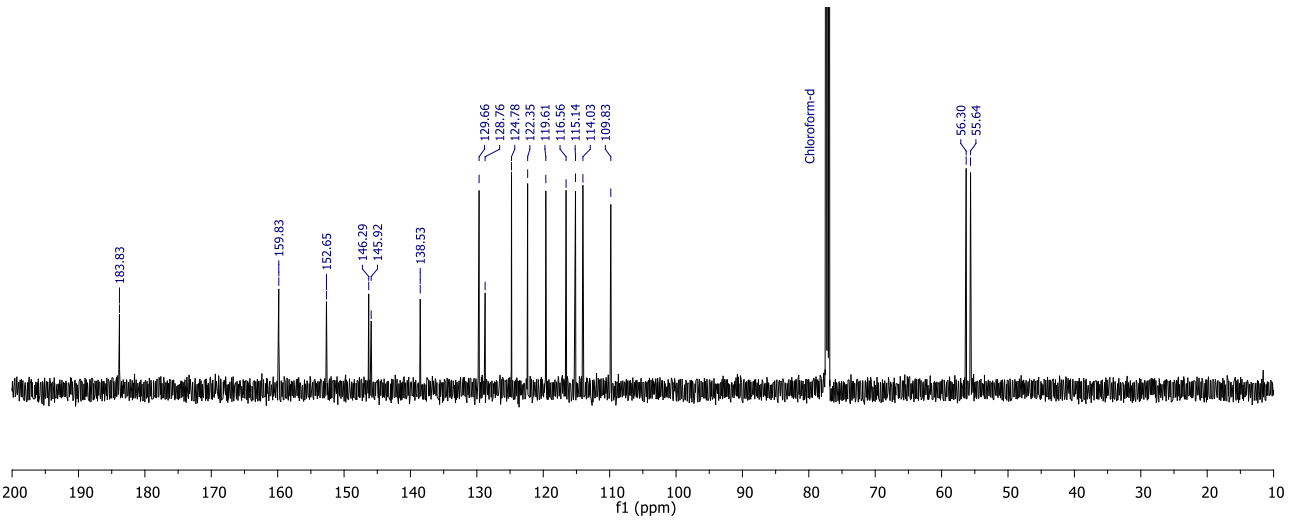
(7-Methoxybenzofuran-2-yl)(3-methoxyphenyl)methanone (**5s**)

^{1}H NMR

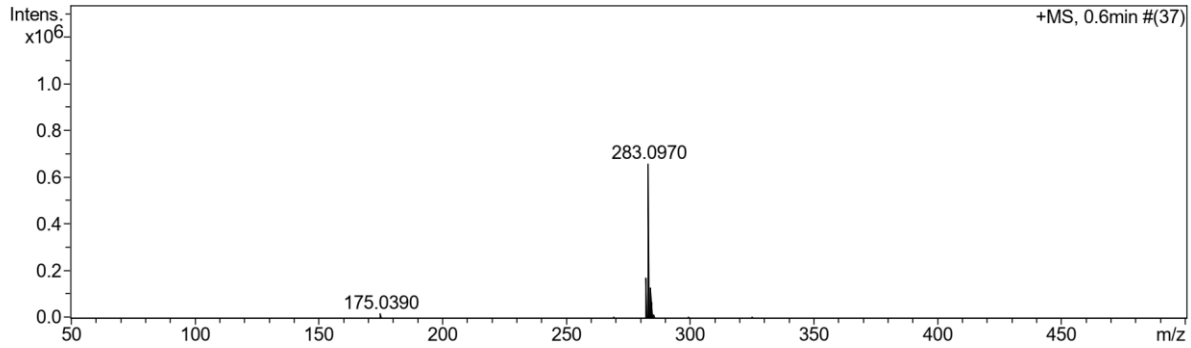




^{13}C NMR



HRMS



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
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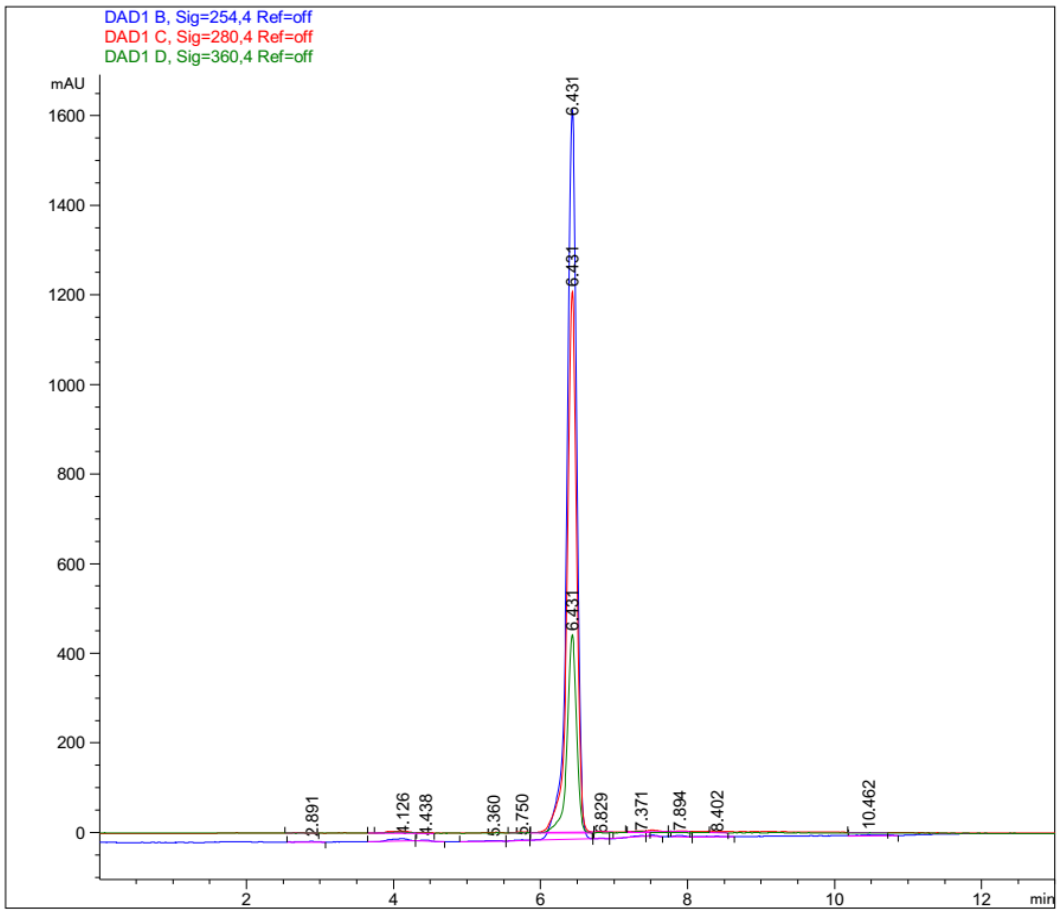
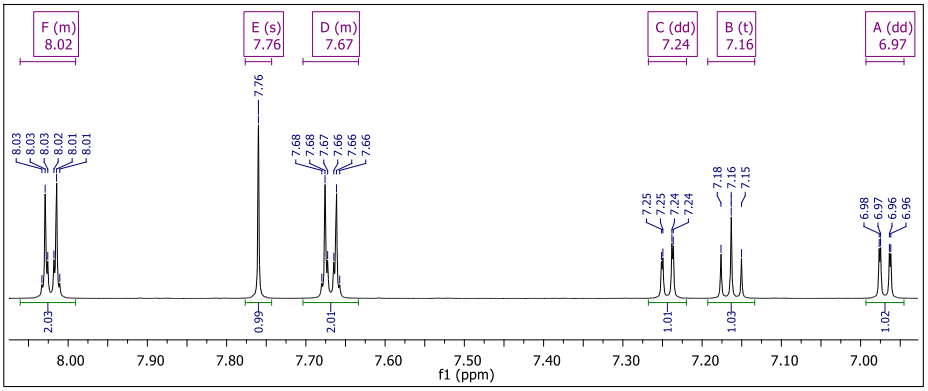
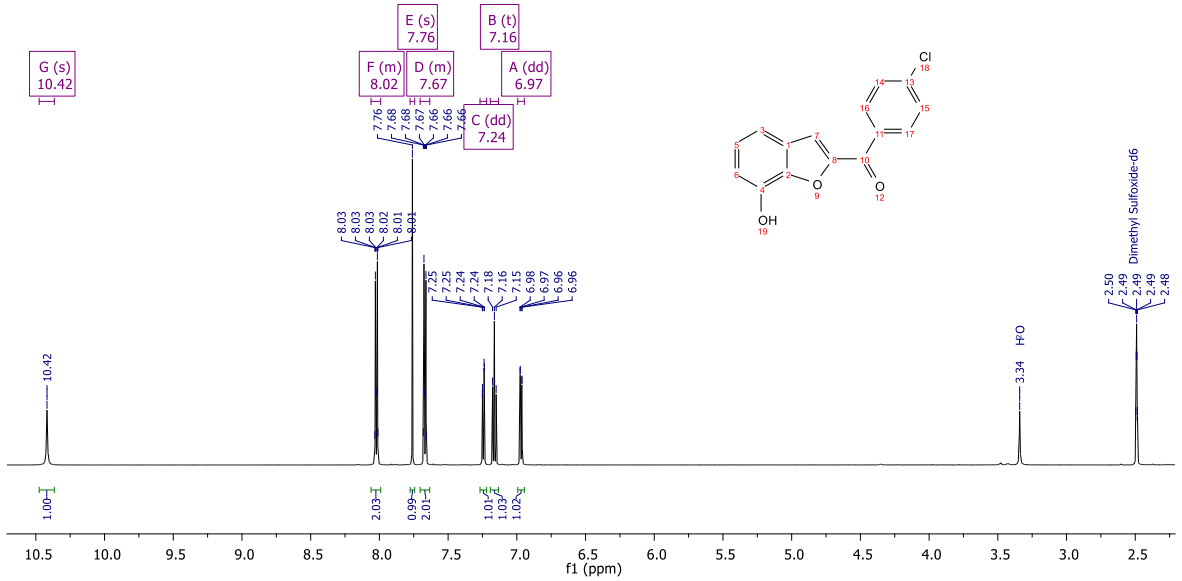
HPLC



