



Design, synthesis, and evaluation of chalcone derivatives as antibacterial agents and monoamine oxidase inhibitors

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PREFACE

The PhD thesis presented in this document is submitted in article format. The first article (chapter 3) has been submitted and published in the journal “Results in Chemistry”. The second article (chapter 4) has been submitted for publication in the journal “Bioorganic & Medicinal Chemistry Letters” in letter format. The third article (chapter 5) has been reported in this thesis, however, it has not been submitted for publication.

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LETTER OF PERMISSION



25 November 2024

To whom it may concern.

CO-AUTHORSHIP OF RESEARCH ARTICLE

The undersigned are co-authors of the research articles listed and hereby give permission to Me. Alize Hoepfner to submit this article as part of the degree Doctor of Philosophy in Pharmaceutical Chemistry at the North-West University (NWU) Potchefstroom campus:

"*In vitro* and *in silico* antibacterial evaluation of nitrocatechol chalcone and pyrazoline derivatives"

"Guaiacol-chalcone and nitroguaiacol-chalcone derivatives: *In vitro* and *in silico* antibacterial evaluation"

Sincerely,



Dr Theunis Cloete



Prof. Anél Petzer



Prof. Jacques Petzer

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ABSTRACT

Antibiotics have been successfully used to treat bacterial infections since the discovery of penicillin in the early 20th century. However, the misuse of these compounds led to the widespread development of antimicrobial resistance in bacterial strains resulting in a drastic increase in morbidity and mortality. This combined with the decrease in the discovery and development of novel antibiotics emphasises the need for novel antibiotic lead compounds.

Drug repurposing is a drug discovering technique which entails identifying novel indications for drugs that were originally designed for a different indication. Globally, Parkinson's disease (PD) is the second most common neurodegenerative disorder with a rising incidence mostly attributed to the increase in aging populations and industrialisation. Levodopa is the first-choice treatment for patients with PD, although its efficacy decreases as the disease progresses with patients commonly experiencing wearing-off periods. It is evident that more novel avenues of symptomatic treatment are needed.

Chalcones are simple natural chemical scaffolds with various biological effects including antibacterial and anti-Parkinson activities. Nitrocatechol-chalcones have previously been shown to target not only catechol *O*-methyl transferase (COMT), but also monoamine oxidase (MAO), both targets for neurodegenerative diseases. Furthermore, chalcone-containing compounds have been shown to have antibacterial activity against a wide variety of bacteria, however, there is no known antibacterial target.

This study aimed to determine whether chalcone-containing compounds with known MAO activity possess antibacterial properties, starting with the screening of these derivatives against *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* using the broth microdilution method. The *in vitro* data were utilised to create and validate a common feature pharmacophore model by determining the rank score, enrichment factor (EF), and area under the receiver operating characteristic – area under the curve (ROC-AUC). The pharmacophore model aided in the design of novel compounds of which the antibacterial activity was determined against the relevant pathogens. A checkerboard assay was performed to indicate a possible antibacterial mode of action. Lastly, the novel derivatives were screened for activity against MAO-A and MAO-B and their mode of inhibition was determined via Lineweaver-Burke plots.

In the first article for this study, it was found that previously synthesised nitrocatechol-chalcone and nitrocatechol-pyrazoline derivatives with activity against MAO-A, MAO-B and/or COMT showed activity against *S. aureus* with compounds **2a**, **2b** and **1b** (1 - 2 µg/ml)

(article 1) having activity comparable to that of tetracycline. A common feature pharmacophore model was created (rank score: 84.02, maximum fit value: 4), which was able to identify active chalcone and pyrazoline derivatives out of a test set ($EF^{10\%}$: 6.7, ROC-AUC: 0.85). The pharmacophore model highlighted that four hydrogen bond acceptors are important for antibacterial activity. Utilising the pharmacophore data, novel nitrocatechol-chalcone and pyrazoline derivatives were synthesised after which the minimum inhibitory concentration (MIC) of each derivative was determined against both the drug-susceptible and resistant *S. aureus* (MRSA) strains. The most active compounds were **3i** (0.5 µg/ml) and **3c** (0.5 µg/ml) (article 1) against the susceptible and resistant strains respectively, which were more active than tetracycline. The most active pyrazoline derivatives were **4a** and **4c** (article 1), with activities of 16 µg/ml and 32 µg/ml against the susceptible and resistant strains, respectively.

In the second article for this study, novel guaiacol-chalcone and nitroguaiacol-chalcone derivatives were synthesised and their antibacterial activity determined against *S. aureus*, MRSA, *K. pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *K. aerogenes*. The most active derivative was nitroguaiacol-chalcone derivative **2c** (8 µg/ml) (article 2), with activity against the susceptible *S. aureus* and MRSA strains. The checkerboard assay demonstrated that the nitroguaiacol-chalcone derivative (**2a**) (article 2) displayed a synergistic effect when combined with chloramphenicol, yielding a fractional inhibitory concentration index (FICI) of 0.45 ± 0.09 . This finding suggests that the antibacterial mechanism of action of **2a** may also involve targeting bacterial protein synthesis. A pharmacophore model was developed and validated (max. fit: 4, rank score: 52.34, $EF^{10\%}$: 8, ROC-AUC: 0.95 ± 0.02). It indicated four features that are important for antibacterial activity *i.e.*, ring aromatic, a hydrophobic moiety and two hydrogen bond acceptors.

In the third article of this study, the novel chalcone and pyrazoline derivatives synthesised for both articles 1 and 2 were evaluated for MAO inhibition by determining their half-maximal inhibitory concentration (IC_{50}) values. Overall, the chalcone derivatives were more potent inhibitors of MAO-B compared to MAO-A and the pyrazoline derivatives had comparable MAO-A and MAO-B activity. The guaiacol-chalcone derivative, **3c** (article 2) had the most potent inhibition against MAO-B with an IC_{50} of 0.06 ± 0.01 µM, which is more potent than the reference inhibitor safinamide ($IC_{50} = 0.12 \pm 0.02$ µM), however, showed less potent inhibition against MAO-A with an IC_{50} of 0.49 ± 0.03 µM. The guaiacol-chalcone derivatives, **3b** and **3f** (article 2) exhibited inhibitory action comparable to safinamide with an IC_{50} -value

of $0.12 \pm 0.01 \mu\text{M}$ against MAO-B. The Lineweaver-Burk graphs indicated that compound **3c** (article 2) competitively inhibited both MAO-A and MAO-B. A pharmacophore model was developed and subsequently validated, showing a maximum fit of 4, a rank score of 62.24, $\text{EF}^{10\%}$ of 7.5, and a ROC-AUC of 1.00 ± 0.002 . This pharmacophore model suggested that two hydrophobic features, along with one hydrogen bond donor and one hydrogen bond acceptor, are important for MAO inhibitory activity.

Keywords: Chalcones; Monoamine oxidase; Antibacterial activity; Common feature pharmacophore model; Checkerboard assay; Antibacterial mode of action; Lineweaver-Burk plots; Minimum inhibitory concentration.

OPSOMMING

Sedert die ontdekking van penisillien in die vroeë 20ste eeu, is antibiotika suksesvol gebruik om bakteriële infeksies te behandel. Die misbruik van hierdie verbindings het egter gelei tot die wydverspreide ontwikkeling van antimikrobiële weerstand in bakteriële stamme en gevolglik tot die drastiese toename in morbiditeit en mortaliteit. Gekombineer met die afname in die ontdekking en ontwikkeling van nuwe antibiotika, is daar duidelik 'n behoefte vir nuwe antibiotiese leidraadverbindinge.

Geneesmiddel hergebruik is 'n geneesmiddel-ontdekkingstegniek wat die identifisering van nuwe indikasies vir medisyne wat oorspronklik vir 'n ander indikasie ontwerp is behels. Parkinson se siekte is wêreldwyd die tweede mees algemene neurodegeneratiewe versteuring, met 'n verhoogde prevalensie wat meestal toegeskryf kan word aan die toename in verouderde bevolkings en industrialisasie. Levodopa is die eerstekeusebehandeling vir pasiënte met Parkinson se siekte, alhoewel die doeltreffendheid daarvan afneem na verlengde gebruik met pasiënte wat sporadiese terugval episodes ervaar. Dit is duidelik dat nuwe benaderings tot simptomatiese behandeling nodig is.

Chalkone is eenvoudige natuurlike chemiese steiers met verskeie biologiese effekte, insluitend antibakteriële en anti-Parkinson aktiwiteite. Daar is voorheen getoon dat nitrokatechol-chalkone nie net katekol O-metieltransferase (KOMT) teiken nie, maar ook monoamienoksidase (MAO), beide teikens vir neurodegeneratiewe siektes. Verder is getoon dat chalkoon-bevattende verbindings antibakteriese aktiwiteit teen 'n wye verskeidenheid bakterieë het, maar daar is geen bekende antibakteriese teiken nie.

Die doel van hierdie studie was om vas te stel of chalkoon-bevattende verbindings met bekende MAO-aktiwiteit ook antibakteriese eienskappe besit, deur eerstens hierdie derivate te toets teen *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* en *Staphylococcus aureus* deur gebruik te maak van die sop-mikroverdunningsmetode. Die *in vitro* data is gebruik om 'n algemene-kenmerk-farmakofoormodel te skep en te valideer deur die rangtelling, verrykingsfaktor (VF) en area onder die ontvanger operateurskenmerk oppervlak-onder-die-kromme (OOK-OOK) te bepaal. Die farmakofoormodel het gehelp met die ontwerp van nuwe verbindings waarvan die antibakteriese aktiwiteit teen die relevante patogene bepaal is. 'n Skaakbordtoets is uitgevoer om 'n moontlike antibakteriese werkingswyse aan te dui. Laastens is die nuwe derivate getoets vir aktiwiteit teen MAO-A en MAO-B en hul meganisme van inhibisie is bepaal met Lineweaver-Burk grafieke.

In die eerste artikel van hierdie studie is gevind dat voorheen gesintetiseerde nitrokatekol-chalkoon en nitrokatekol-pirazolien derivate met aktiwiteit teen MAO-A, MAO-B en/of KOMT, ook aktiwiteit teen *S. aureus* gehad het en verbindings **2a**, **2b** en **1b** (1 - 2 µg/ml) (artikel 1) het aktiwiteit vergelykbaar met dié van tetrasiklien gehad. 'n Algemene-kenmerk-farmakofoormodel is geskep (rang telling: 84.02, maksimum paswaarde: 4), wat in staat was om aktiewe chalkoon en pirazolien derivate uit 'n toetsstel te identifiseer (VF^{10%}: 6.7, OOK-LOOK: 0.85). Die farmakofoormodel het uitgelig dat vier waterstofbinding-ontvangers belangrik is vir antibakteriese aktiwiteit. Nuwe nitrokatekol-chalkoon en pirazolien derivate is toe gesintetiseer deur gebruik te maak van die farmakofoor data, waarna die minimum inhiberende konsentrasie (MIK) van elke derivaat bepaal is teen beide die geneesmiddel vatbare en weerstandbiedende *S. aureus* (MRSA) stamme. Die mees aktiewe verbindings was **3i** (0.5 µg/ml) en **3c** (0.5 µg/ml) (artikel 1) teen onderskeidelik die vatbare en weerstandbiedende stamme, en was meer aktief as tetrasiklien. Die mees aktiewe pirazolien derivate was **4a** en **4c** (artikel 1), met aktiwiteite van onderskeidelik 16 µg/ml en 32 µg/ml teen die vatbare en weerstandbiedende stamme.

In die tweede artikel van hierdie studie is nuwe guaiakol-chalkoon en nitroguaiakol-chalkoon derivate gesintetiseer en hul antibakteriese aktiwiteit is teen *S. aureus*, MRSA, *K. pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* en *K. aerogenes* bepaal. Die mees aktiewe derivaat was nitroguaiakol-chalcone derivaat **2c** (8 µg/ml) (artikel 2), met aktiwiteit teen die vatbare *S. aureus* en MRSA-stamme. Die skaakbordtoets het getoon dat die nitroguaiakol-chalkoon-derivaat (**2a**) (artikel 2) 'n sinergistiese effek vertoon wanneer dit gekombineer word met chlooramfenikol, wat 'n fraksionele inhiberende konsentrasie-indeks (FIKI) van 0.45 ± 0.09 het. Hierdie bevinding dui daarop dat die antibakteriese werkingsmeganisme van **2a** ook moontlik bakteriële proteïensintese kan teiken. 'n Farmakofoormodel is ontwikkel en gevalideer (maks. pas: 4, rangtelling: 52.34, VF^{10%}: 8, OOK-LOOK: 0.95 ± 0.02). Dit het aangedui dat vier kenmerke belangrik is vir antibakteriese aktiwiteit, d.w.s. 'n aromatiese ring, 'n hidrofobiese entiteit en twee waterstofbinding-ontvangers.

In die derde artikel van hierdie studie is die nuwe chalkoon- en pirasolienderivate wat vir beide artikels 1 en 2 gesintetiseer is, vir MAO-inhibisie geëvalueer deur hul halfmaksimale inhiberende konsentrasie (IC₅₀) waardes te bepaal. Oor die algemeen was die chalkoon-derivate kragtiger inhibeerders van MAO-B in vergelyking met MAO-A en die pirazolien derivate het vergelykbare MAO-A- en MAO-B-aktiwiteit gehad. Die guaiakol-chalkoon

derivaat, **3c** (artikel 2) het die kragtigste inhibisie teen MAO-B gehad met 'n IC_{50} van $0.06 \pm 0.01 \mu M$, wat sterker is as die verwysingsinhibeerder safinamied ($IC_{50} = 0.12 \pm 0.02 \mu M$). Dit het egter minder kragtige inhibisie teen MAO-A met 'n IC_{50} van $0.49 \pm 0.03 \mu M$ gehad. Die guaiakol-chalkoon derivate, **3b** en **3f** (artikel 2) het inhiberende werking getoon wat vergelykbaar is met safinamied met 'n IC_{50} -waarde van $0.12 \pm 0.01 \mu M$ teenoor MAO-B. Die Lineweaver-Burk grafieke het aangedui dat verbinding **3c** (artikel 2) beide MAO-A en MAO-B kompetierend inhibeer. 'n Farmakofoormodel is ontwikkel en daarna gevalideer, wat 'n maksimum paswaarde van 4, 'n rangtelling van 62.24, $VF^{10\%}$ van 7.5 en 'n OOK-OOK van 1.00 ± 0.002 toon. Hierdie farmakofoormodel dui daarop dat twee hidrofobiese kenmerke, saam met een waterstofbindingskenker en een waterstofbinding-ontvanger, belangrik is vir MAO-inhiberende aktiwiteit.

Sleutelwoorde: Chalkone; Monoamienoksidase; Antibakteriële aktiwiteit; Algemene kenmerk farmakofoormodel; Skaakbordtoets; Antibakteriële werkingswyse; Lineweaver-Burk grafieke; Minimum inhiberende konsentrasie.

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ABBREVIATIONS

Abbreviation	Definition
λ_{max}	Lambda-max
$\mu\text{g/ml}$	Microgram per millilitre
μl	Microliter
μM	Micromolar
A	Hydrogen Bond Acceptors
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
AlCl_3	Aluminium Chloride
AMR	Antimicrobial Resistance
APCI	Atmospheric Pressure Chemical Ionisation
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
AUC	Area Under Curve
BBB	Blood-Brain Barrier
^{13}C	Carbon 13 NMR Spectra
CADD	Computer-Aided Drug Design
CDCL3	Deuterated Chloroform
CFU/ml	Colony Forming Unit per millilitre
CLSI	Clinical and Laboratory Standards Institute
CNS	Central Nervous System
COMT	Catechol O-Methyl Transferase
d	Doublet
dd	Doublet of Doublets
ddd	Doublet of Doublet of Doublets

Abbreviation	Definition
D	Hydrogen Bond Donors
dH ₂ O	Distilled Water
Ddl	D-alanine-D-alanine ligase
DMSO	Dimethyl Sulfoxide
DMSO-d ₆	Deuterated Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DS	Discovery Studio
DSC	Differential Scanning Calorimetry
eDNA	Environmental DNA
EF	Enrichment Factor
ESKAPE	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Enterobacter</i> species
EtOAc	Ethyl Acetate
EtOH	Ethanol
FAD	Flavin Adenine Dinucleotide
FDA	Food and Drug Administration
FICI	Fractional Inhibitory Concentration Index
GABA	Gamma-Aminobutyric Acid
GC	Growth Control
GlcN-6-P	Glucosamine-6-phosphate synthase
h	Hours
H	Hydrophobic
¹ H	Hydrogen 1 NMR Spectra
HH	Hydrazine Hydrate

Abbreviation	Definition
HNO ₃	Nitric Acid
H ₂ O ₂	Hydrogen Peroxide
HOAc	Acetic Acid
HPLC	High-Performance Liquid Chromatography
HR	Hit Rate
HRMS	High-Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
HTS	High-Throughput Screening
Hypos	Hypothesis
IC ₅₀	Half-Maximal Inhibitory Concentration
J	Joule
KCL	Potassium Chloride
K _i	Enzyme Inhibition Constant
KOH	Potassium Hydroxide
LBDD	Ligand-Based Drug Design
L-dopa	L-dihydroxyphenylalanine
m	Multiplet
MAO	Monoamine Oxidase
MaxOmitFeat	Maximum Omitted Feature
MBC	Minimum Bactericidal Concentration
MDR	Multidrug-Resistant
MH ⁺	Positive Ion Mode
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MHz	Megahertz

Abbreviation	Definition
MIC	Minimum Inhibitory Concentration
MOA	Mechanism Of Action
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-Sensitive <i>Staphylococcus aureus</i>
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NO ₂ ⁻	Nitrogen Dioxide
OD ₆₀₀	Optical Density at a wavelength of 600 nm
PD	Parkinson's Disease
ppm	Parts Per Million
Py	Pyridine
QS	Quorum Sensing
QSAR	Quantitative Structural-Activity Relationship
R	Aromatic Rings
RCHO	Relevant Aromatic Aldehyde
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RNAP	RNA polymerase
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
s	Singlet
SAR	Structure-Activity Relationship
SBDD	Structure-Based Drug Design
SNpc	Substantia Nigra pars compacta
SNpr	Substantia Nigra pars reticulata

Abbreviation	Definition
t	Triplet
TB	Tuberculosis
TLC	Thin-Layer Chromatography
U.S.	United States
UV	Ultraviolet
WHO	World Health Organization
XDR	Extensively Drug-Resistant

AFKORTINGS

Afkortings	Definisie
FIKI	Fraksionele Inhiberende Konsentrasie-Indeks
IC ₅₀	Halfmaksimale Inhiberende Konsentrasie
KOMT	Katechol O-Metieltransferase
MAO	Monoamienoksidase
MIK	Minimum Inhiberende Konsentrasie
OOK-OOK	Ontvanger Operateurskenmerk Oppervlak-Onder-Die-Kromme
VF	Verrykingsfaktor

CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1 Drug discovery

The drug discovery process involves identifying, evaluating and optimising compounds with desired activity for clinical application. Identifying and evaluating a target, most commonly a gene product with predicted pharmacological activity modulation, is the first step in this process. The initial identification of these candidate compounds or leads can involve high-throughput screening (HTS) of small molecule libraries, collections or compounds selected based on their structural significance with predetermined or theoretical activity against the desired target (Verkman, 2004:C465). Other drug discovery methodologies also include the utilisation of natural sources like medicinal plants, computer-aided drug design (CADD) and drug repurposing (Berdigaliyev & Aljofan, 2020:343).

1.1.1.1 High-throughput screening (HTS)

HTS is a process where a large number of compounds are tested for antagonistic or agonistic activity against a specific biological target. This process aims to identify lead compounds or hits that successfully interact with the target at low concentrations (Broach & Thorner, 1996:14). HTS has become a pharmaceutical industry standard in discovering new drug leads for human diseases and is commonly used in the early stage of drug discovery (Berdigaliyev & Aljofan, 2020:343).

1.1.1.2 Computer-aided drug design (CADD)

CADD is an efficient and cost-effective tool in the drug discovery and development process (Macalino *et al.*, 2015:1686). CADD is a computational tool utilised to store, manage, analyse and model compounds (Song *et al.*, 2009:580) to simulate drug-target interactions (Kore *et al.*, 2012:142). This approach can be divided into two main methods: structure-based drug design (SBDD) and ligand-based drug design (LBDD).

SBDD focuses on the interaction between the binding site of the target, *i.e.*, an enzyme or receptor, and an active ligand (Macalino *et al.*, 2015:1687). SBDD methods like molecular docking and *de novo* drug design rely on knowledge of the structure of the target molecule, obtained mainly from nuclear magnetic resonance (NMR), X-ray crystallography or homology models (Ou-Yang *et al.*, 2012:1131). In contrast, LBDD is utilised in the absence of knowledge regarding the target protein structure, however, the structures of active ligands

are known and subsequently serve as the starting point for drug design (Lee *et al.*, 2011:5305). The rationale for LBDD is the assumption that ligands with similar structures will interact with the same target (Peng *et al.*, 2019:10). The LBDD approach is most commonly used to create either common feature pharmacophore models or quantitative structure-activity relationship (QSAR) models that have the potential to identify potentially active compounds (Lee *et al.*, 2011:5305; Tintori *et al.*, 2010:1019).

1.1.1.3 Drug repurposing

Drug repurposing/repositioning is the process of assigning a new indication for existing drugs, other than the original indication it was developed for (Tanoli *et al.*, 2021:1656). Repurposing drugs can reduce the drug discovery time and financial liability significantly as the compounds being considered already have known absorption, distribution, metabolism, excretion and toxicity (ADMET) profiles (Sam & Athri, 2019:299). The drug repurposing approach will commonly use public databases to identify new leads. The data is obtained from primary and translational research, previous clinical trials, off-label uses and human research data (Kulkarni *et al.*, 2023:159). Systematic repurposing approaches can overall be divided into experimental screening and *in silico* strategies that utilise existing data that identify new drug-disease associations (Cha *et al.*, 2018:169).

1.1.2 Parkinson's disease

1.1.2.1 Pathology of Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease characterised by the early death of dopaminergic neurons in the substantia nigra pars compacta (Lew, 2007:155S; Tysnes & Storstein, 2017:901). This neuron death leads to dopamine deficiency in the basal ganglia resulting in a movement disorder that is commonly characterised by Parkinson's related motor symptoms. PD is considered a progressive neurodegenerative disorder involving multiple neuroanatomical areas resulting from a combination of genetic and environmental factors. The gold standard in diagnosing PD is firstly the presence of substantia nigra pars compacta degeneration, characterised by the presence of at least two symptoms, *i.e.*, resting tremor, bradykinesia, rigidity or postural imbalance, and second, the identification of Lewy bodies, which are abnormal aggregates of α -synuclein protein, in post-mortem examinations (Kalia & Lang, 2015:896; De Lau & Breteler, 2006:525). This Lewy pathology involves not only the dopaminergic neurons but also cholinergic, noradrenergic, serotonergic, histaminergic and glutaminergic cells and leads to the wide clinical

presentation of the disease. As Lewy bodies develop and spread through the brain, it was also observed that these aggregates have the ability to spread trans-synaptically through anatomically connected regions of the brain (Williams-Gray & Worth, 2016:543).

1.1.2.2 Treatment of Parkinson's disease

As PD is a multifactorial disease, there is currently no treatment that will cure or stop the progression of the disease and current treatment options focus mainly on the symptomatic treatment of the motor disturbances associated with the disease. The health industry standard is the administration of levodopa, a prodrug of dopamine, with patients showing a good initial response when treated. However, with disease progression, the brain loses its capacity to store dopamine and patients will commonly endure wearing-off symptoms with alternating phases of good and poor responses, on-off symptoms and other motor complications (De Bie *et al.*, 2020:454; Sveinbjornsdottir, 2016:318). Other dopaminergic medications include monoamine oxidase (MAO) type B inhibitors, catechol O-methyl transferase inhibitors (COMT) and dopamine receptor agonists (Jankovic & Stacy, 2007:678; Sveinbjornsdottir, 2016:319).

The enzyme MAO is commonly found in the central nervous system, but also the peripheral tissues and has two isoenzymes, viz., MAO-A and MAO-B (Youdim & Bakhle, 2006:S287). This enzyme is responsible for metabolising biogenic amines resulting in the subsequent inactivation of neurotransmitters (Shih, 2018:1554; Youdim & Bakhle, 2006:S287; Youdim *et al.*, 2006:295). Hydrogen peroxide is often produced as a byproduct during MAO metabolising reactions. It is believed that hydrogen peroxide is an active contributor to oxidative stress and subsequent neural degradation. Neurodegenerative disorders such as PD are often attributed to the increased production of hydrogen peroxide (Berman & Hastings, 1999:1127). Inhibiting MAO will decrease hydrogen peroxide production and subsequently decrease or relieve oxidative stress in the nervous system (Naoi & Maruyama, 2010:2800; Sturza *et al.*, 2019:1).

1.1.3 Antibiotics and antibiotic resistance

1.1.3.1 Antibiotic targets and classification

The United States Food and Drug Administration (FDA) defines an antimicrobial agent, or antibiotic, as a drug that can kill or slow down the growth of a targeted microorganism that causes infections (U.S. FDA, 2023). Antibiotics can be divided into different classes based on their unique mechanism of action and based on their ability to prevent the growth of

bacteria (bacteriostatic) or cause bacterial cell death (bactericidal) (Da Cunha *et al.*, 2019:1; Hancock, 2005:209). The four major classes of antibiotics are cell wall synthesis inhibitors, protein synthesis inhibitors, nucleic acid synthesis inhibitors and inhibitors of metabolism (Dowling *et al.*, 2017:536).

1.1.3.2 Antibiotic resistance

According to the World Health Organization (WHO), antibiotic resistance is one of the top threats to global health with an estimated 4.95 million deaths associated with bacterial antibiotic resistance in 2019. The highest death rate was estimated and recorded for western sub-Saharan Africa at 27.3 deaths per 100,000 people (Murray *et al.*, 2022:629; WHO, 2023). The development of antimicrobial resistance (AMR) can most commonly be attributed to antibiotic overuse, inappropriate prescribing practices, extensive use of antibiotics in agriculture, a decreased availability of novel antibiotics and barriers in antibiotic regulation (Ventola, 2015:278). From a microbiological perspective, resistance can be classified as either intrinsic, caused by inherent genes, or acquired where a novel resistance mechanism developed (Culyba *et al.*, 2015:3573; Davies & Davies, 2010:422; Hasan *et al.*, 2021:6; Reygaert, 2018:485). The WHO classified the ESKAPE pathogens, *i.e.*, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, as priority bacteria for which new antibiotics are urgently needed (WHO, 2017). A review of their clinical and economic impact showed that these bacteria are associated with a high mortality rate, thereby increasing the financial burden of treatment (Mulani *et al.*, 2019:2).

1.1.3.3 Drug discovery and antibiotic resistance

Based on reports from 2017, novel drug development phases I to III were predicted to produce 39 novel antibiotics, however, more research has shown that the present pipeline is insufficient in meeting the current and future clinical needs. Only 13 (33%) of the proposed 39 antibiotics are likely to be registered (Muteeb *et al.*, 2023:10). The main reasons for the lack of antibiotic discovery are threefold: drug approval regulations, market forces and scientific limitations (Wright, 2014:147). Even though the complete bacterial genome was sequenced as early as 1995, scientists have struggled to screen targeted genes and identify novel compounds, therefore the focus shifted to developing compounds that inhibited already validated targets. Discovering compounds safe for human consumption is a difficult process. Furthermore, targeting and destroying Gram-negative bacteria is more challenging than Gram-positive bacteria, because of the presence of a protective outer membrane

(Anderson *et al.*, 2023:4; Miller, 2016:1; Payne *et al.*, 2007:29). Given that natural scaffolds are crucial in drug development, various methods for discovering natural bioactive compounds have garnered significant interest (Baranova *et al.*, 2023:1).

1.1.4 Bioactive compounds

1.1.4.1 Chalcones

Chalcones (Figure 1.1) are naturally occurring flavonoids present in a wide variety of plant species (Bozic *et al.*, 2014:264). These natural compounds have numerous biological activities *e.g.*, antimicrobial, anti-inflammatory, anticancer and antioxidant (Achanta *et al.*, 2006:426; Friis-Møller *et al.*, 2002:416; Janković *et al.*, 2020:1; Zhong *et al.*, 2015:1230). Chalcones are also privileged scaffolds for the development of drugs for the treatment of neurodegenerative disorders (Guglielmi *et al.*, 2020:2). Furthermore, chalcone derivatives have been found to have antimicrobial activity against numerous bacteria such as *Bacillus subtilis*, *Salmonella typhi*, *Aspergillus niger*, and *P. aeruginosa* to name a few (Okolo *et al.*, 2021:21781).

1.1.4.2 Nitro-containing compounds

The catechol moiety is an *ortho*-hydroxylated phenol with proven activity against microorganisms (Cowan, 1999:565). The nitrocatechol moiety (Figure 1.1), contains a nitro-group, and is present in existing PD treatments, *e.g.* tolcapone, entacapone and opicapone. It was found that both tolcapone and entacapone also have antibacterial activity, and both of these drugs can be repurposed as potential antibiotics to treat *Mycobacterium tuberculosis*, where they act on the same target as the tuberculosis drug isoniazid (Maitra *et al.*, 2015:53). Further studies have identified that tolcapone and entacapone can also be utilised to possibly treat both multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) (Kinnings *et al.*, 2009:1). Tolcapone has also been shown to act synergistically in combination with vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA) (Moore *et al.*, 2020:283). A study done on the synthesis and evaluation of nitroguaiacol (Figure 1.1) ether derivatives of the antibiotic streptomycin has reported that these derivatives can be used as streptomycin analogues to treat infections caused by streptomycin-sensitive bacteria (Abad & Amils, 1990:1908). In the current study, guaiacol-chalcone derivatives were also synthesised, in an effort to determine the importance of the nitro-group for activity against MAO and bacteria.

1.1.4.3 Pyrazoline-containing compounds

The pyrazoline moiety (Figure 1.1) is a five-membered nitrogen-containing heterocyclic compound with numerous documented biological activities (Haider *et al.*, 2022:1). This broad-spectrum of biological activities includes anti-inflammatory, antioxidant, antimicrobial, analgesic, anti-diabetic and anticancer (Haider *et al.*, 2022:1; Monga *et al.*, 2014:2020; Nehra *et al.*, 2020:2). Additionally, pyrazoline compounds were reported to have activity against MAO, and thus have the potential to serve as treatment for neurodegenerative disorders such as PD (Gökhan-Kelekçi *et al.*, 2009:675; Karuppasamy *et al.*, 2010:1875).