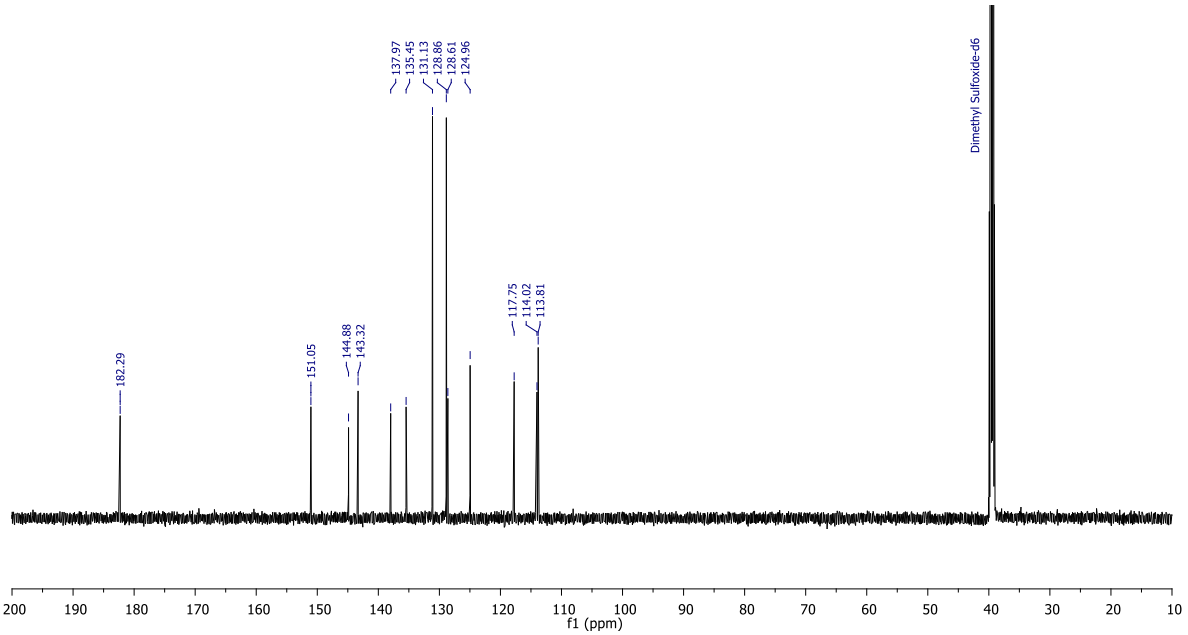
(4-chlorophenyl)(7-hydroxy-1-benzofuran-2-yl)methanone (**5t**)

^{1}H NMR

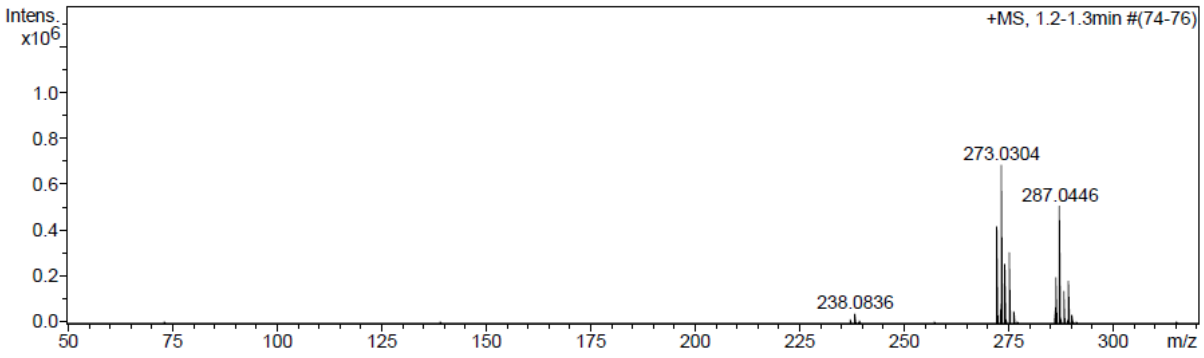




^{13}C NMR

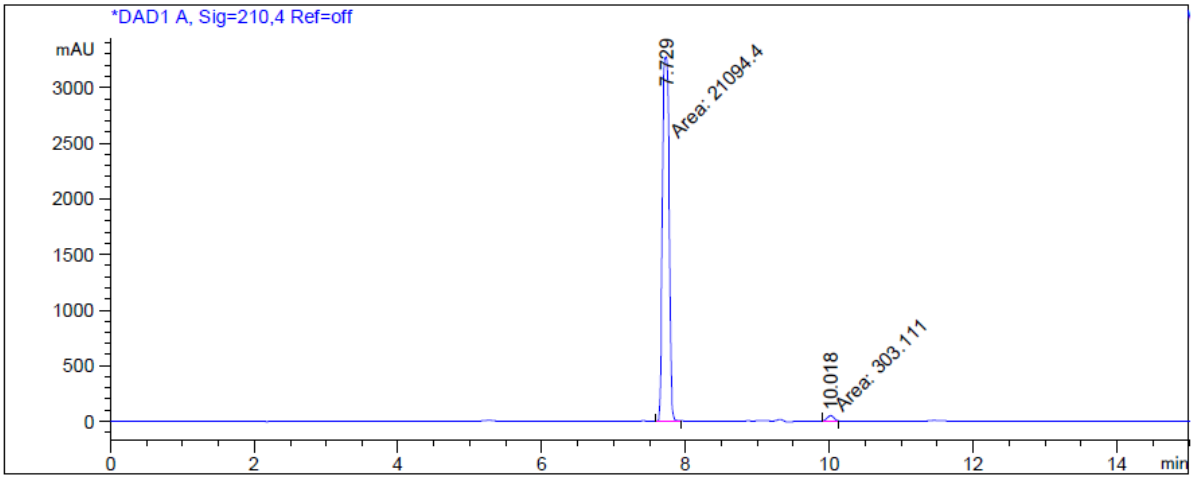


HRMS



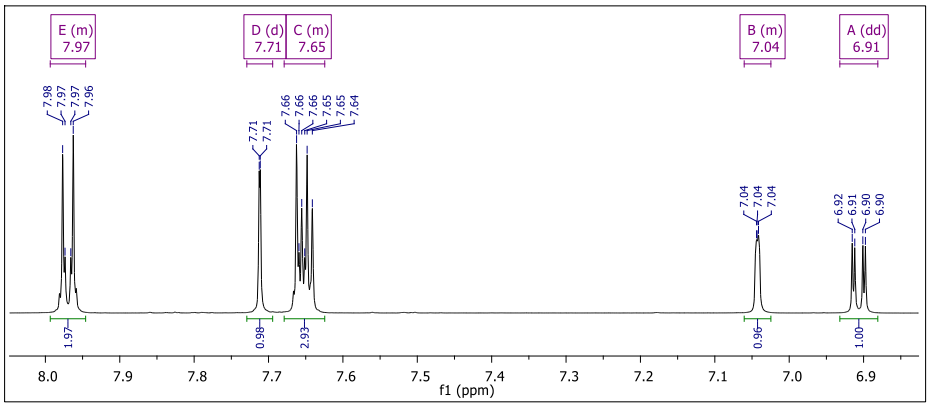
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
273.0304	1	C 18 H 8 Cl N	22.74	273.0340	3.5	12.9	90.9	15.0	ok	odd
	2	C 17 H 6 Cl N 2	0.00	273.0214	-9.0	-33.1	94.0	15.5	ok	even
	3	C 15 H 10 Cl O 3	100.00	273.0313	0.9	3.1	105.4	10.5	ok	even
	4	C 21 H 5 O	0.00	273.0335	3.0	11.2	255.3	19.5	ok	even
	5	C 20 H 3 N O	0.00	273.0209	-9.5	-34.9	257.5	20.0	ok	odd
	6	C 19 H 3 N 3	0.00	273.0321	1.7	6.2	258.6	20.0	ok	odd
	7	C 16 H 5 N 2 O 3	0.00	273.0295	-1.0	-3.6	267.0	15.5	ok	even