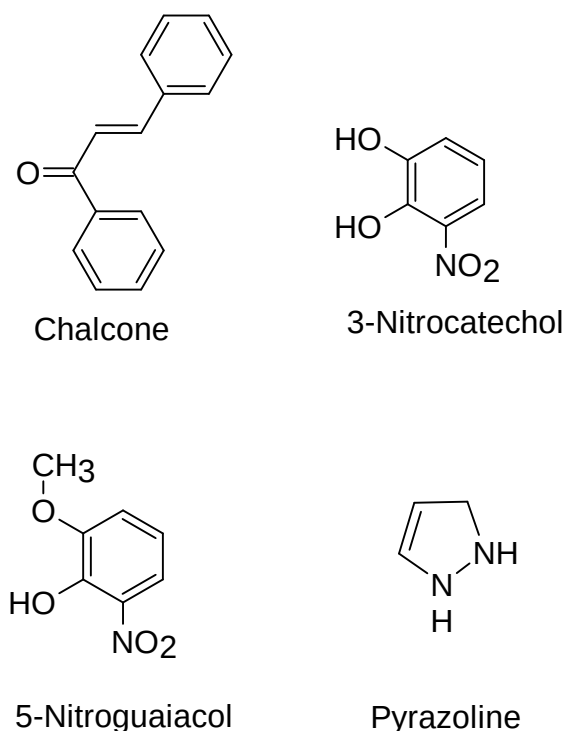


Figure 1.1: Structures of the chalcone, 3-nitrocatechol, 5-nitroguaiacol and pyrazoline moieties.

1.2 Research problem

The use and subsequent misuse of antibiotics have contributed to the development and existence of AMR leading to high morbidity and mortality rates worldwide (Aminov, 2010:4; Frieri *et al.*, 2017:371; Zaman *et al.*, 2017:1). Common bacterial pathogens that cause serious diseases like the ESKAPE pathogens are especially prone to develop AMR (Boucher *et al.*, 2009:1; Carlet *et al.*, 2011:369; Collignon, 2015:1109; De Kraker, Davey, *et al.*, 2011:1; De Kraker, Wolkewitz, *et al.*, 2011:398). As the development pipeline for new antibiotic treatments dwindles, bacteria continue to evolve resistance to existing therapies (Macgowan & Macnaughton, 2017:622). This has created a clear disparity between the high

demand for new antibiotic compounds and the limited supply capable of effectively treating bacterial infections in humans.

PD is the second most common neurodegenerative disease in the world with a global prevalence of 6 million people in 2016 and is one of the leading causes of neurological disability (Dorsey *et al.*, 2018:S3; Ou *et al.*, 2021:2; Tolosa *et al.*, 2021:385). Although PD is non-infectious, Dorsey *et al.* (2018) argue that its current prevalence resembles that of a pandemic. This is due to the rising incidence of PD across all major regions globally, the migration and shifting patterns of the disease in response to ageing populations and industrialisation, and the fact that no one is immune to the condition (Dorsey *et al.*, 2018:S4). It is evident that given the rise in the global prevalence of PD, novel compounds are needed to treat and relieve symptoms related to the disease.

The drug repurposing approach aims to identify new uses for existing compounds. In this study, we aim to use the same concept and determine the antibacterial activity of compounds previously synthesised for MAO inhibition. The repurposing of derivatives with known activity against other conditions, *e.g.*, PD and Alzheimer's disease, may allow the rapid identification of antibacterial lead compounds and subsequent development and synthesis of novel derivatives with more potent activity.

1.3 Aims and objectives

1.3.1 Aim

This study aims to screen previously synthesised nitrocatechol-chalcone and nitrocatechol-pyrazoline derivatives, with proven *in vitro* activity against MAO-A, MAO-B and/or COMT, for antibacterial activity against some of the ESKAPE pathogens. The minimum inhibitory concentration (MIC) will be determined for each lead compound identified during screening. CADD will be utilised to design novel compounds which will subsequently be synthesised, and their antibacterial activity will again be determined. A checkerboard assay will also be conducted to shed light on a possible antibacterial mode of action.

Furthermore, the novel derivatives will be screened for MAO-A and MAO-B activity by determining their half-maximal inhibitory concentration (IC₅₀) values. Lineweaver-Burk plots will be constructed to determine the mode of MAO inhibition. The *in vitro* data will then be utilised to generate a common pharmacophore model.

1.3.2 Objectives

- Screen previously synthesised compounds for activity against some of the ESKAPE pathogens using an adapted broth microdilution method.
- Determine the MIC of the hit compounds identified during screening with the broth microdilution method.
- Use LBDD to design novel antibacterial derivatives.
- Synthesise the novel derivatives and characterise them with nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS), and determine the purity with high-performance liquid chromatography (HPLC).
- Determine the MIC of the novel derivatives with the broth microdilution method.
- Determine the possible antibacterial mode of action of the novel derivatives using the checkerboard assay.
- Evaluate the novel derivatives as inhibitors of MAO by determining their IC_{50} values.
- Determine the mode of inhibition for MAO by constructing Lineweaver-Burk plots.
- Use LBDD to create a pharmacophore model for MAO inhibition.

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CHAPTER 2: LITERATURE STUDY

2.1 Drug discovery

Drug discovery involves the identification and evaluation of a small synthetic molecule or a large biomolecule as a drug candidate (Sinha & Vohora, 2018:19). The drug discovery process (Figure 2.1) commences with target identification, followed by screening method development to assess libraries of natural products or pure compounds against biological materials. A chemical or mixture exhibiting significant activity referred to as the hit, is then identified. This hit/lead compound or mixture is utilised to synthesise similar compounds to improve activity and establish potential structure-activity relationships. Once one or more lead compounds with robust pharmacological activity are discovered, their animal toxicology, pharmacokinetic, and pharmaceutical properties are assessed. Additional similar compounds are frequently synthesised to produce a compound that fulfils all the requirements for clinical development. The clinical candidate undergoes regulatory testing before being approved for clinical trials and subsequent market registration (Salazar & Gormley, 2017:720; Southey & Brunavs, 2023:2).

Drug discovery programs are initiated based on the need for medical products to treat an important disease or clinical condition (Hughes *et al.*, 2011:1239). During the hit identification and lead discovery stage, compound screening assays are developed (Hughes *et al.*, 2011:1242). Lead identification is a crucial step in the drug discovery process, with several computational and experimental techniques being utilised such as high-throughput screening (HTS) and virtual screening *i.e.*, computer-aided drug design (CADD) (Reddy *et al.*, 2007:330; Yi *et al.*, 2023:4187).

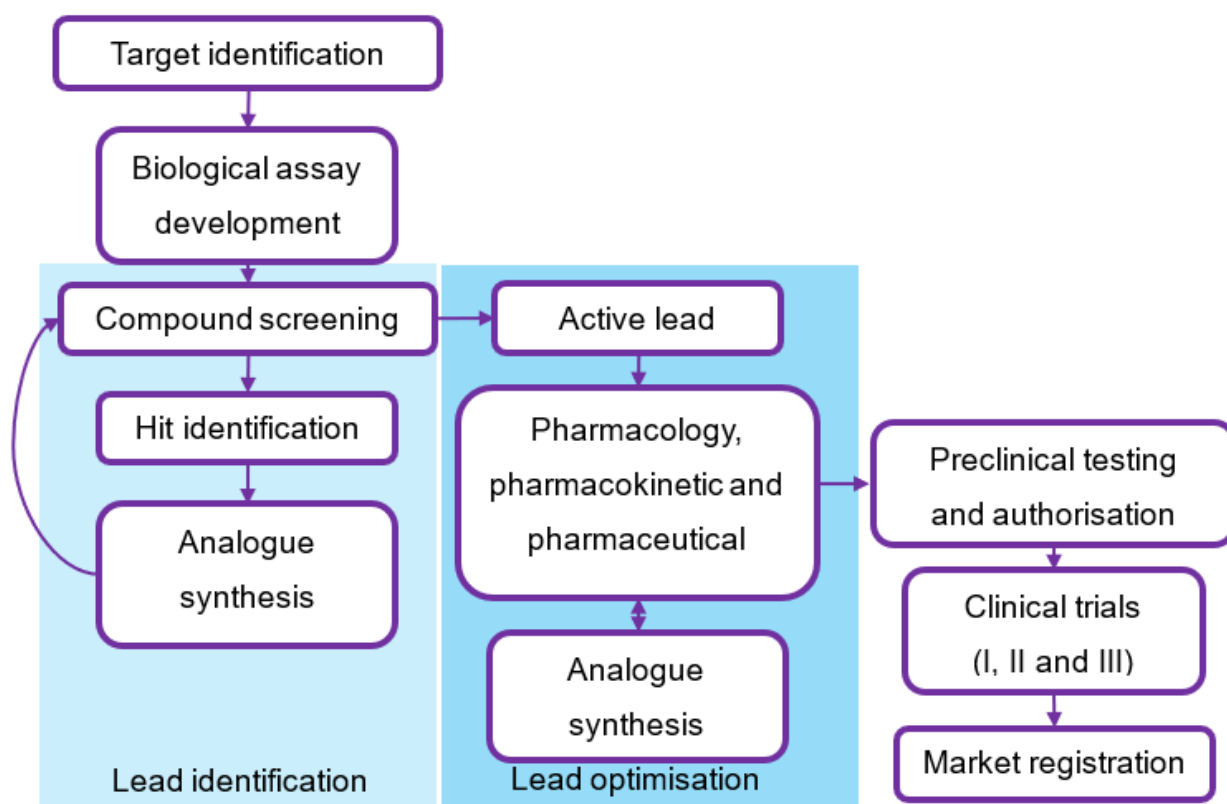


Figure 2.1: The drug discovery process as adapted from Salazar & Gormley (2017:721).

2.1.1 High-throughput screening (HTS)

HTS refers to the screening of a large number of compounds to determine their activity against a biological target to identify active compounds that will be used as candidates in additional biological and pharmacological experiments (An & Tolliday, 2010:180). The goal of HTS is to accelerate the process of drug discovery and can even be utilised to determine and characterise metabolic, pharmacokinetic and toxicological data of novel compounds (Fernandes *et al.*, 2009:342; Martis *et al.*, 2011:2; Szymański *et al.*, 2011:428). A knowledge-based approach involves utilising the knowledge of the target or chemical classes that have activity against the target to select smaller subsets of molecules from chemical libraries (Hughes *et al.*, 2011:1242). HTS has the advantage that limited prior knowledge of the drug compound is required and no drug design is initially involved, however, it is not considered cost-effective as the hit rate is low (Sliwoski *et al.*, 2014:336).

Recently, HTS conducted by Blasco and co-workers was able to identify antibacterial compounds active against multidrug-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Blasco *et al.*, 2024:1).

In this study, the high cost associated with HTS precluded its use and a more targeted approach was used to identify hit compounds with activity against the biological targets. A smaller targeted library of compounds with possible biological activity, supported by prior research, was screened.

2.1.2 Computer-aided drug design (CADD)

CADD leverages computational power to accelerate and enhance the efficiency of drug discovery and development (Kapetanovic, 2008:165). CADD can be utilised to design and select compounds as potential drug candidates before they are synthesised and tested for biological activity (Giordano *et al.*, 2022:1; Song *et al.*, 2009:579). Because drug development is expensive and time-consuming, CADD (Figure 2.2) cannot only decrease the time and costs associated with developing drugs but also minimise final-stage failures (Macalino *et al.*, 2015:1689; Song *et al.*, 2009:579). CADD can be divided into two main methods, *viz.*, structure-based drug design (SBDD) which is used when the three-dimensional target structure is known, and ligand-based drug design (LBDD) which is used when the structures and biological activity of the ligands that interact with the biological target is known (Huang *et al.*, 2010:624).

The process of SBDD starts with target protein structure purification and elucidation through nuclear magnetic resonance (NMR), X-ray crystallography or homology modelling (Kalyaanamoorthy & Chen, 2011:831; Macalino *et al.*, 2015:1687). Once the structure of the biological target is resolved, a computer algorithm is used to position compounds into the target binding site, whereafter the compounds are ranked and scored according to their interactions with the target site. The biological activity of promising compounds identified during SBDD can be determined or the docking/scoring cycle can be repeated with novel compounds in an effort to increase their *in silico* activity before determining their biological activity (Anderson, 2003:787; Singh *et al.*, 2006:316).

Some of the most common methods used in LBDD are common feature pharmacophore modelling and quantitative structural-activity relationship (QSAR) studies (Lee *et al.*, 2011:5305). QSAR studies are a form of mathematical modelling that reveals the relationship between the structural properties of a compound and the observed biological activity (Kwon *et al.*, 2019:1; Verma *et al.*, 2010:95). QSAR modelling involves 4 standard steps: model building, internal test set validation, model selection and finally external test set validation (Kausar & Falcao, 2018:2). Besides virtual screening, pharmacophore modelling is also employed in identifying drug targets, ligand profiling, docking studies and

pharmacological investigation studies (Muhammed & Aki-Yalcin, 2021:752). Common feature pharmacophore models contain features, represented as spheres, organised in a three-dimensional sequence (Qing *et al.*, 2014:83). To successfully generate a pharmacophore model, all identified ligands are divided into a training set which is used to create the pharmacophore, and a test set which is used to validate the pharmacophore model (Qing *et al.*, 2014:84). A pharmacophore is validated to ensure the generated pharmacophore can successfully estimate the activity of new ligands introduced to the model (John *et al.*, 2011:S4). The metrics used to validate the pharmacophore are the enrichment factor (EF) and receiver operating characteristic (ROC) curve (Pascual *et al.*, 2019:5).

As the biological target of the tested compounds in this study is unknown, a ligand-based approach to computer modelling was utilised to design and synthesise novel compounds with desired activity.