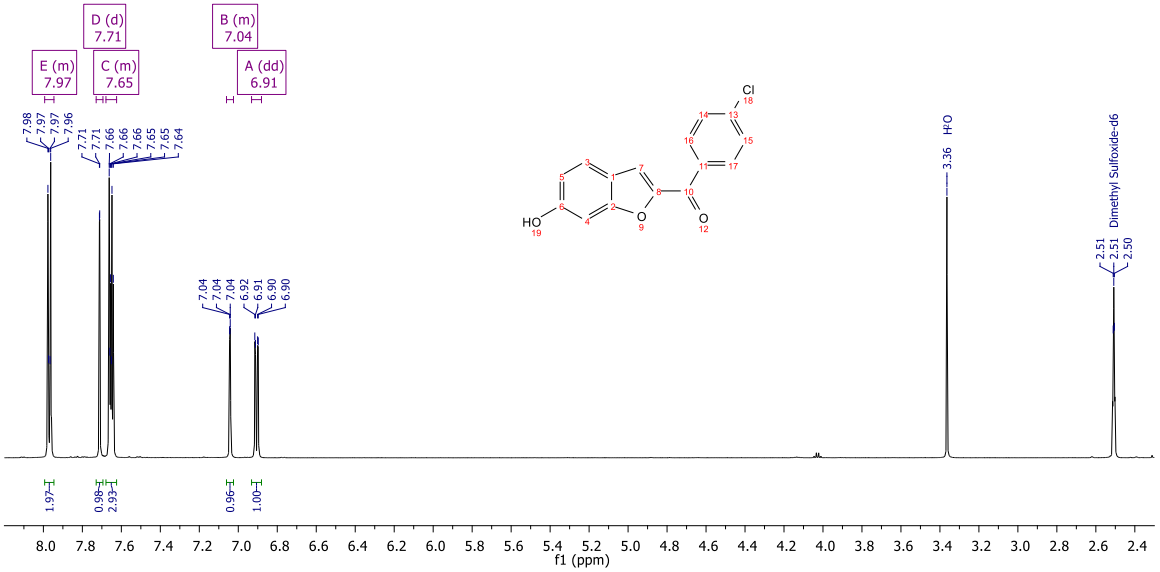
HPLC



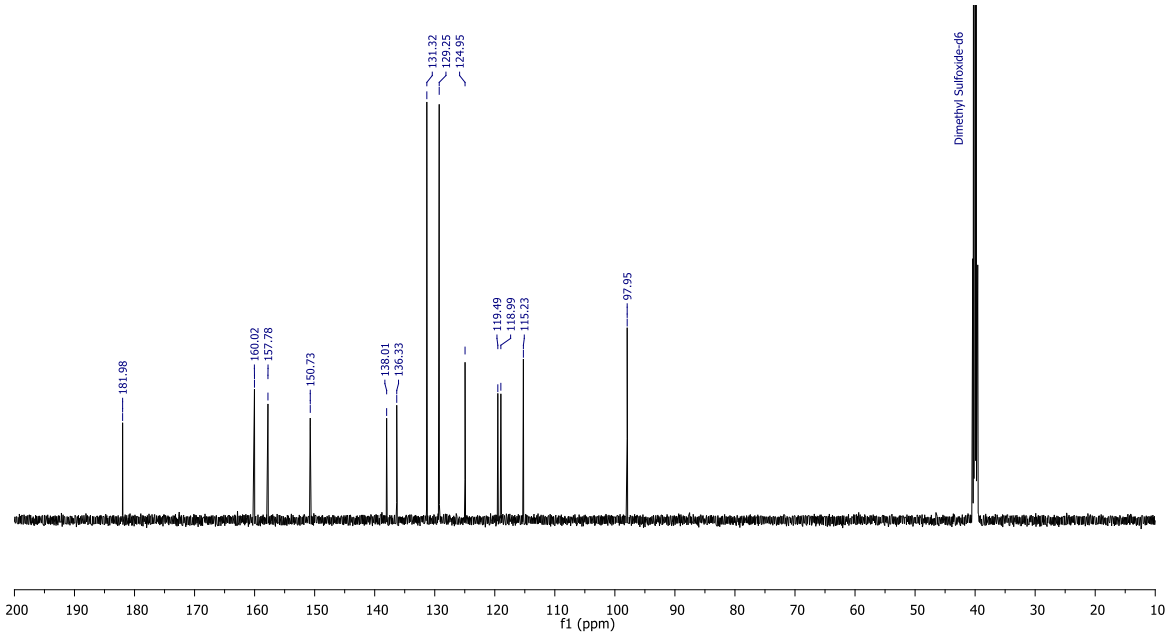
(4-chlorophenyl)(6-hydroxy-1-benzofuran-2-yl)methanone (**5u**)

^{1}H NMR

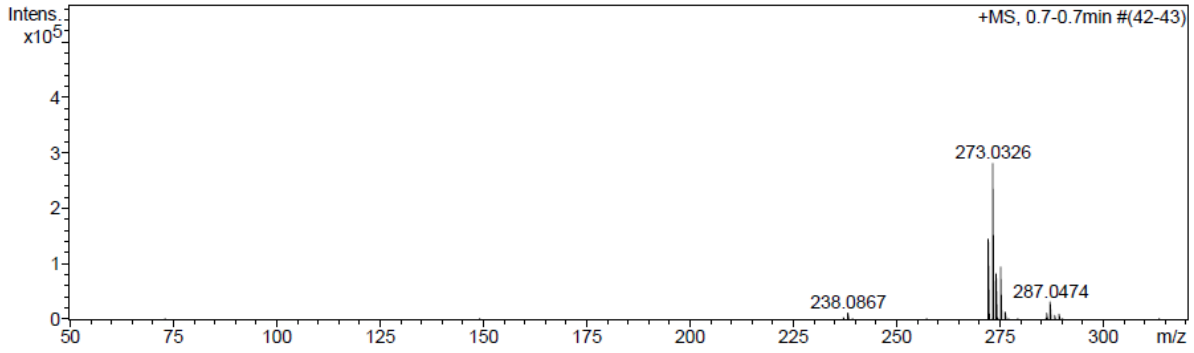




^{13}C NMR

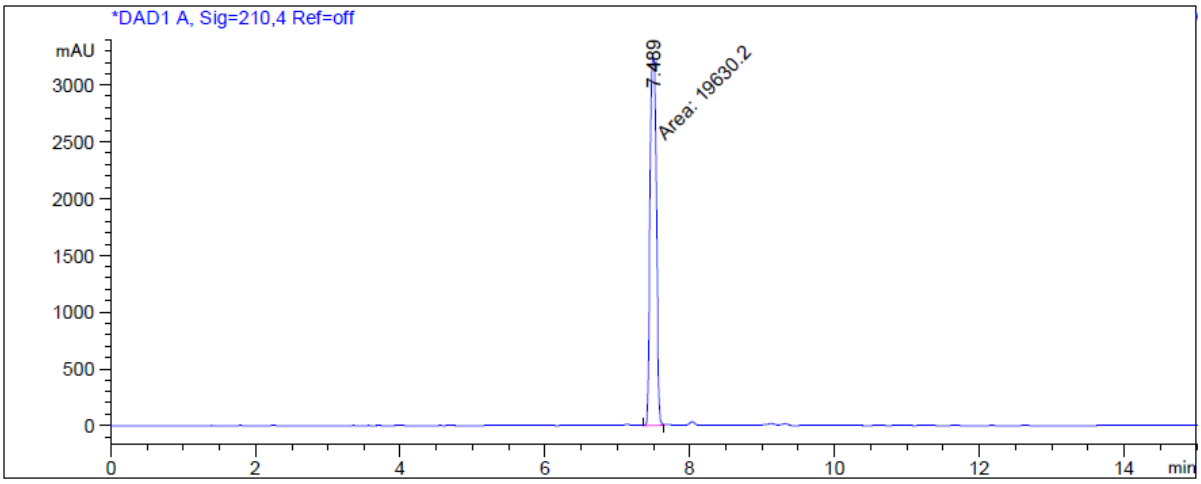


HRMS



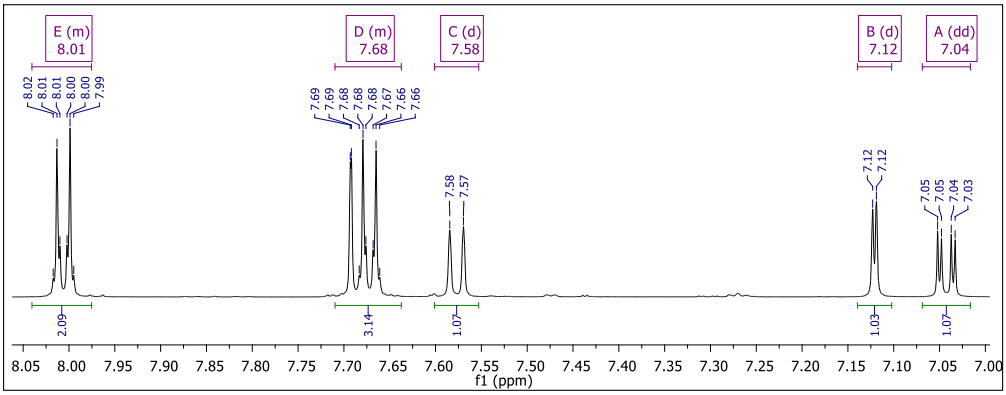
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
273.0326	1	C 18 H 8 Cl N	100.00	273.0340	1.4	5.2	42.5	15.0	ok	odd
	2	C 15 H 10 Cl O 3	68.32	273.0313	-1.3	-4.6	58.4	10.5	ok	even
	3	C 17 H 7 N O 3	0.00	273.0420	9.5	34.8	62.2	15.0	ok	odd
	4	C 21 H 5 O	0.08	273.0335	0.9	3.4	183.5	19.5	ok	even
	5	C 19 H 3 N 3	0.09	273.0321	-0.4	-1.5	186.1	20.0	ok	odd
	6	C 16 H 5 N 2 O 3	0.01	273.0295	-3.1	-11.3	192.6	15.5	ok	even
	7	C 15 H 5 N 4 O 2	0.00	273.0407	8.1	29.8	194.7	15.5	ok	even