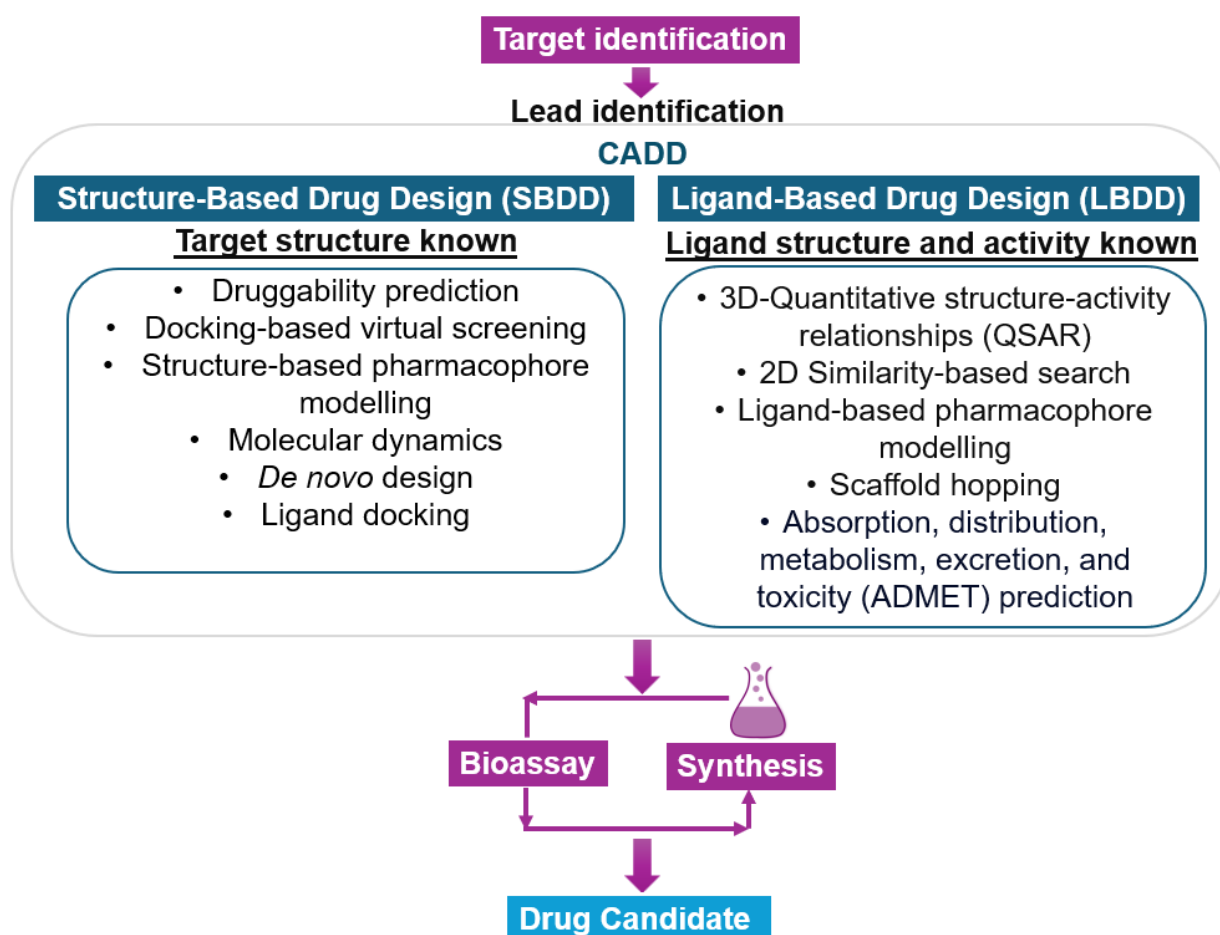


Figure 2.2: The process of CADD in drug discovery and development as adapted from Lu *et al.* (2018:3), Niazi & Mariam (2023:2) and Sliwoski *et al.* (2014:338).

2.1.3 Drug repurposing

Drug repurposing, or drug repositioning, is a modern drug discovery technique that aims to identify novel indications for drugs that have undergone extensive safety and efficacy testing and include approved, discontinued, shelved and experimental drugs (Begley *et al.*, 2021:1; March-Vila *et al.*, 2017:1; Talevi & Bellera, 2020:397). An advantage of drug repurposing is the economic benefit as it represents a cost-effective method compared to novel drug discovery and development, as it offers the best risk versus reward trade-off (Ashburn & Thor, 2004:673; Dinić *et al.*, 2020:1; Parvathaneni *et al.*, 2019:2076). Registering an existing drug for a new indication is quicker than developing a new drug as it advances through preclinical testing and clinical trials more rapidly reducing both risks and costs (Jin & Wong, 2014:637; Kulkarni *et al.*, 2023:157). The rationale behind this technique is two-fold; firstly, various drugs have cryptic biological activities as made evident by their many side-effects; secondly, certain diseases share common molecular pathways or genetic factors (Farha & Brown, 2019:565).

A recent example of drug repurposing is tolcapone, initially approved for treating Parkinson's disease (PD), which has the potential to also be used to combat methicillin-resistant *Staphylococcus aureus* (MRSA) (Moore *et al.*, 2020:283). Although the definition of drug repurposing does not include compounds that have not been clinically investigated (Jourdan *et al.*, 2020:1145), the concept can be expanded to include in-house compound libraries which will help accelerate the lead compound identification.

2.2 Parkinson's disease

2.2.1 Brief history of Parkinson's disease

James Parkinson first described the disease as a neurological syndrome in 1817, however earlier descriptions of rest tremor by Sylvius de la Boë were already documented in 1680 (Goetz, 2011:1). The publication of Parkinson's "An Essay on the Shaking Palsy" began a 200-yearlong study into the disease that would later be known as PD (Toodayan, 2018:384). It was only in 1893 that Georges Marinesco and Paul Blocq suggested the link between what is now known as PD and pathology in the substantia nigra (Hostiuc *et al.*, 2016:1; McDonald *et al.*, 2018:210). In 1895, the French neurological school reported further clinical descriptions and pathological changes related to PD. These studies not only provide the clinical and morphological details associated with the progressive stages of PD but also drawings and statutes to support these findings (Goetz, 2011:2). A complete pathological

analysis of PD was conducted in 1953 by Greenfield and Bosanquet (Goetz, 2011:2; Greenfield & Bosanquet, 1953:213). The deficiency of striatal dopamine in PD was first described in 1960 and led to the implementation of levodopa therapy shortly after in 1961 (Tolosa *et al.*, 1998:S2).

2.2.2 Pathology of Parkinson's disease

PD is a movement disorder and progressive neurodegenerative disease that results in motor problems and mental disabilities (Baweja *et al.*, 2024:1823). The disease is caused primarily by the death of neurons critical to producing dopamine. Dopamine is a neurotransmitter produced mainly in the substantia nigra, but also in the ventral tegmental area and hypothalamus in the brain (Latif *et al.*, 2021:114). This disease is multifunctional with genetic and environmental factors that contribute to the genesis (Ball *et al.*, 2019:1). The most common mechanisms of pathology associated with the development of PD include oxidative stress, mitochondrial damage, genetic mutation, misfolded protein aggregates, protein clearance pathway failure and neuroinflammation (Baweja *et al.*, 2024:1824).

The substantia nigra can be divided into two regions, the pars compacta (SNpc) which contains the dopaminergic neurons and the pars reticulata (SNpr) containing the gamma-aminobutyric acid (GABA) containing neurons (Sonne *et al.*, 2024). In PD the degeneration of the dopaminergic neurons within the substantia nigra SNpc triggers functional changes leading to the whole basal ganglia network being affected. The basal ganglia are responsible for processing signals that flow from the brain cortex and bring forth the execution of voluntary movements (Blandini *et al.*, 2000:63). Dopaminergic neuron loss leads to the presentation of the known clinical motor symptoms used to diagnose PD, *viz.*, resting tremor, rigidity and bradykinesia, with postural instability occurring at a later stage (Figure 2.3) (Kouli *et al.*, 2018:3). The loss of dopaminergic neurons is however not the only pathway involved in PD pathomechanism, as noradrenergic, serotonergic and cholinergic pathways are also associated with PD (Dezsi & Vecsei, 2017:425).

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify the reactive intermediates. There are several pathways capable of generating ROS, such as direct interactions of oxygen species and redox-active metals or direct pathways that activate enzymes such as nitric oxide synthase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, all dependent on the activation of molecular oxygen (Dias *et al.*, 2013:462). Mitochondria are the major site of ROS generation with several in-cell sites where mitochondrial electron

transport chains, in particular complex I and II, take place (Cadenas & Davies, 2000:222; Jin *et al.*, 2014:1283). As the brain consumes 20% of oxygen in the body, a large amount of oxygen can be converted to ROS in the brain. The generation of ROS in neurons and glial mitochondria via the electron transport chain can be attributed to several sources including flavoenzymes like monoamine oxidase (MAO) and nitrogen oxide (Dumont & Beal, 2011:1015; Johnson *et al.*, 2012:1410; Yan *et al.*, 2013:92).

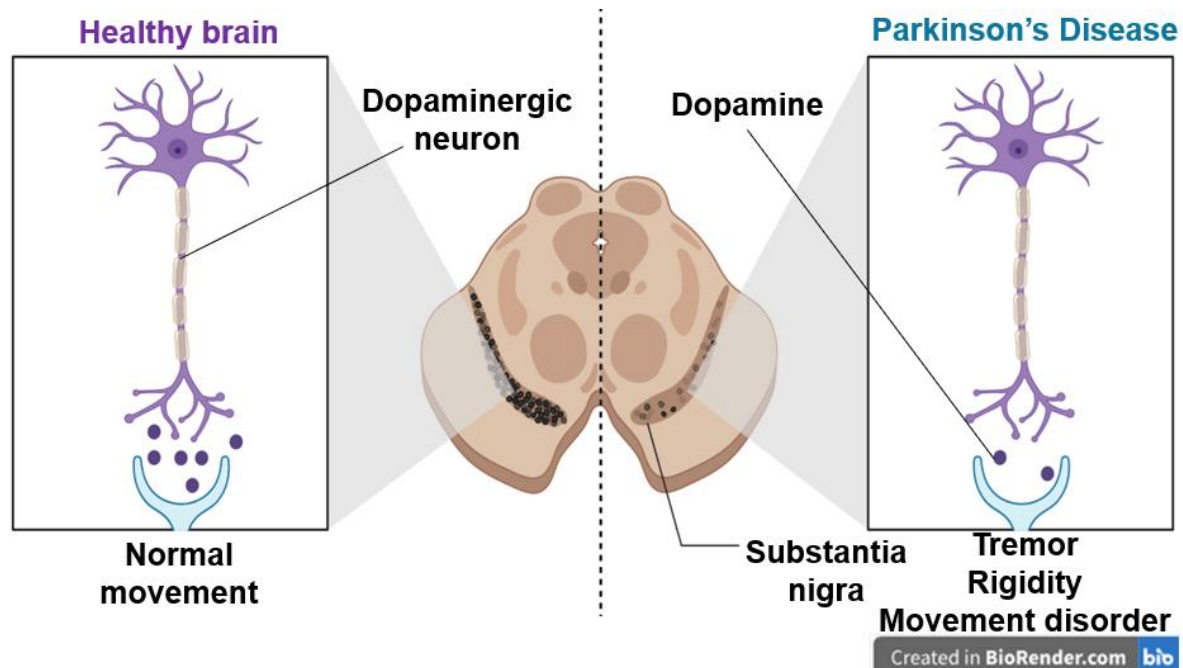


Figure 2.3: Comparison between healthy and abnormal dopaminergic neuron density and dopamine production in the substantia nigra as adapted from Baweja *et al.* (2024:1824).

The synthesis of dopamine involves the hydroxylation of the amino acid tyrosine to L-dihydroxyphenylalanine (levodopa) and the decarboxylation of levodopa to dopamine (Figure 2.4). This reaction is catalysed by tyrosine hydroxylase and aromatic amino acid decarboxylase (Muñoz *et al.*, 2012:1). During the excitation of dopaminergic neurons, dopamine is emptied into the synaptic cleft to interact with the postsynaptic dopamine receptors or regulatory presynaptic dopamine autoreceptors. To stop this reaction, dopamine needs to be removed from the synaptic cleft by reuptake into the dopaminergic neurons or degradation after uptake by glial cells (Meiser *et al.*, 2013:7; Zhang & Sulzer, 2012:5). The surplus dopamine taken in by the glial cells is degraded by MAO and catechol-O-methyl transferase (COMT) (Figure 2.5) (Meiser *et al.*, 2013:8). During oxygen-dependent enzymatic degradation, MAO deaminates dopamine to form hydrogen peroxide (H_2O_2) and 3,4-dihydroxyphenylacetaldehyde. The latter is then oxidised by aldehyde dehydrogenase to 3,4-dihydroxyphenylacetic acid, which subsequently is methylated by COMT to form

homovanillic acid (Hermida-Ameijeiras, 2004:104; López-Pérez *et al.*, 2015:1). The excessive production of H_2O_2 , resulting from a loss of dopaminergic neurons is linked to an increase in oxidative stress (Hermida-Ameijeiras, 2004:104; Olgún *et al.*, 2016:3). Dopamine can also be directly metabolised by COMT to 3-methoxytyramine and subsequently reduced to homovanillic acid by MAO (Olgún *et al.*, 2016:3).

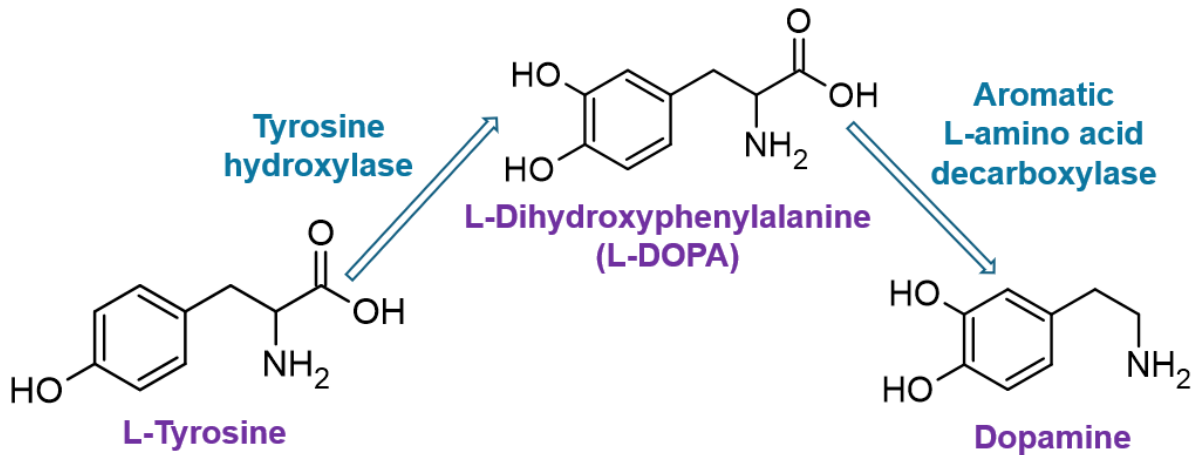


Figure 2.4: Synthesis of dopamine as adapted from Olgún *et al.* (2016:2).

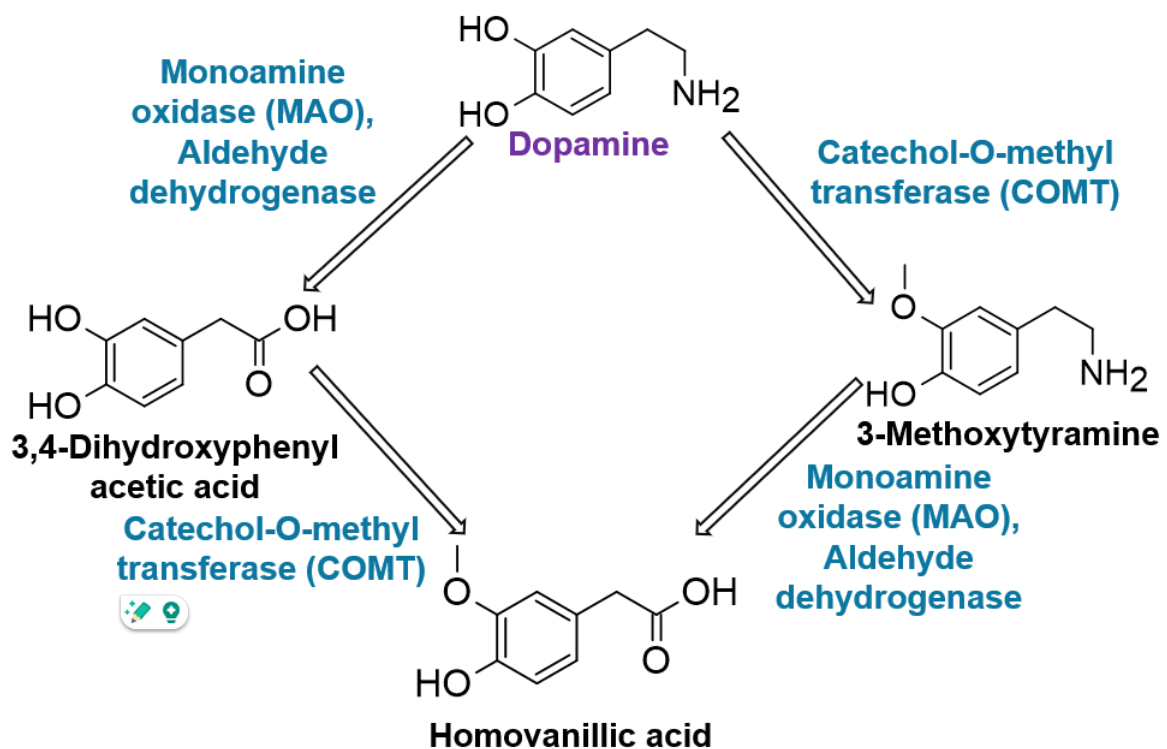


Figure 2.5: Dopamine metabolism as adapted from Olgún *et al.* (2016:3).