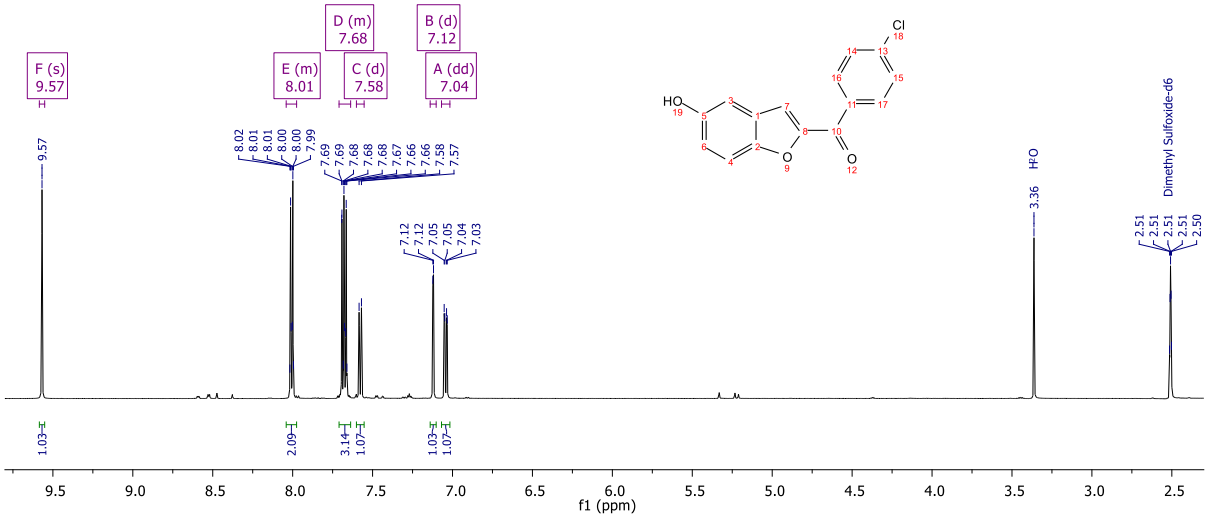
HPLC



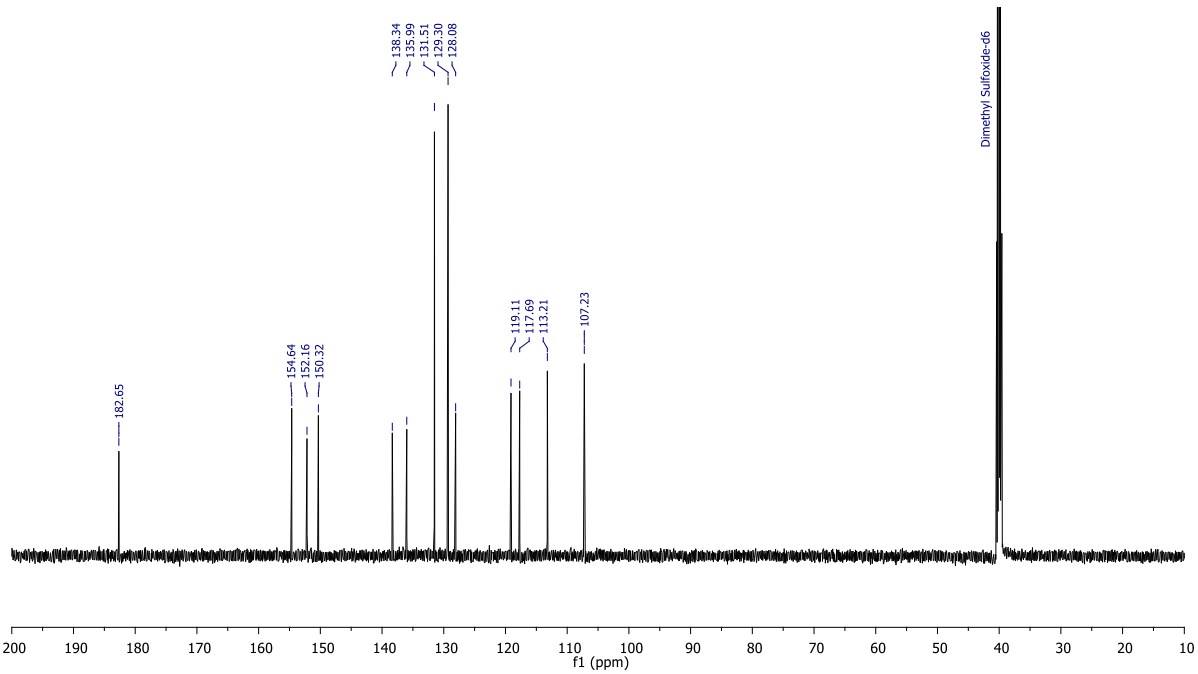
(4-chlorophenyl)(5-hydroxy-1-benzofuran-2-yl)methanone (**5v**)

^{1}H NMR

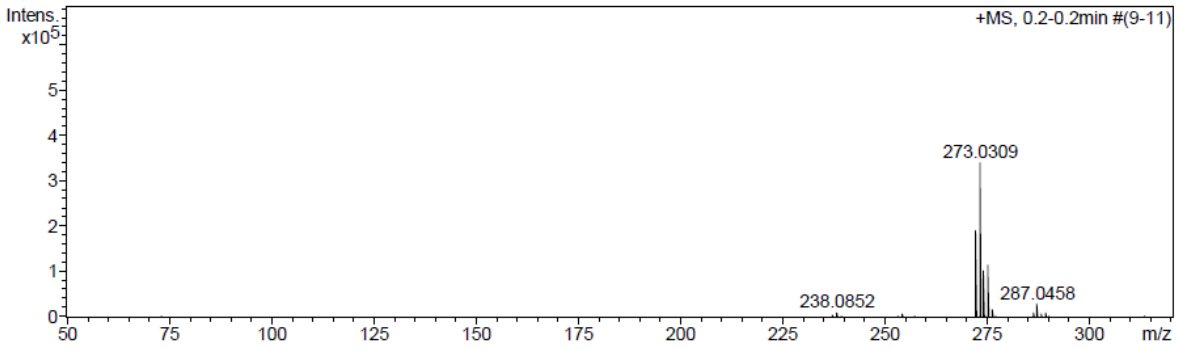




^{13}C NMR

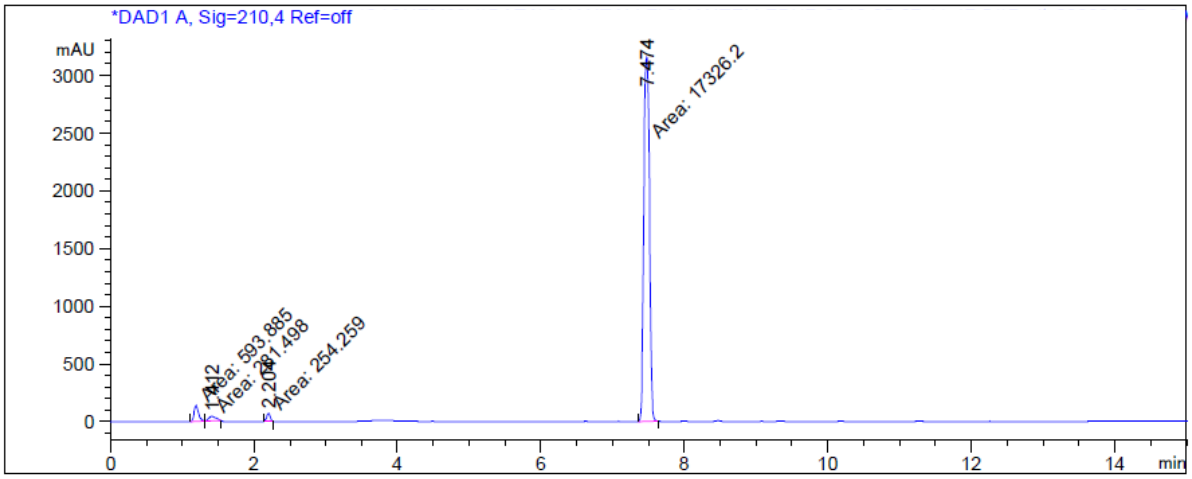


HRMS



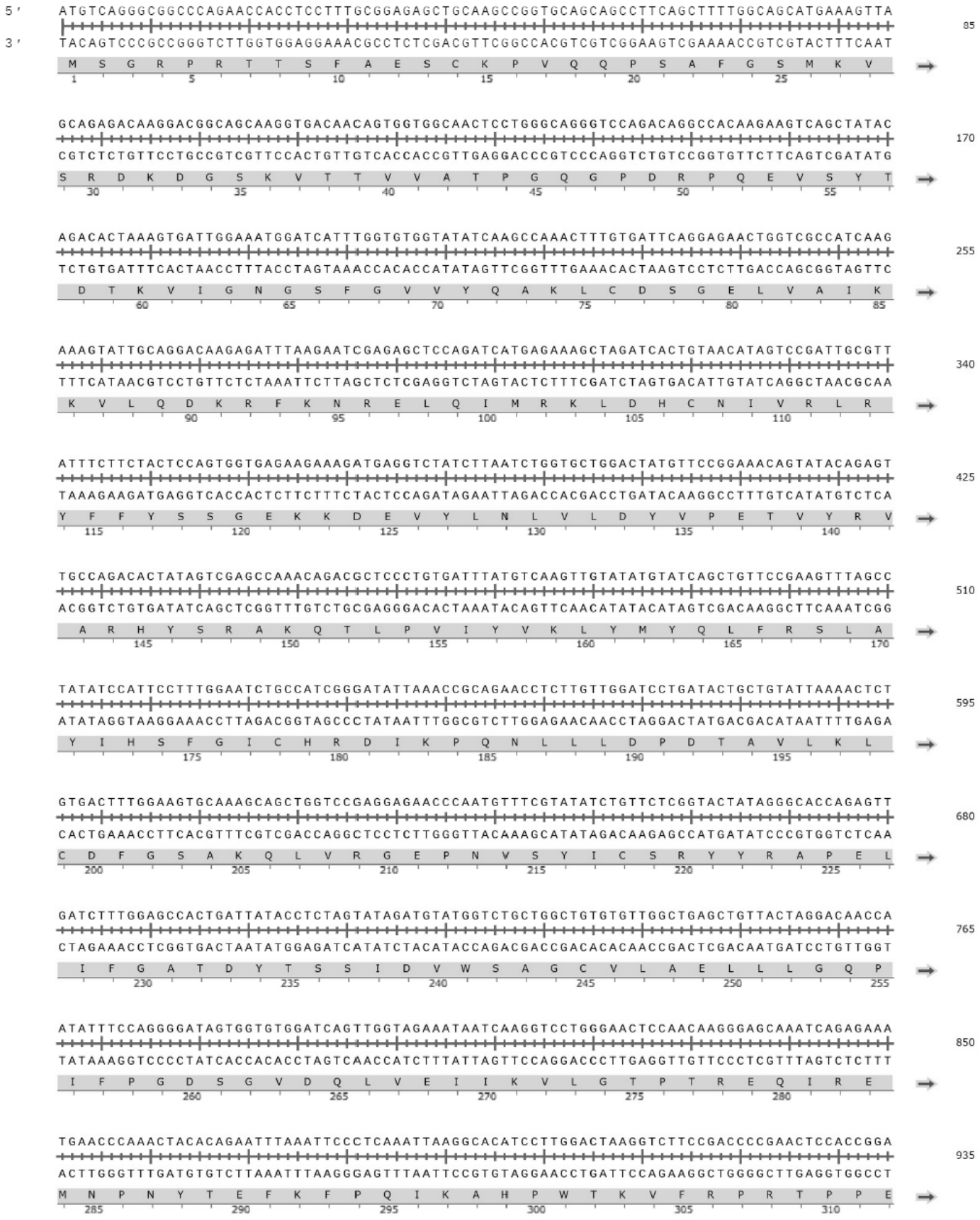
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
273.0309	1	C 18 H 8 Cl N	24.92	273.0340	3.1	11.2	47.8	15.0	ok	odd
	2	C 17 H 6 Cl N 2	0.00	273.0214	-9.5	-34.9	50.9	15.5	ok	even
	3	C 15 H 10 Cl O 3	100.00	273.0313	0.4	1.4	63.7	10.5	ok	even
	4	C 15 H 5 N 4 O 2	0.00	273.0407	9.8	35.8	74.5	15.5	ok	even
	5	C 21 H 5 O	0.02	273.0335	2.6	9.4	187.6	19.5	ok	even
	6	C 19 H 3 N 3	0.04	273.0321	1.2	4.5	190.5	20.0	ok	odd
	7	C 16 H 5 N 2 O 3	0.02	273.0295	-1.5	-5.3	197.5	15.5	ok	even

HPLC



2. Biology

2.1. *Hs*GSK-3β gene and protein sequences



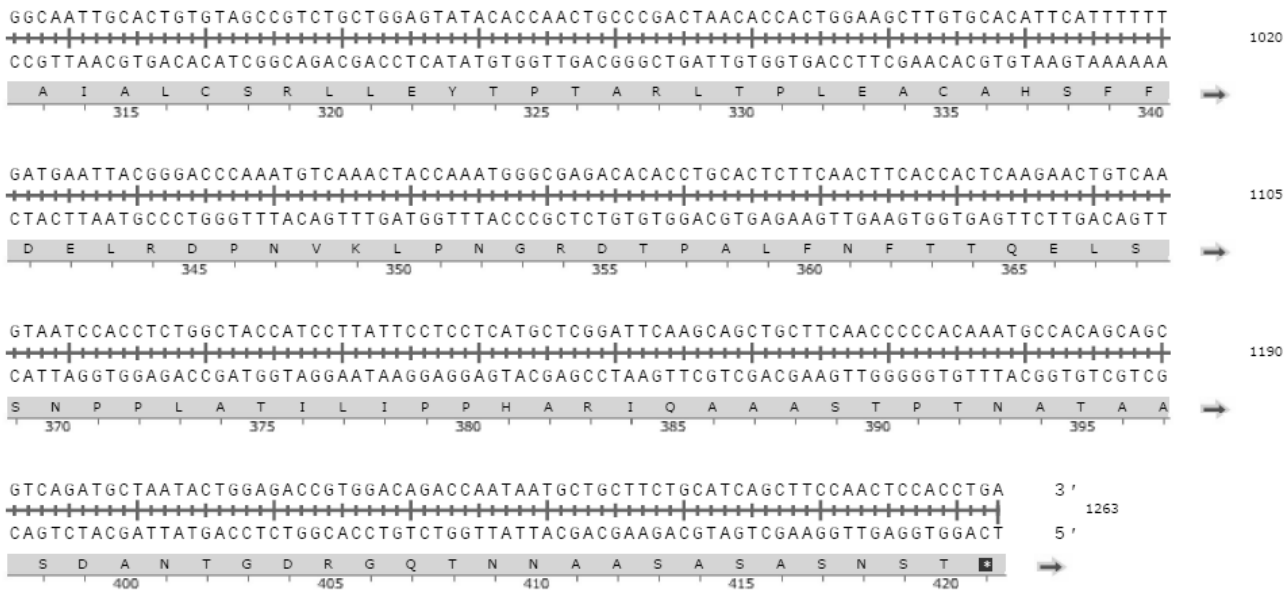
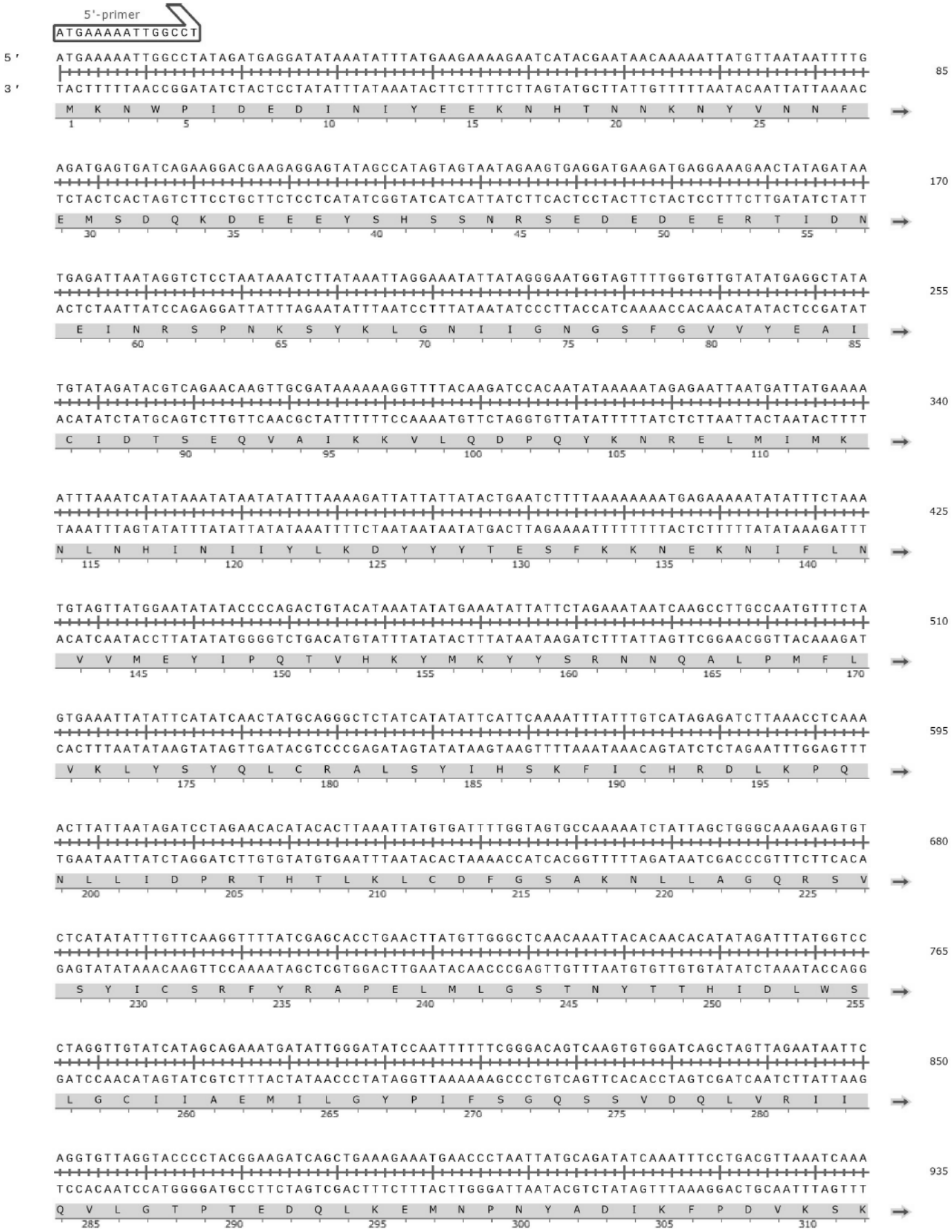


Figure A. Full-length open reading frame of the *Hs*GSK-3β gene (1263 bp, Ensembl Genome ID: ENST00000264235.12) and the translated protein sequence (420 aa).