To power neural activity, mitochondria use the process of oxidative phosphorylation to form adenosine triphosphate (ATP) from the energy released from the oxidation (Blesa *et al.*, 2015:2; Hall *et al.*, 2012:8940). The mitochondrion is believed to be the most important

cellular source of free radicals as it generates superoxide radicals that are converted to H_2O_2 by superoxide dismutase. The H_2O_2 reacts with transition metals to generate hydroxyl radicals that contribute to cell damage (Beal, 2003:120). The increase in ROS formation is closely related to mitochondrial dysfunction. It was found that some familial occurrences of PD can also be attributed to mitochondrial dysfunction-related oxidative stress caused by genetic mutations of proteins (Blesa *et al.*, 2015:2; Van der Merwe *et al.*, 2015:1; Norris *et al.*, 2015:13862).

The loss of dopaminergic neurons is often accompanied by cellular inclusions called Lewy bodies. These α -synuclein aggregates form insoluble fibrils that infiltrate the neurons of the substantia nigra and locus coeruleus (Baba *et al.*, 1998:879; Schulz-Schaeffer, 2010:132; Wakabayashi *et al.*, 2007:494). These aggregates are assumed to be harmful to dopaminergic neurons in the substantia nigra as the formation of Lewy bodies has the potential to trigger the transmission of toxic α -synuclein between affected cells and adjacent healthy cells resulting in a cascade of Lewy body formation and cell death. Cell death leads to more dopaminergic cell loss as the aggregates spread from cell to cell. It was found that the aggregation of α -synuclein can be initiated within the receiver cell as non-aggregated α -synuclein in the cell can act as a seed. This seed will then induce the aggregation of soluble α -synuclein in a prion-like fashion (Angot & Brundin, 2009:S143; Bernis *et al.*, 2015:2; Emamzadeh & Surguchov, 2018:2; Steiner *et al.*, 2018:162).

2.2.3 Treatment of Parkinson's disease

The main focus of PD treatment is to alleviate symptoms by increasing the level of dopamine, mimicking the role of dopamine, inhibiting the oxidative metabolism of dopamine that leads to the generation of ROS, and inhibiting the formation of protein aggregates that lead to cell death (Figure 2.6) (Emamzadeh & Surguchov, 2018:5). Dopaminergic medication is currently the most effective treatment for PD, however, nonmotor complications can be common and for this reason, some medication also focuses on targeting nondopaminergic pathways to treat PD (Freitas & Fox, 2016:250).

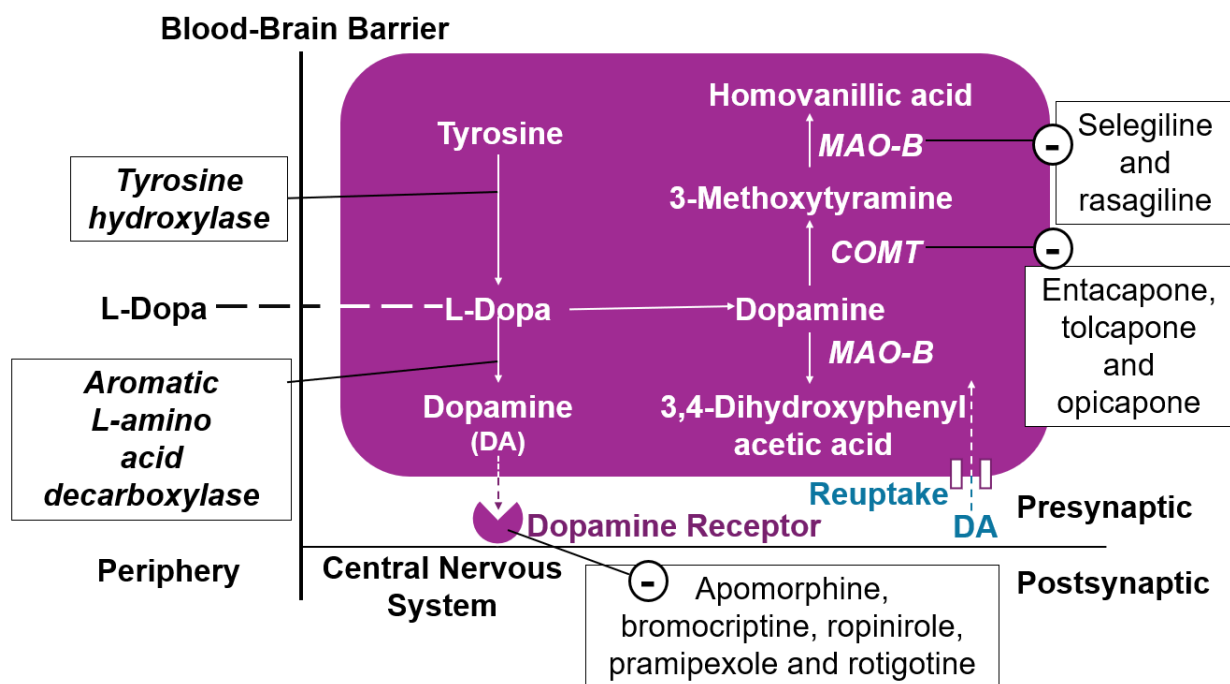


Figure 2.6: Dopaminergic treatment of Parkinson's disease adapted from Dezsi & Vecsei (2017:426).

2.2.3.1 Dopaminergic treatment

Dopamine precursors such as levodopa (L-dopa) are commonly used in the treatment of PD as it is biosynthetically transformed to dopamine by the enzyme L-amino acid decarboxylase (Ellis & Fell, 2017:4248; Emamzadeh & Surguchov, 2018:6). Levodopa was introduced in 1967 and has since been utilised as the gold standard for the treatment of PD, however, it tends to lose its efficacy over time leading to patients experiencing periodic periods and dyskinesia (Olanow *et al.*, 2004:997; Singh *et al.*, 2007:32). Carbidopa and benserazide, which are amino acid decarboxylase inhibitors, are commonly combined with levodopa treatment as they reduce the systemic metabolism of levodopa maintaining its efficacy and reducing side-effects (Ellis & Fell, 2017:4248; Singh *et al.*, 2007:33).

The brain contains five types of dopamine receptors, *viz.*, D1-D5. These five receptors can be divided into two groups based on their function, *i.e.*, D1-like and D2-like receptors (Ellis & Fell, 2017:4248; Missale *et al.*, 1998:190). The D1-like receptors play an important role in regulating the reward system, locomotor activity, learning, memory and signal transduction pathways. The D2-like receptors on the other hand are most commonly related to postsynaptic receptor-mediated behavioural and extrapyramidal activity as they can decrease neuronal excitability or reduce dopamine synthesis and release (Mishra *et al.*, 2018:2; Pothos *et al.*, 1998:5575). Dopamine agonists bind to dopamine receptors to amplify

the effects of dopamine. Approved agonists include apomorphine (Figure 2.7), bromocriptine, ropinirole, pramipexole and rotigotine. These compounds tend to be more potent agonists of D2-like receptors, although some have D1-like receptor agonism and serotonin, α -adrenergic and β -adrenergic antagonism (Ellis & Fell, 2017:4248; Jankovic & Aguilar, 2008:746).

The MAO enzymes are flavoenzymes expressed in humans and other mammals as two isoforms, MAO-A and MAO-B. These isoforms differ in tissue distribution, substrate specificities and overall physiological roles. Some amine compounds such as dopamine, epinephrine, norepinephrine and tyramine are substrates of both isoforms (Shetnev *et al.*, 2024:1010). Although both isoforms metabolise dopamine, it is mainly metabolised by MAO-B in the substantia nigra, where MAO-B is the main isoform distributed in the glial cells. The expression of MAO-B increases as one ages leading to a higher production of H_2O_2 and other ROS leading to neural death (Tzvetkov *et al.*, 2017:471; Yeung *et al.*, 2019:2). The incorporation of MAO-B inhibitors would subsequently decrease dopamine metabolism and increase dopamine concentrations to reduce motor symptoms (Ellis & Fell, 2017:4248). A recent *in vitro* study reported that MAO-B inhibition delays the degeneration of dopaminergic neurons in the brain and abolishes the formation and transmission of α -synuclein aggregates (Króllicka *et al.*, 2022:5; Nakamura *et al.*, 2021:7479). Approved MAO-B inhibitors include selegiline and rasagiline (Figure 2.7), both are irreversible inhibitors of the enzyme (Fowler *et al.*, 2015:650). Safinamide on the other hand is a multitarget compound as it exhibits a reversible inhibition of MAO-B and nondopaminergic mechanisms of action that include the selective sodium channel blockage and calcium channel modulation (Fabbri *et al.*, 2015:481; Tzvetkov *et al.*, 2017:471).

As COMT also metabolises dopamine in the brain, the inhibition of the enzyme leads to the increase of dopamine levels in the brain, reducing motor symptoms (Ellis & Fell, 2017:4248). In dopaminergic systems, COMT also metabolises levodopa, thereby inhibiting the synthesis of dopamine (Króllicka *et al.*, 2022:10). The most common inhibitors are entacapone, tolcapone (Figure 2.7) and opicapone. Entacapone is the most common reversible peripherally acting inhibitor and tolcapone is used when other inhibitors are deemed ineffective due to a rare hepatotoxic side-effect (Salamon *et al.*, 2022:1123).

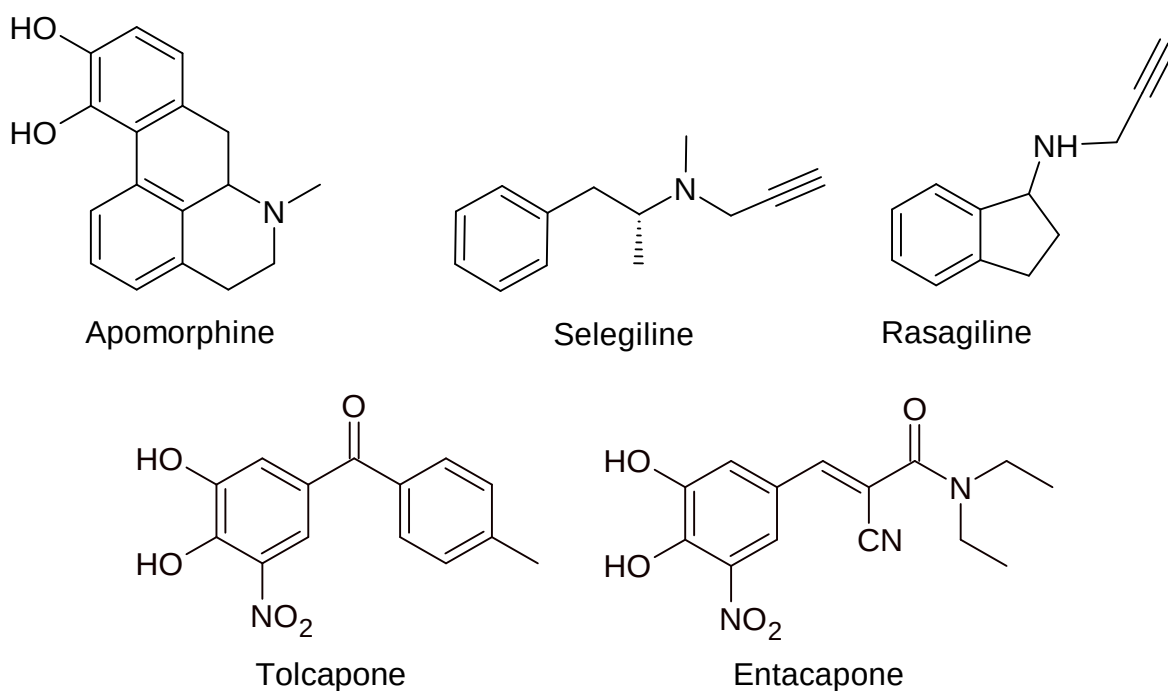


Figure 2.7: Drugs used in the dopaminergic treatment of PD.

2.2.3.2 Nondopaminergic treatment

Adenosine, a neuromodulator released from the neurons and glial cells, is a metabolic intermediate formed from adenosine triphosphate, adenosine diphosphate and adenosine monophosphate (Kanda & Jenner, 2020:S22). Adenosine receptors are widely distributed throughout the central nervous system (CNS). The adenosine A_{2A} receptor in particular is selectively localised in the brain, specifically in the basal ganglia and the limbic region of the brain. These receptors have the ability to modulate the function of several neurotransmitter systems, making them valuable as a nondopaminergic approach to PD treatment (Mori *et al.*, 2022:1). The inhibition of A_{2A} receptors has in the past decade been studied as a potential way to treat motor fluctuations in PD. There are several theories on how adenosine receptor inhibition contributes to PD treatment, one theory suggests that the A_{2A} receptors form heterocomplexes with dopamine D_2 receptors (Pourcher & Huot, 2015:331). Istradefylline (Figure 2.8) is a potent competitive A_{2A} receptor inhibitor recently approved for the management of PD motor fluctuations, based on clinical study results (Jenner, 2005:729; Jenner *et al.*, 2021:322).

Trihexyphenidyl hydrochloride (Figure 2.8), procyclidine hydrochloride, diphenhydramine hydrochloride, and benzotropine mesylate are anticholinergic drugs and were the first-line treatment of the motor symptoms of PD before the introduction of levodopa (Barrett *et al.*, 2021:1181; Shetti & Venkatachalam, 2010:S299). These compounds are competitive

antagonists of muscarinic receptors. It has been theorised that PD causes an imbalance between dopaminergic and cholinergic neurological pathways. By reducing the neurotransmission mediated by neostriatal acetylcholine, anticholinergic compounds can correct this imbalance in early PD (Brocks, 1999:40).