2.2. *Pf*GSK-3 gene and protein sequences



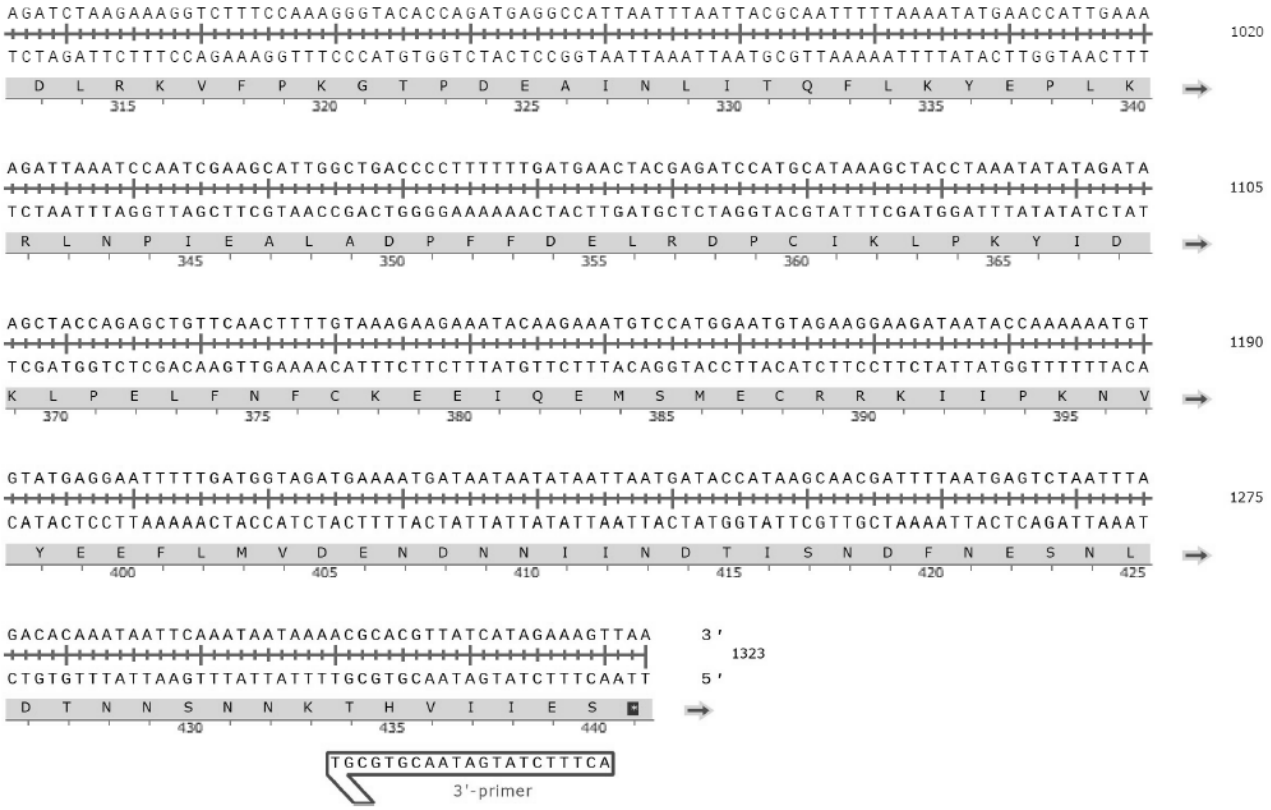


Figure B. Full-length open reading frame of the *Pf*GSK-3 gene (1323 bp, Ensembl Genome ID: PF3D7_0312400) and the translated protein sequence (440 aa). The forward (5') and reverse (3') primers used for PCR amplification of the *Pf*GSK-3 gene are also indicated.

2.3. Optimisation of *Pf*GSK-3 purification and enzyme storage

2.3.1. Protease inhibitors

Droucheau *et al.* (2004:189) reported possible proteolytic degradation of *Pf*GSK-3. Therefore, protease inhibitors were added to the extraction agent (1x Complete Ultra Protease Inhibitor Mini Tablet dissolved in 10 ml B-PER™ Reagent). However, SDS-PAGE analysis showed that the number of proteins that co-elute with *Pf*GSK-3 remain the same with or without added protease inhibitors. As a precautionary measure, protease inhibitors were still added for all subsequent *Pf*GSK-3 extractions.

2.3.2. Protino® Ni-TED column wash protocol

The wash protocol recommended by the Protino® Ni-TED column supplier, was optimised to remove the co-eluting proteins prior to eluting the *Pf*GSK-3 protein. After loading the soluble fraction onto the Protino® Ni-TED column, the column was washed successively with elution buffer containing different concentrations of imidazole (1, 2, 3, 4, 5, 10, 20, 50 and 250 mM). The flow-through from each wash step was collected separately for SDS-PAGE analysis. Imidazole concentrations of 1-5 mM removed some of the co-eluting proteins while still retaining the *Pf*GSK-3 protein on the column. At 10 mM imidazole the *Pf*GSK-3 protein started to elute from the column. Based on these observations, the wash protocol for *Pf*GSK-3 was adapted to include three wash steps with 5% imidazole.

2.3.3. Amicon® Ultra-15 centrifugal device

The Amicon® Ultra-15 centrifugal device was used as a second method of purification in order to remove the co-eluting proteins (<50 kDa) that still remained in the sample after Protino® Ni-TED column purification. The centrifugation time was optimised to ensure that the *Pf*GSK-3 protein was retained in the concentrate. The elution fraction from the Protino® Ni-TED column was divided into five aliquots and each aliquot was diluted with LEW buffer to a final volume of 12 ml (recommended volume for use with fixed-angle rotors). Each 12 ml sample was loaded onto a separate Amicon® Ultra-15 centrifugal device and centrifuged (5000 x g, 4 °C) for either 10, 15, 20, 40 or 60 min. SDS-PAGE analysis of the samples indicated that the *Pf*GSK-3 protein remained in the concentrate regardless of centrifugation time. The co-eluting proteins <50 kDa were no longer present in the concentrate after centrifuging for 10 min. Thus a centrifugation time of 10 min was chosen for subsequent *Pf*GSK-3 purifications.

2.3.4. Storage buffer

Purified *Pf*GSK-3 and *Hs*GSK-3β enzymes (in elution buffer containing 25% glycerol) were snap-frozen in liquid nitrogen and stored at -80 °C. The activity of the frozen enzymes was analysed three days after freezing using the ADP-Glo™ Kinase Assay. The luminescence values obtained for the frozen enzymes were much lower than that of the enzymes before freezing. This indicated that the enzymes were unstable when frozen and

thus required a different storage buffer. Based on the storage buffer used by SignalChem, a commercial supplier of active recombinant *Hs*GSK-3β, the following storage buffer was prepared for both enzymes: 50 mM Tris-HCl pH 8.0 (with added protease inhibitors - Complete Ultra Protease Inhibitor Mini Tablets); 150 mM NaCl; 0.5 mM EDTA; 0.25 mM DTT; 25% glycerol. In addition to purification and concentration, the Amicon® Ultra-15 centrifugal device was also used to exchange the elution buffer with the new storage buffer. Both enzymes remained stable in this storage buffer for all subsequent assays. Stability was confirmed each time a new aliquot of frozen enzyme was used, by including a control reaction (containing enzyme, substrate, ATP and kinase reaction buffer) on the plate.

Reference

Droucheau, E., Primot, A., Thomas, V., Mattei, D., Knockaert, M., Richardson, C., . . . Meijer, L. 2004. Plasmodium falciparum glycogen synthase kinase-3: molecular model, expression, intracellular localisation and selective inhibitors. *Biochimica et Biophysica Acta*, 1697(1-2):181-196. https://doi.org/10.1016/j.bbapap.2003.11.023

2.4. Intra- and inter-assay variation of the ADP-Glo™ Kinase Assay

Intra-assay coefficient of variation (CV) is a measurement of the variance between reaction replicates on the same plate. Generally, an intra-assay CV of less than 10% is considered as acceptable. To assess the intra-assay variation of the ADP-Glo™ Kinase Assay, two different reactions were prepared for each enzyme (Reaction 1 & 2 for *Hs*GSK-3β, and Reaction 3 & 4 for *Pf*GSK-3). Each 20 µl reaction contained the optimal amount of kinase (*Hs*GSK-3β: 8 ng; *Pf*GSK-3: 32 ng), substrate (*Hs*GSK-3β: 0.8 mg/ml; PfGSK-3: 0.6 mg/ml) and 10 µM ATP. Reaction 1 and 3 contained only kinase reaction buffer, while DMSO (1% final concentration) was added to Reaction 2 and 4 (to simulate compound screening conditions). All four reactions were performed in duplicate.

Inter-assay CV is a measurement of the variance between reaction replicates on different plates and is used to assess plate-to-plate consistency. Generally, an inter-assay CV of less than 15% is considered as acceptable. The inter-assay CV of the ADP-Glo™ Kinase Assay was assessed by preforming the same four reactions (in duplicate) on three consecutive days.

Based on the results (Table A), the ADP-Glo™ Kinase Assay demonstrated acceptable intra- (<10%) and inter-assay (<15%) variation.

Table A. Intra- and inter-assay variation for *Hs*GSK-3β and *Pf*GSK-3 reactions using the ADP-Glo™ Kinase Assay.

Reaction	**Recombinant enzyme**	**Intra-assay variation**			**Inter-assay variation**		
		Mean of Luminescence (n=2)[a]	**SD**	**CV** (%)	**Mean of Luminescence** (n=6)[b]	**SD**	**CV** (%)
1	*Hs*GSK-3β	3383.5	47.4	1.4	3223.8	223.0	6.9
2		1358.5	51.6	3.8	1322.5	126.4	9.6
3	*Pf*GSK-3	6204.5	207.2	3.3	6194.3	370.4	6.0
4		3226.0	113.1	3.5	4130.3	327.5	7.9

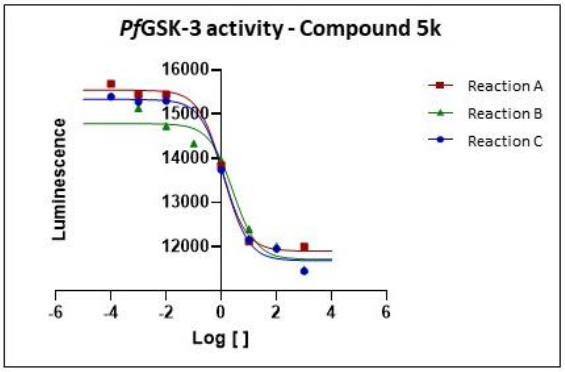
[a] Mean value calculated from duplicate reactions.