Oxidative stress can also be targeted directly with antioxidant therapy. Mitochondria-targeted antioxidant therapy involves the administration of coenzyme Q10, a parabenzoquinone also known as ubiquinone, and menaquinone (Crane, 2001:591; Duarte-Jurado *et al.*, 2021:4). Ubiquinone acts as a primary scavenger of free radicals, subsequently avoiding lipid peroxidation, protecting biological membranes and DNA from oxidative stress-related damage (Ernster & Dallner, 1995:195; Mischley *et al.*, 2012:72; Silva *et al.*, 2022:1847). The endogenous levels of ubiquinone depend on the consumption and production rates of an organism. The *in vitro* levels can also be altered by oxidative stress-associated diseases such as PD (Duarte-Jurado *et al.*, 2021:4). Menaquinone can quickly cross the blood-brain barrier (BBB) and cell membranes to enter mitochondria where it is reduced to ubiquinol, an active antioxidant, where it serves the same purpose as ubiquinone, *i.e.*, stimulating oxidative phosphorylation and protecting against oxidative stress (Duarte-Jurado *et al.*, 2021:5; James *et al.*, 2005:21295; Murphy & Smith, 2007:629). The supplementation of these compounds is an attractive therapeutic strategy in diseases associated with oxidative stress (Duarte-Jurado *et al.*, 2021:5; James *et al.*, 2005:21296). Studies furthermore suggest that certain vitamins and minerals can act as antioxidants by binding to antioxidant enzymes as cofactors, regulating genes that control intracellular antioxidant systems, and directly scavenging for ROS (Park & Ellis, 2020:4; Virmani *et al.*, 2013:353).

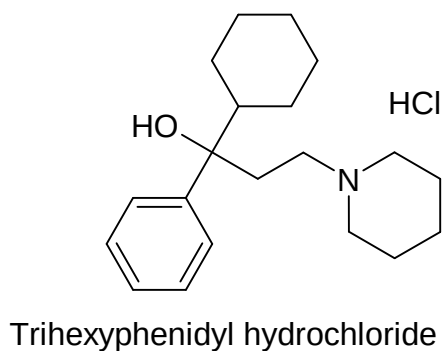
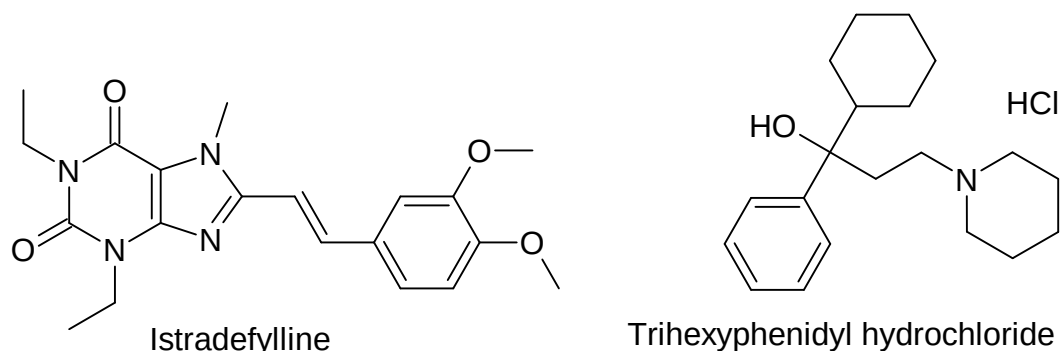


Figure 2.8: Drugs used in the nondopaminergic treatment of PD.

2.2.4 Drug discovery and Parkinson's disease

Drug discovery is a time-consuming and costly process requiring an estimated investment of 2.8 billion United States dollars and 12-15 years to register a new drug on the market (Singh *et al.*, 2023:3). Overall, 96% of drug development efforts fail, with failure rates being the highest for drugs with new mechanisms of action (Hingorani *et al.*, 2019:2).

PD is the second most common neurodegenerative disorder with dopamine replacement drugs remaining the most effective treatment. To this day levodopa remains the first-choice treatment for PD, but as the disease progresses and more dopaminergic neurons are lost the efficacy of levodopa greatly diminishes. Other dopamine replacement therapies do not address the other degenerative effects in the brain (Finkbeiner & Skibinski, 2011:15; Müller, 2020:405). More novel avenues of PD treatment are emerging such as the control of the symptoms without the side-effects and reducing neural loss with the focus on gene therapy and cell-based treatments. Drug repurposing has also become an attractive strategy for identifying ways to attenuate the course of PD (Stoker & Barker, 2020:1).

2.3 Antibiotics and antibiotic resistance

2.3.1 Brief history of antibiotics

Antibiotics can inhibit the growth or kill bacteria and other microorganisms, are of natural or synthetic origins and can even be produced by microorganisms (Lietman, 1986:824). The discovery of antibiotics is famously attributed to Alexander Fleming who discovered penicillin in 1928. However, antibiosis, the process of antibacterial material secretion by microbes, was already described by Louis Pasteur (Brunel, 1951:291; Hutchings *et al.*, 2019:73). Furthermore, the first synthetic antibiotic, salvarsan, was discovered by Paul Ehrlich and subsequently synthesised and biologically tested by Alfred Bertheim and Sahachiro Hata respectively in 1909 (Figure 2.9) (Mohr, 2016:345). Inspired by their work, Gerhard Domagk discovered the sulphonamide prodrug prontosil leading to the study and use of the first broad-spectrum antimicrobials, that is still in use today (Hutchings *et al.*, 2019:72; Otten, 1986:689). Howard Florey and Ernst Chain clarified the structure of penicillin G in 1939 leading to the large-scale production and clinical use of penicillin in 1941 (Mohr, 2016:350). After the discovery and elucidation of penicillin, Selman Waksman and his research group at Rutgers University were confident that soil microorganisms had antibacterial potential (Sköld, 2011:17). This study led to the discovery of streptomycin and their findings were subsequently published in 1944 (Schatz *et al.*, 1944:66). This work with antimicrobials

ushered in the golden age of antibiotics from the 1940s to the 1960s (Hutchings *et al.*, 2019:74; Schatz *et al.*, 1944:67). During this period numerous antibiotic discoveries were made *i.e.* (i) natural antibiotics, *e.g.*, macrolides, aminoglycosides, amphenicols, glycopeptides, ansamycins, lacosamides, streptogramins, tetracyclines and cycloserine, (ii) synthetic antibiotics, *e.g.*, quinolones, sulfones, nitrofurans, ethambutol, phenazines, azoles, and thioamides, and (iii) fungal antibiotics, *e.g.*, penicillins and cephalosporins (Pancu *et al.*, 2021:2).

After 1962 two novel antibiotic classes, *i.e.*, fluoroquinolones and trimethoprim, were approved before the year 2000 (Coates *et al.*, 2011:184; Powers, 2004:24) and most other antibiotics were derivatives of existing antibiotics (Aminov, 2010:3). The turn of the century marked the end of the golden era of antibiotics as the discovery and approval of novel antibiotic classes declined drastically (Gould, 2016:575). Since 2000, only two novel antibiotic classes, *i.e.*, lipopeptides and oxazolidinones, were approved by the FDA to treat Gram-positive bacterial infections. The quinolone antibiotics were the last antimicrobial agents approved for treating Gram-negative bacterial infections (Powers, 2004:24; Tacconelli *et al.*, 2018:318).

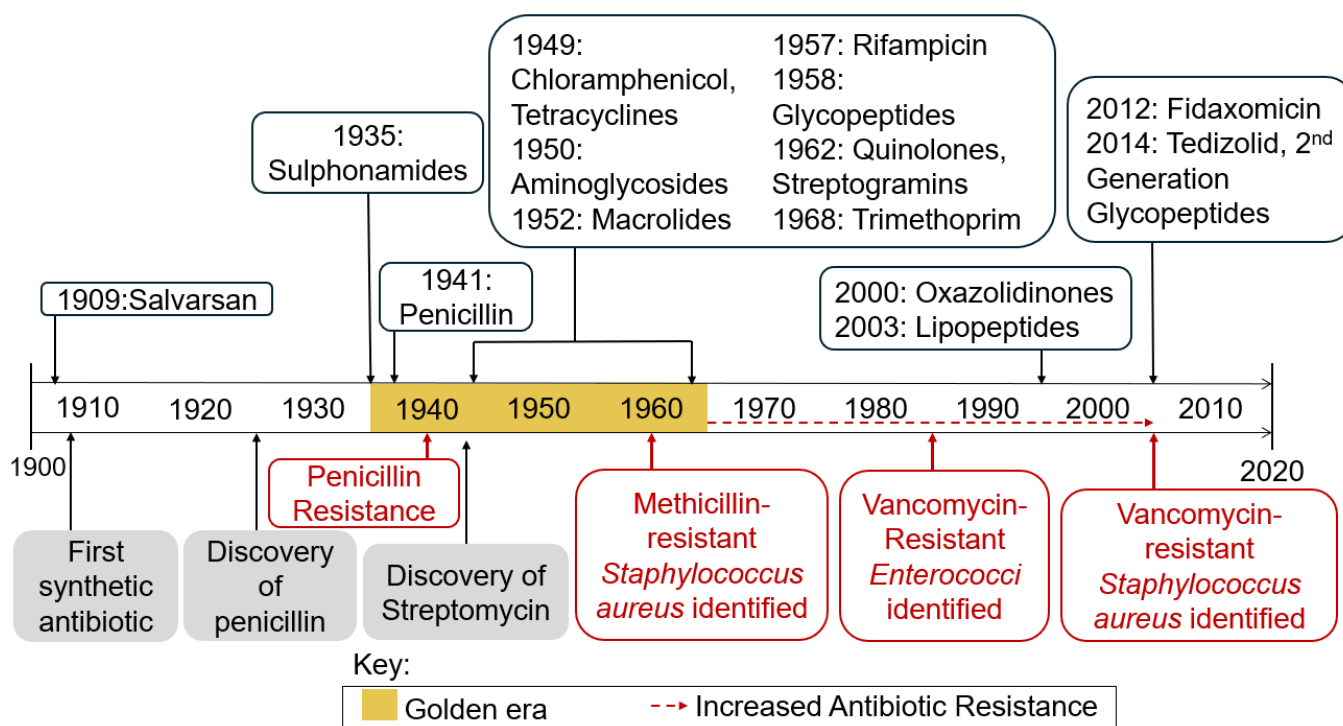


Figure 2.9: The discovery pipeline of antibiotics adapted from Hutchings *et al.* (2019:73), Davies & Davies (2010:417), Walsh & Wencewicz (2017) and Powers (2004:24).

2.3.2 Antibiotic targets and classification

Antimicrobial compounds, or antibiotics, each possess unique mechanisms of action in which they interfere with the survival of bacteria (Da Cunha *et al.*, 2019:1). These agents would traditionally be classified based on their mechanism of action, chemical structure, or spectrum of activity. Furthermore, they can be described as bacteriostatic if they inhibit the growth of the organism, or bactericidal if they cause bacterial cell death (Calderón & Sabundayo, 2007:9; Hancock, 2005:209; Pankey & Sabath, 2004:864). The various bacterial targets are, the bacterial cell wall or membrane, protein synthesis, nucleic acid synthesis and metabolic compound synthesis (Figure 2.10) (Kirmusaoğlu *et al.*, 2019:1). Another classification of importance is the ability of the antibiotic to target Gram-positive and Gram-negative bacteria. Gram-negative bacteria are more challenging to treat as they are equipped with an additional unique lipopolysaccharide outer membrane and efflux pumps among other resistance mechanisms (Saxena *et al.*, 2023:1).

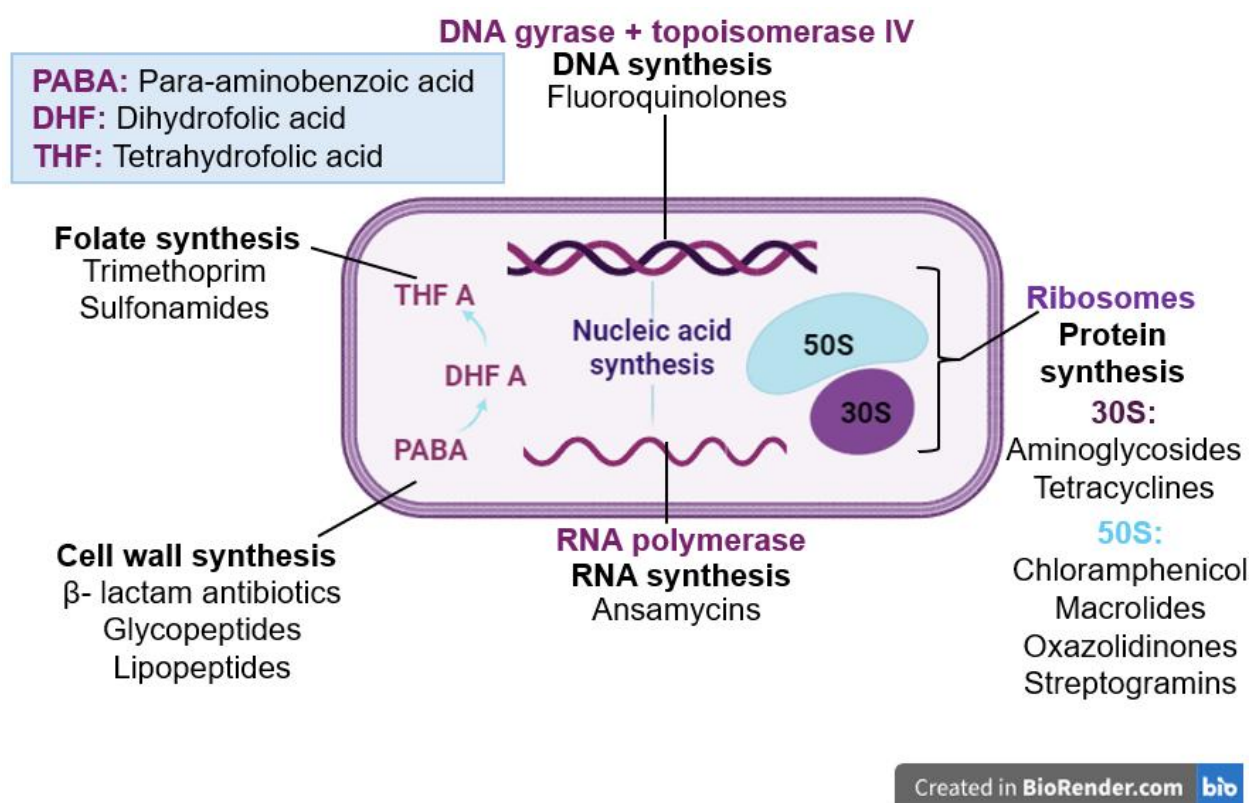


Figure 2.10: The antibiotic classes based on their targets adapted from Kapoor *et al.* (2017:301) and Eyler & Shvets (2019:1082).

Glycopeptides (vancomycin, teicoplanin, telavancin, *etc.*), lipopeptides (daptomycin, surfactin, iturin, *etc.*) and β -lactams (penicillins, cephalosporins, carbapenems and monobactams) (Figure 2.11) (Blaskovich *et al.*, 2018:715; Meena & Kanwar, 2015:1;

Palzkill, 2013:17) are cell wall synthesis inhibitors (Alanis, 2005:699). As the name suggests, these compounds target the synthesis of the bacterial cell wall *i.e.*, the production of peptidoglycan (Hubschwerlen, 2007:481). Glycopeptides, like vancomycin (Rotschafer *et al.*, 1988:214), specifically target Gram-positive bacteria by binding to the end of the growing peptidoglycan chain and subsequently reducing the binding affinity of the enzyme transpeptidase that is responsible for catalysing the final step of peptidoglycan synthesis (Binda *et al.*, 2014:572; Hubschwerlen, 2007:482). Daptomycin is the most common lipopeptide that has activity against Gram-positive bacteria and is known for its unique calcium-dependent binding to the cell membrane leading to potassium efflux and cell death (Hair *et al.*, 2007:1484; Hancock, 2005:211). The β -lactam antibiotics are characterised by their β -lactam ring that inhibits peptidoglycan synthesis by competitively inhibiting transpeptidase (Dowling *et al.*, 2017:536). This group of antibiotics possess a broad-spectrum of activity against both Gram-positive and Gram-negative bacteria (Hubschwerlen, 2007:481; Sykes & Bonner, 1985:S580).