[b] Mean value of duplicate reactions from 3 different assays (i.e. 6 reactions in total).

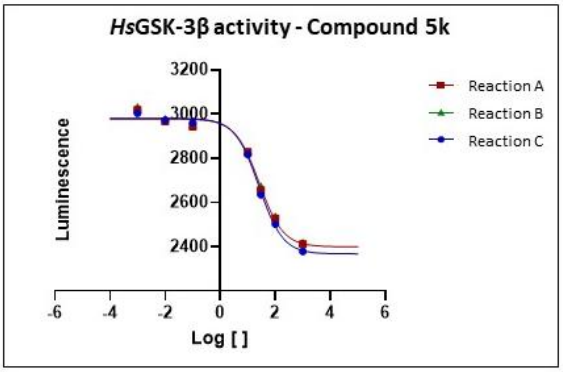
SD: Standard deviation; CV: Coefficient of variation.

2.5. Dose-response curves

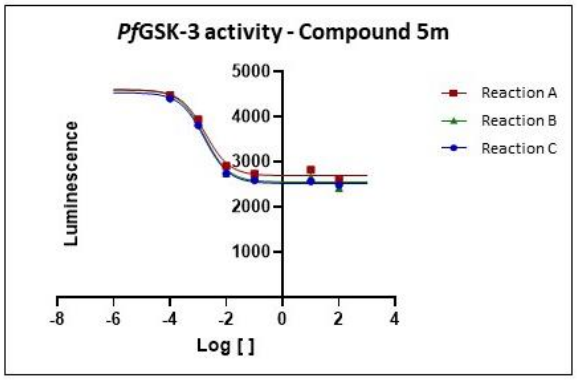
Dose-response curves (luminescence value vs logarithm of inhibitor concentration) were performed for all compounds that demonstrated inhibitory activity at 10 µM. Each reaction was done in triplicate (Reaction A, B and C) and the IC_{50} values were calculated using nonlinear regression (Graphpad Prism Software). The IC_{50} values are expressed as the mean of the three reactions (µM) ± the standard deviation (SD).



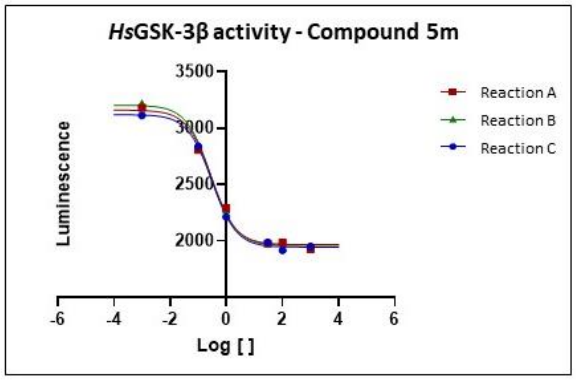
Reaction	A	B	C	Average	SD
IC50 (µM)	1,333	1,081	2,663	**1,692**	**0,850**



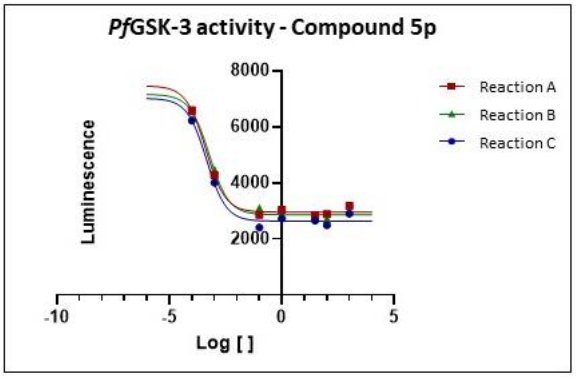
Reaction	A	B	C	Average	SD
IC50 (µM)	25,410	25,980	26,060	**25,817**	**0,354**



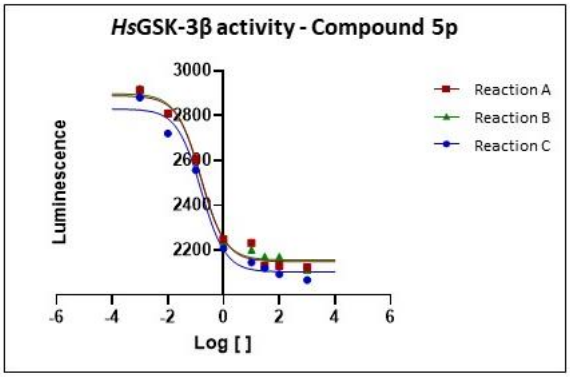
Reaction	A	B	C	Average	SD
IC50 (µM)	0,00166	0,00173	0,00165	**0,00168**	**0,00005**



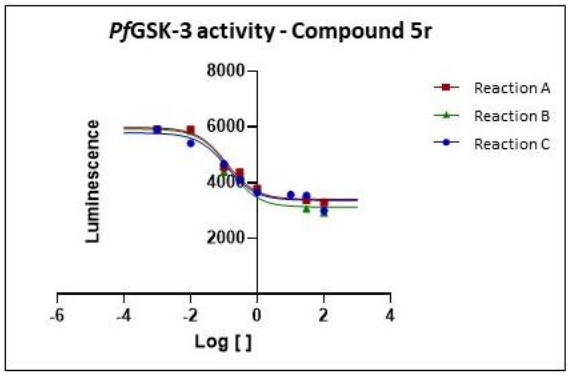
Reaction	A	B	C	Average	SD
IC50 (µM)	0,308	0,295	0,279	**0,294**	**0,014**



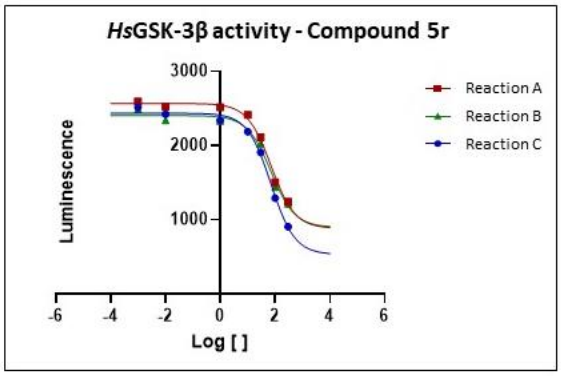
Reaction	A	B	C	Average	SD
IC50 (µM)	0,00045	0,00042	0,00060	**0,00049**	**0,00010**



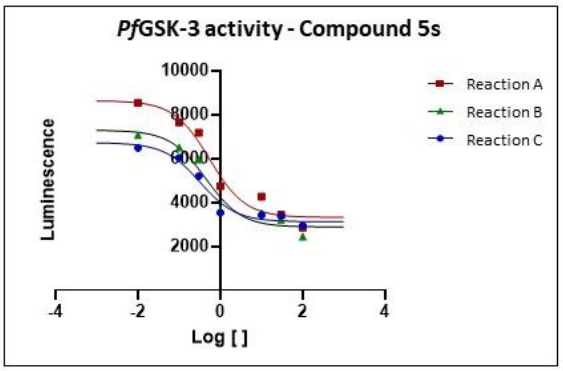
Reaction	A	B	C	Average	SD
IC50 (µM)	0,158	0,159	0,149	**0,155**	**0,005**



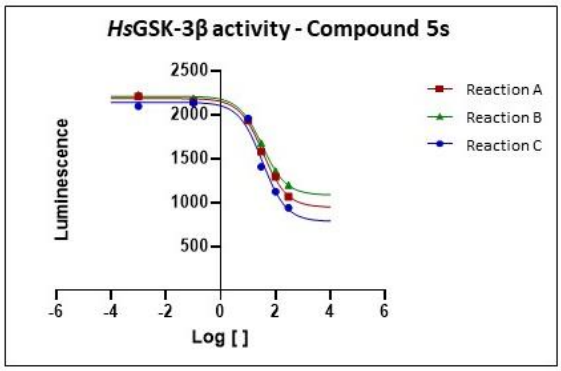
Reaction	A	B	C	Average	SD
IC50 (µM)	0,120	0,122	0,138	**0,127**	**0,010**



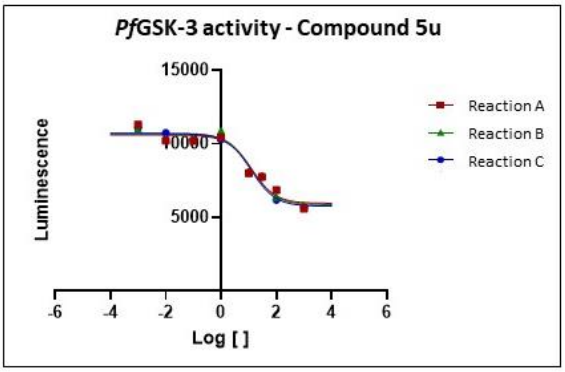
Reaction	A	B	C	Average	SD
IC50 (µM)	70,850	70,560	70,580	**70,997**	**0,506**



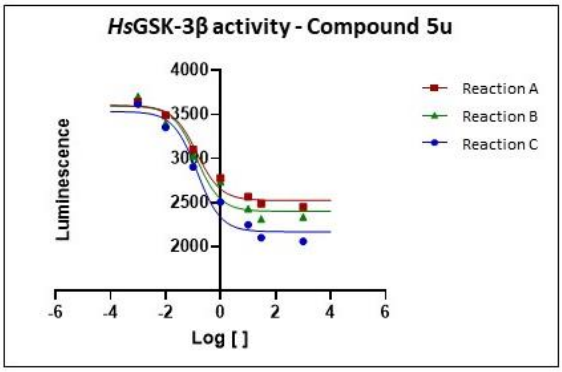
Reaction	A	B	C	Average	SD
IC50 (µM)	0,324	0,550	0,447	**0,440**	**0,113**