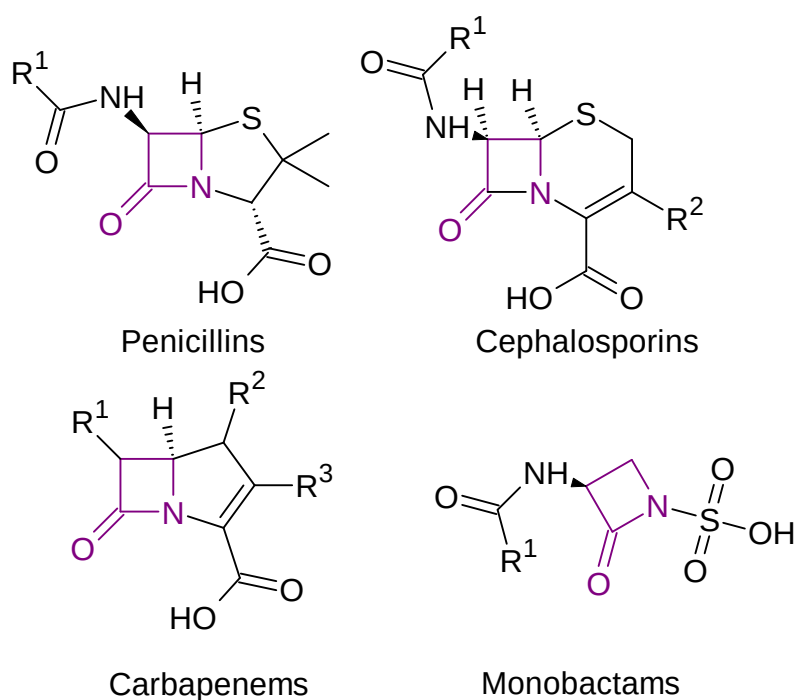


Figure 2.11: The basic structures of β -lactam antibiotics (Palzkill, 2013:17).

As protein synthesis takes place at the ribosomal 50S and 30S subunits in the bacterial cell cytoplasm, these subunits serve as the targets of the protein synthesis inhibitor antibiotics (Champney, 2020:787; Garneau-Tsodikova & Labby, 2016:12). Aminoglycosides (streptomycin, amikacin, neomycin *etc.*) and tetracyclines (oxytetracycline, doxycycline *etc.*) (Figure 2.12) (Fuoco, 2012:2; Markley & Wencewicz, 2018:2; Serio *et al.*, 2018:6) target the

A-site of the 30S subunit, subsequently effecting translocation (Bauer *et al.*, 2004:592; Kirmusaoğlu *et al.*, 2019:7; Krause *et al.*, 2016:4; Nguyen *et al.*, 2014:561). Aminoglycosides and tetracyclines are broad-spectrum antibiotics with activity against both Gram-positive and Gram-negative bacteria (Chopra & Roberts, 2001:232; Garneau-Tsodikova & Labby, 2016:11). Chloramphenicol, macrolides (clarithromycin, erythromycin, azithromycin *etc.*) (Figure 2.13), oxazolidinones (linezolid, delpazolid, radezolid *etc.*) and streptogramin antibiotics (pristinamycin, quinupristin-dalfopristin *etc.*) (Figure 2.14) all target the 50S subunit of the bacterial ribosome to inhibit protein synthesis. These agents are all broad-spectrum antibiotics with activity against Gram-positive bacteria (Diekema & Jones, 2000:8; Dinos, 2017:2967; Foti *et al.*, 2021:2; Gaillard *et al.*, 2016:3; Mast & Wohlleben, 2014:44; Mukhtar & Wright, 2005:534; Myers & Clark, 2021:1635).

Some antibiotics can inhibit bacterial nucleic acid synthesis, replication and transcription (Kirmusaoğlu *et al.*, 2019:4). Fluoroquinolones (nalidixic acid, levofloxacin, moxifloxacin *etc.*), metronidazole and rifampin are nucleic acid synthesis inhibitors (Figure 2.15) (Dingsdag & Hunter, 2018:265; Fengxian & Reti, 2017:111). Fluoroquinolones are bactericidal inhibitors of two enzymes *viz.* DNA gyrase and topoisomerase IV, preventing DNA replication (Bernatová *et al.*, 2013:13190; Ezelarab *et al.*, 2018:2; Hooper & Jacoby, 2016:1). Fluoroquinolones possess a broad-spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria (Pham *et al.*, 2019:1719). The nitro-group from metronidazole plays an important role in its mode of action, as it reacts to DNA, subsequently breaking the strand and destabilising the helix (Bradley & Sauberan, 2018:1524). Metronidazole is active against Gram-positive and Gram-negative anaerobic bacteria (Löfmark *et al.*, 2010:S16). Rifampin binds to RNA polymerase and blocks the initiation of transcription (Kirmusaoğlu *et al.*, 2019:5).

Other antibiotics target certain metabolic pathways in bacterial cells *i.e.*, the folic acid synthesis pathway. Sulphonamides (sulfamethoxazole, sulfasalazine, sulfisoxazole *etc.*) and trimethoprim (Figure 2.16) (Ovung & Bhattacharyya, 2021:259; Sköld, 2011:51) are examples of antibiotics that inhibit folic acid formation. Sulphonamides act as a competitive inhibitor by binding to the dihydropteroate enzyme, ultimately preventing dihydrofolic acid formation (Igwe & Okoro, 2014:1; Lavanya, 2017:1). Trimethoprim acts as a sulphonamide potentiator, subsequently inhibiting the dihydrofolate reductase enzyme and preventing tetrahydrofolic acid formation (Kirmusaoğlu *et al.*, 2019:4; Masters *et al.*, 2003:402).

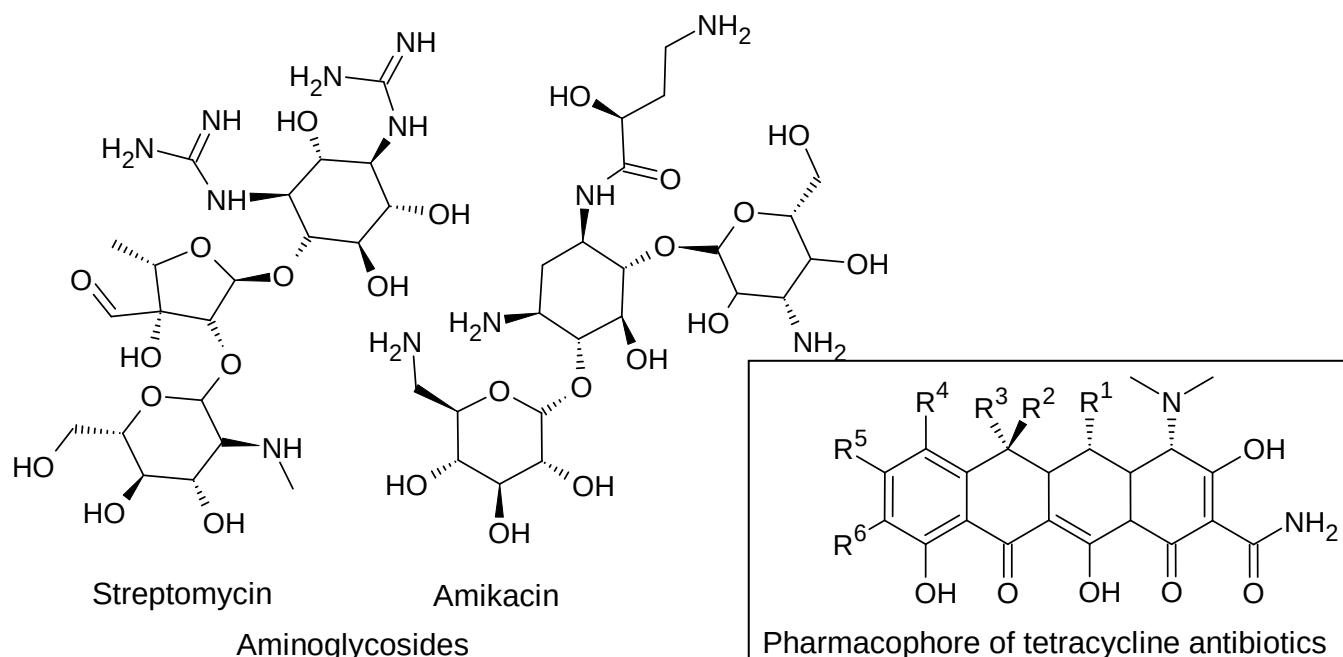


Figure 2.12: Structures of antibiotics that target the 30S subunit of ribosomes (Fuoco, 2012:7; Markley & Wencewicz, 2018:2; Serio *et al.*, 2018:6).

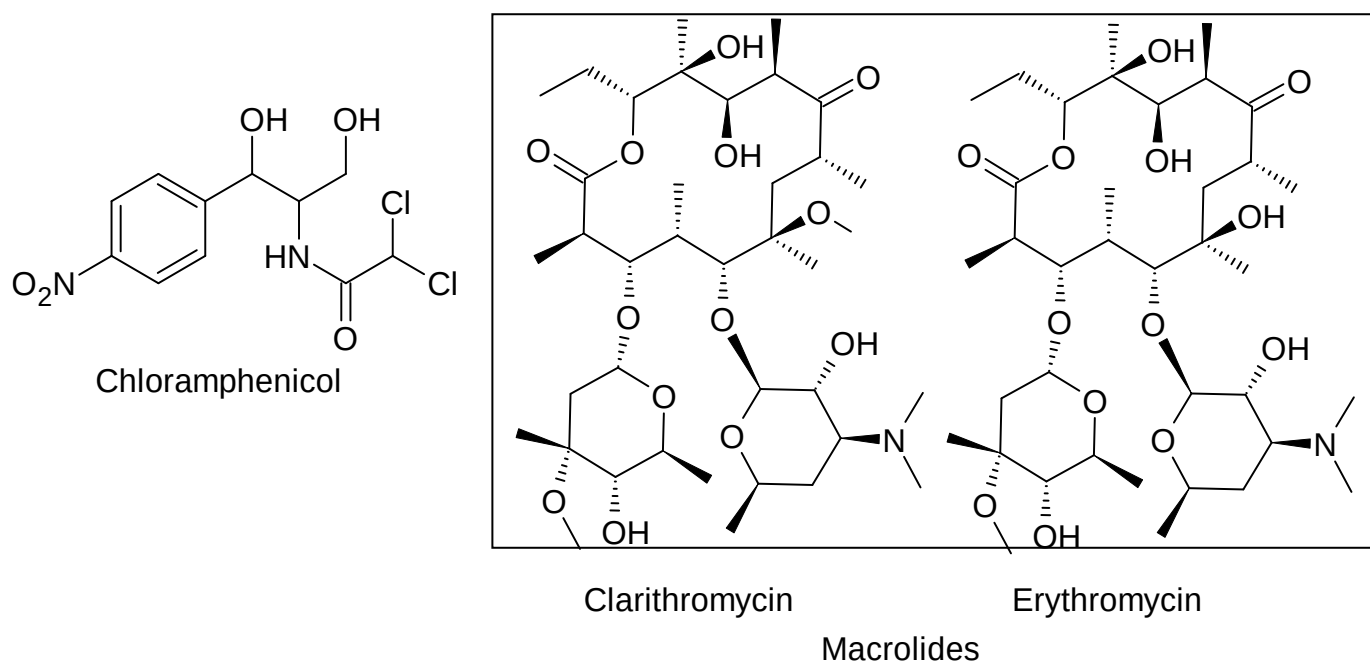


Figure 2.13: Structures of antibiotics that target the 50S subunit of ribosomes; chloramphenicol and macrolides (Dinos, 2017:2968; Ma *et al.*, 2024:3; Tevyashova, 2021:1).

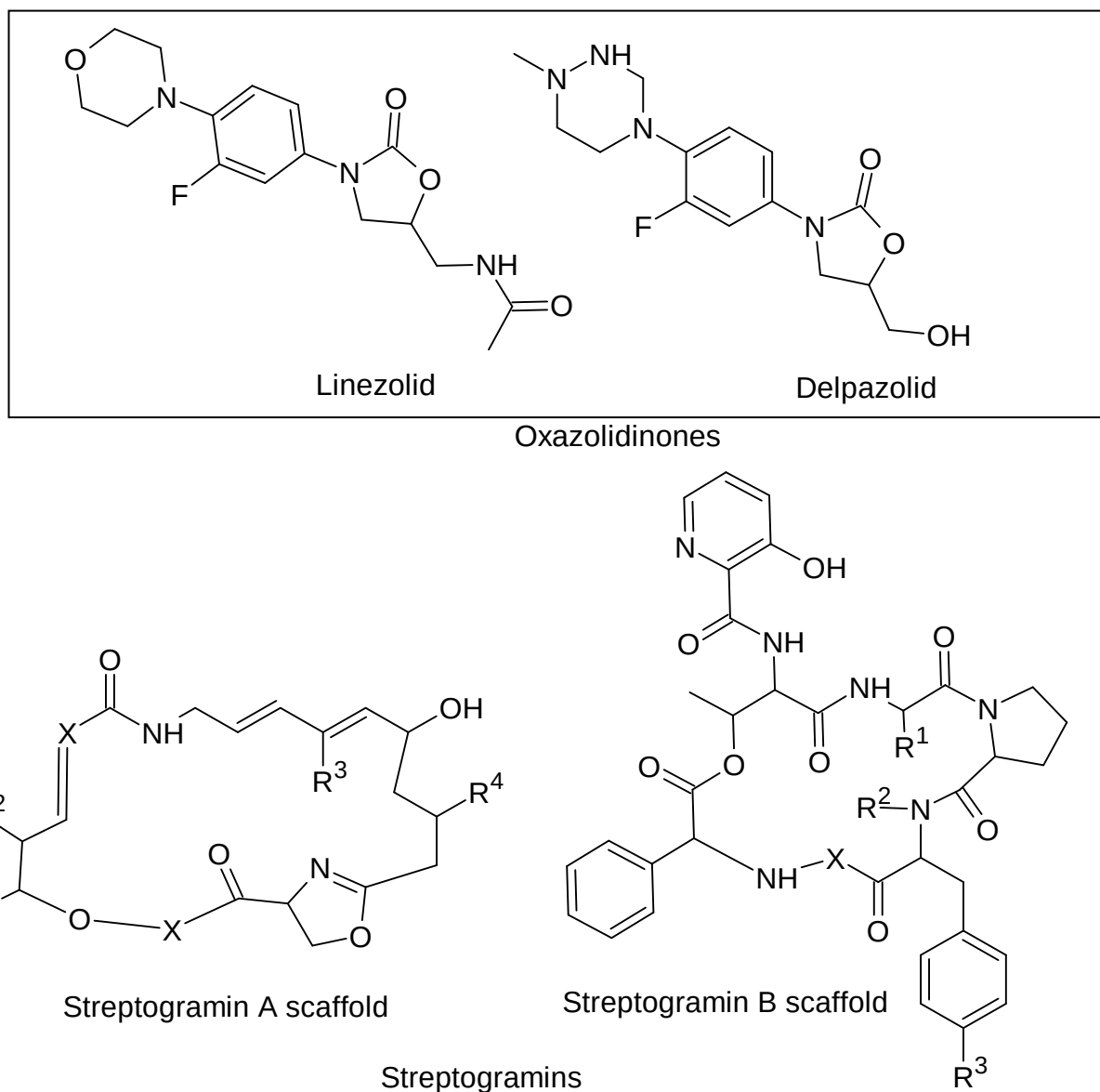


Figure 2.14: Structures of antibiotics that target the 50S subunit of ribosomes; oxazolidinones (Foti *et al.*, 2021:3) and streptogramins (Mukhtar & Wright, 2005:532).