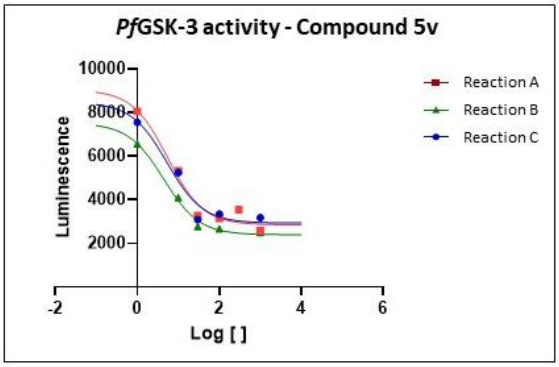
Reaction	A	B	C	Average	SD
IC50 (µM)	33,700	33,120	33,060	**33,960**	**1,054**



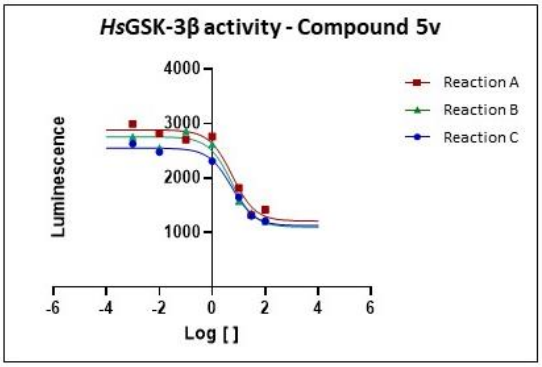
Reaction	A	B	C	Average	SD
IC50 (µM)	12,190	12,600	12,890	**12,560**	**0,352**



Reaction	A	B	C	Average	SD
IC50 (µM)	0,139	0,137	0,134	**0,137**	**0,002**



Reaction	A	B	C	Average	SD
IC50 (µM)	5,377	5,496	4,437	**5,103**	**0,580**



Reaction	A	B	C	Average	SD
IC50 (µM)	5,396	5,639	5,311	**5,449**	**0,170**

Annexure D: Poster Presentation

Work from this study was presented as a poster at the SASBMB-FASBMB Conference, Potchefstroom, 8-11 July 2018.

Exploration of benzofuran and benzothiophene derivatives as potent & selective *Pf*GSK-3 inhibitors: New antimalarial agents

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[b] Centre for Human Metabolomics, Biochemistry, North-West University, Potchefstroom, South Africa

Introduction

- Widespread multi-drug resistant malaria has created an urgent need to develop additional antimalarial agents with novel modes of action.
- *Plasmodium falciparum* glycogen synthase kinase-3 (*Pf*GSK-3) has been identified as a potential therapeutic target, as it is essential for survival of the parasite during its erythrocytic stages [1].
- Development of new *PfGSK-3* selective inhibitors are challenging due to limited availability of previous work done on this target [2,3], absence of the *Pf*GSK-3 crystal structure [4], and the high degree of structural similarity of *Pf*GSK-3 with its human orthologue (*Hs*GSK-3β) [2].
- *In vitro* antimalarial activity of chalcone-based compounds, has been demonstrated previously [3].
- The goal of this study is to explore the potential of other chalcone-like scaffolds for the design of potent and selective *Pf*GSK-3 inhibitors.

Methods

- A series of 2-aroylbenzofuran, 3-substituted 2-aroylbenzofuran and 3-substituted 2-aroylbenzothiophene derivatives were synthesized and characterized through NMR, HPLC, MS, and IR analyses.

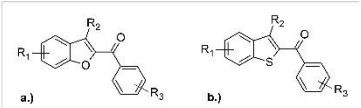

Figure 1: a.) Benzofuran; and **b.)** benzothiophene scaffolds substituted with a variety of functional groups (R1, R2 and R3).

- *Hs*GSK-3β and *Pf*GSK-3 reference sequences were synthesized by GeneArt (ThermoFisher Scientific).
- The *Hs*GSK-3β gene was subcloned into the pET32a(+) plasmid and transformed into Origami cells that already contained the pGro7 chaperone plasmid.
- Expression of the pGro7 chaperone was initially induced using arabinose, followed by *Hs*GSK-3β expression induced with IPTG.
- Recombinant *Hs*GSK-3β was expressed as a His-tag fusion protein, thus immobilized metal ion affinity chromatography (Protino® Ni-TED columns, Macherey-Nagel) was used for purification.
- The *Pf*GSK-3 gene was cloned into the pBAD/Thio-TOPO plasmid and transformed into One Shot™ TOP10 cells.

Results

Expression of recombinant *Hs*GSK-3β

- The *Hs*GSK-3β protein was successfully co-expressed with the pGro7 chaperone in the soluble fraction.
- The *Hs*GSK-3β protein and the GroEL subunit of pGro7 are roughly the same size (64kDa and 60kDa, respectively) and resulted in one prominent band seen on the SDS-PAGE gel (Figure 2).

References

[1] Sulyakov, L., Halbert, J., Alam, M.M., Serrano, J.P., Dorin Semblat, D., Reininger, L., Bollrill, A.R., Misty, S., Abd, A., Fennell, C., Holland, Z., Demarta, C.; Bouza, Y; Sicard, A.; Nivez, M.P.; Eschenbuer, S.; Lama, T.; Thomas, D.C.; Sharma, P; Agarwal, S.; Kern, S.; Pradel, G.; Graciotti, M.; Tobin, A.B. & Doerig, C. 2011. Global kinomic and phospho-proteomic analyses of the human malaria parasite *Plasmodium falciparum*. *Nature communications*. 2(355):1-12.
[2] Doerig, C. & Meijer, L. 2004. *Plasmodium falciparum* glycogen synthase kinase-3: molecular model, expression, intracellular localisation and selective inhibitors. *Biochimica biophysica acta*. 1697(1-2):191-96.
[3] Fugol, W.; Oberholzer, A.E.; Gschloessl, B.; Dzikowski, R.; Pressburger, N.; Prou, L.; Pearl, L.H.; Berdo, B.; Ratin, M.; Okun, I.; Doerig, C.; Kruggel, S.; Lemcke, T.; Meijer, L. & Kunick, C. 2013. 3,6-Dianino-4-(2-halophenyl)-2-benzoylthieno[2,3-b]pyridine-5-carbonitriles are selective inhibitors of *Plasmodium falciparum* glycogen synthase kinase-3. *Journal of medicinal chemistry*. 56(1):264-275.
[4] Rafiuddeen Aher, R. & Roy, K. 2015. First report on exploring classification and regression based QSAR modeling of Plasmodium falciparum glycogen synthase kinase (PfGSK-3) inhibitors. *SAR & QSAR in environmental research*. 26(11):959-976.

Results

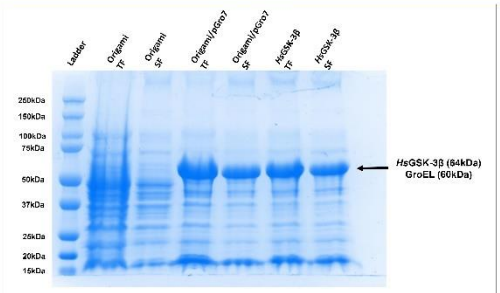

Figure 2: 10% SDS-PAGE analysis of the *Hs*GSK-3β protein, co-expressed with the pGro7 chaperone (GroEL and GroES proteins). The Origami TF and SF served as negative controls, and the Origami/pGro7 TF and SF served as positive controls. TF = Total Fraction; SF = Soluble Fraction.

Purification of recombinant *Hs*GSK-3β

- The *Hs*GSK-3β-His-tag fusion protein was successfully purified using immobilized metal ion affinity chromatography (Figure 3).

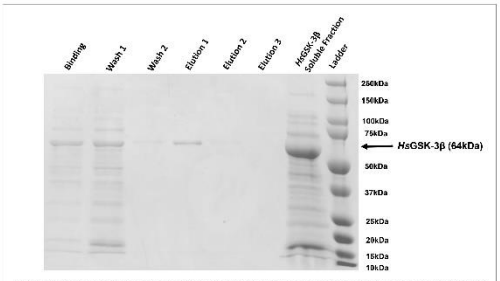

Figure 3: 10% SDS-PAGE analysis of the different fractions collected during purification of the recombinant GSK-3β protein. The *Hs*GSK-3β soluble fraction was loaded onto a Protino® Ni-TED column. The flow-through from the binding, washing, and elution steps were collected. The purified *Hs*GSK-3β protein was collected during the first elution.

Discussion & Future Research

- 2-Aroylbenzofurans (22 compounds), 3-substituted 2-aroylbenzofurans (20 compounds) and 3-substituted 2-aroylbenzothiophenes (14 compounds) were synthesized with acceptable purity (≥ 95%).
- The recombinant *Hs*GSK-3β was successfully expressed in the soluble fraction, and purified.
- Once the *Pf*GSK-3 protein has been expressed and purified, enzymatic activity of the two proteins will be evaluated using a luciferase-based kinase assay (ADP-Glo™ Kinase Assay, Promega).
- The synthesized compounds will be screened against the two enzymes, generating dose-response curves and IC_{50} values.
- Molecular docking studies will be carried out on all *Pf*GSK-3 selective compounds.
- This will provide invaluable insight into the structural features that are essential for potent and selective *Pf*GSK-3 inhibition.

Funding

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