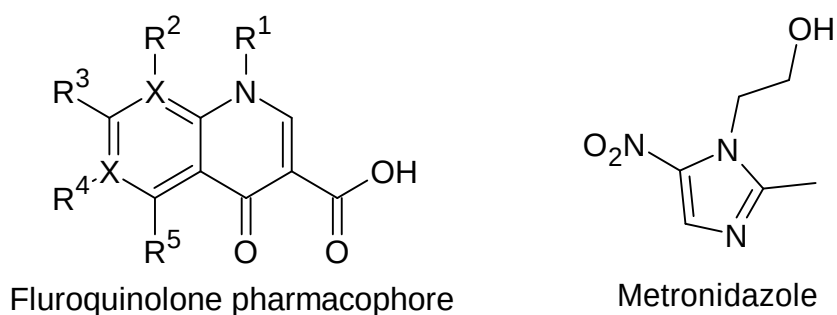


Figure 2.15: Structures of the fluoroquinolone pharmacophore (Fengxian & Reti, 2017:115) and metronidazole (Dingsdag & Hunter, 2018:266).

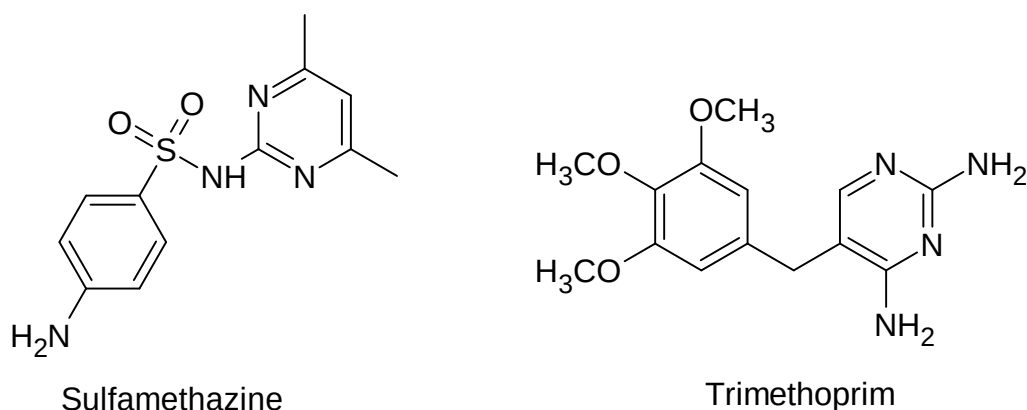


Figure 2.16: Structures of antibiotic compounds sulfamethazine (Ovung & Bhattacharyya, 2021:261) and trimethoprim (Sköld, 2011:52).

2.3.3 Antibiotic resistance

Bacteria possess genetic plasticity that allows them to respond to various environmental threats including antibiotic compounds (Munita & Arias, 2016:3). Resistance to antibiotic compounds can either be acquired through mutation or acquisition of new DNA, or it can be intrinsic naturally occurring biological traits in an organism (Hawkey, 1998:659). Antibiotic resistance occurs when an antibiotic compound loses the ability to inhibit the growth of bacteria, subsequently allowing the bacteria to multiply even in the presence of this once-effective compound (Zaman *et al.*, 2017:2). The most noteworthy of these bacteria is known as the ESKAPE pathogen, *i.e.*, *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. These pathogens are notorious for their ability to acquire resistance and have been identified as priority bacteria against which novel antibiotics are desperately needed (Miller & Arias, 2024:598).

Since the introduction of sulphonamide treatment in 1937, resistance mechanisms in bacteria have been observed. Resistance was identified against penicillin before its therapeutic introduction to the market in 1942 with the discovery of bacterial penicillinase (β -lactamases) (Davies & Davies, 2010:418; Uddin *et al.*, 2021:1754). This discovery supported the finding that some bacteria possess naturally occurring *i.e.*, intrinsic, antibiotic-resistance genes (D'costa *et al.*, 2011:457). Bacteria can share genetic components with other bacteria such as resistant DNA through horizontal gene transfer (Habboush & Guzman, 2023). To counteract the effects of antibiotics, bacteria utilise three primary mechanisms *viz.*, prevention of antibiotic accumulation, alteration of the antibiotic target, enzymatic inactivation of the antibiotic and acquisition of alternative metabolic pathways (Figure 2.17) (Halawa *et al.*, 2024:6; Mutuku *et al.*, 2022:152).

Bacteria possess the ability to change the permeability of their outer membranes, decreasing the accumulation of antibiotic compounds (Kapoor *et al.*, 2017:303). There are two main methods how bacteria can establish this *viz.* through efflux pumps and limiting the entrance of antibiotics into the cell (Halawa *et al.*, 2024:6; Kapoor *et al.*, 2017:303; Muteeb *et al.*, 2023:8). Efflux pumps play a critical role in antibiotic resistance as they allow the bacterium to regulate its internal environment by removing toxic substances, *i.e.*, antibiotics, before they can reach their target (Halawa *et al.*, 2024:7; Pearson *et al.*, 1999:1203; Soto, 2013:223; Wise, 1999). Since efflux pumps can expel a wide range of antibiotics, it is often the cause of multi-drug resistance, for example, *P. aeruginosa* possess efflux pumps that can remove fluoroquinolones, tetracyclines and aminoglycosides (Lorusso *et al.*, 2022:8; Muteeb *et al.*, 2023:8; Quddus *et al.*, 2023:1). Porin channels, present in Gram-negative bacteria, serve as makeshift gatekeepers only allowing certain antibiotics to enter the bacterial cell. The subsequent reduction in the number of porins in the bacterial outer membrane can lead to a decline in the number of antibiotic molecules that can enter the cell (Choi & Lee, 2019:2; Halawa *et al.*, 2024:7; Kapoor *et al.*, 2017:303). The OmpA porin channel in *A. baumannii* has been shown to reduce the sensitivity of the bacterium to chloramphenicol, trimethoprim and numerous other antibiotics (Smani *et al.*, 2014:1806; Zhou *et al.*, 2023:2).

Some bacteria can interfere with the antibiotic target, preventing the binding of the antibiotic molecule and leading to subsequent resistance (Halawa *et al.*, 2024:7). This can be achieved by protecting the target by preventing the antibiotic molecule from reaching the target, modifying the target site thereby decreasing its affinity for the molecule, point mutations, mosaic gene acquisition, target overproduction or target substitution (Munita & Arias, 2016:494; Rice, 2009:75). Target protection does not result in a permanent alteration of the target; instead, it requires continuous or repeated interactions between the target protection protein and the target to develop resistance. On the other hand, target modification only requires a single interaction between the resistance protein and the target to effect a chemical change (Wilson *et al.*, 2020:637). There are numerous instances where target modification has been observed. For example, the mutation of the A-site on the ribosomal 30S subunit led to the reduction of aminoglycoside and tetracycline affinity (Garneau-Tsodikova & Labby, 2016:13; Halawa *et al.*, 2024:7; Wilson *et al.*, 2020:638). Point mutations in the RNA polymerase (RNAP) β -subunit target in *Mycobacterium tuberculosis* and other mycobacteria have decreased the binding affinity of rifampin (Qi *et al.*, 2016:3808).

Biofilm formation is another unique way in which bacteria can protect themselves against antibiotics and contribute to antibiotic resistance (Zhao *et al.*, 2023:1). Biofilms are clusters of bacteria that attach themselves to surfaces or themselves to form a self-produced matrix with their own proteins, polysaccharides and environmental DNA (eDNA). The biofilm acts as a makeshift shield, allowing the bacteria to remain dormant and hidden from the host immune system, subsequently causing tissue damage and acute infections. The biofilm allows the bacteria to adapt to environmental stressors enabling bacteria to become resistant to antimicrobial therapy by inactivating their targets or reducing cellular function requirements (Mann *et al.*, 2021:2; Vestby *et al.*, 2020:1). The formation of biofilms by bacteria is a dynamic process involving three steps: surface attachment mediated by van der Waals interactions and irreversible interactions, development of micro-colonies, macro-colony formation and biofilm maturation and then finally cell dispersion (Dufour *et al.*, 2010:3; Guzmán-Soto *et al.*, 2021:3).

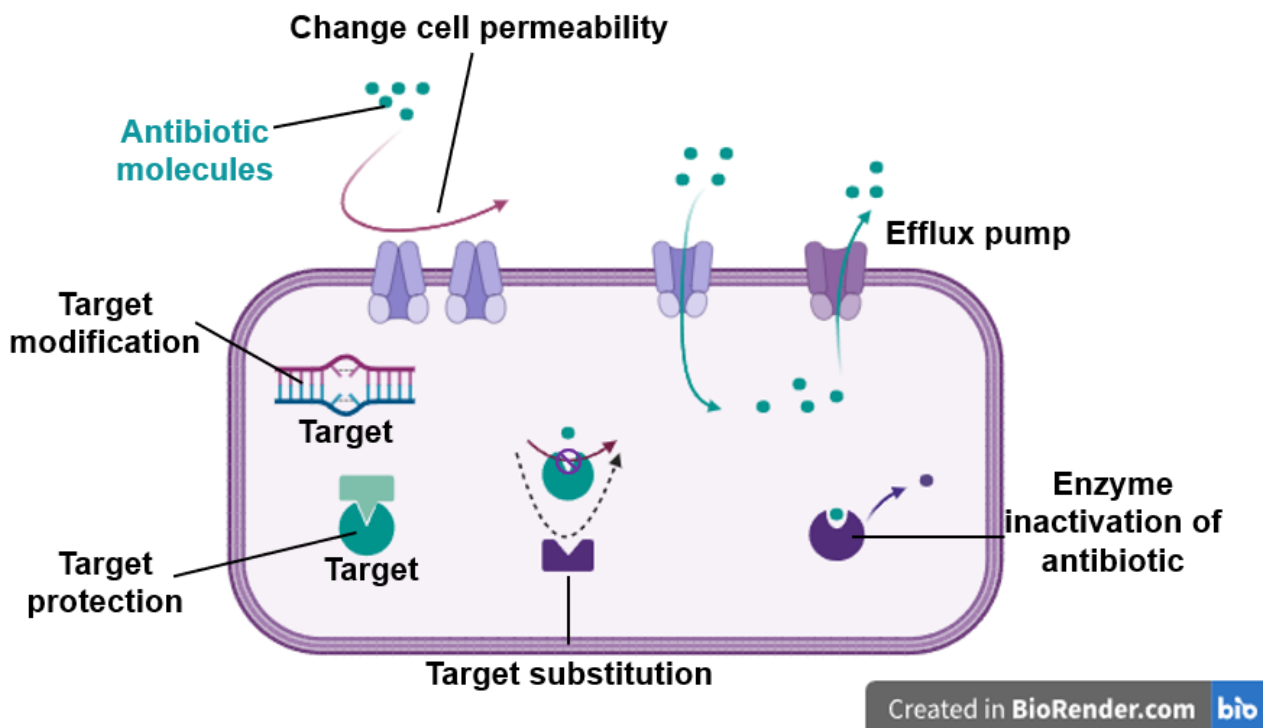


Figure 2.17: Antibiotic resistance strategies employed by bacteria adapted from Mutuku *et al.* (2022:11).

Inactivation of antibiotics by enzymes is a common resistance strategy utilised by bacteria. The enzymes involved in the destruction or modification of the antibiotic structure are characterised based on the reaction they catalyse *viz.* hydrolases, transferases and oxidoreductases (Egorov *et al.*, 2018:40). The production of β -lactamases, a hydrolase enzyme, is a well-known resistance mechanism against β -lactam antibiotics. The enzyme

degrades the amide bonds in the β -lactam ring, inactivating the antibiotic (Egorov *et al.*, 2018:40; Kirmusaoğlu *et al.*, 2019:3).

2.3.4 Drug discovery and antibiotics resistance

According to a recent study by Citeline Clinical (Citeline Clinical, 2024), as of January 2024, there has been a 7.2% increase in the number of novel drugs in the drug research and design pipeline. However, the progression of drugs to the phase III clinical stage has declined, with only a 1.5% increase since 2023. These statistics cover all therapeutic areas. Additionally, there has been almost no growth in the anti-infective drug pipeline, with only a 0.8% increase since 2023. The rise in antibacterial-resistant pathogens outweighs the development of antibiotics with new modes of action. Less than 25% of current drugs in the pipeline represent a novel antibiotic class with none targeting Gram-negative pathogens (Miethke *et al.*, 2021:727).

Initially, most antibiotics were of natural origin. This initial strategy was efficient and productive; however, the identification of new leads had steadily come to a halt (Hegemann *et al.*, 2023:1). Natural products are structurally diverse and offer numerous scaffolds that play an important role in new compound library design (Davison & Brimble, 2019:1). The structural diversity of natural products makes them a good source for drug and drug lead identification that serves as inspiration for the design and synthesis of synthetic and semi-synthetic drugs (Berdigaliyev & Aljofan, 2020:341; Newman & Cragg, 2016:629) like amoxicillin (α -amino-p-hydroxybenzyl penicillin), a semi-synthetic broad-spectrum penicillin (Berdigaliyev & Aljofan, 2020:342; Sutherland *et al.*, 1972:13). In recent years pharmaceutical companies scaled down their natural product research, rather focusing on modern advances to generate a vast number of synthetic small molecules (Shen, 2015:1297). However, this shift did not affect the number of approved drugs with natural scaffold origins (Stratton *et al.*, 2015:4802). To address the issue of antibiotic resistance, two possible approaches to drug discovery have been proposed: developing entirely new scaffolds or, modifying existing natural antibiotic scaffolds (Rossiter *et al.*, 2017:12419). Natural products and their scaffolds still have much to offer regarding drug design and development.

2.4 Bioactive compounds

2.4.1 Chalcones

A chalcone is a simple naturally occurring chemical scaffold and flavonoid found in vegetables, fruits, teas, and other plants (Batovska & Todorova, 2010:1; Henry *et al.*, 2020:300; Sahu *et al.*, 2012:209; Rajendran *et al.*, 2022:2; Zhuang *et al.*, 2017:7762). They are synthesised by plants and contribute to the colour of flowers to attract pollinators, protect the plant from fungal infections and ultraviolet radiation, and play a key role in cellular processes such as energy transfer, respiration, and photosynthesis (Henry *et al.*, 2020:2). The common chalcone derivative, 1,3-diaryl-2-propen-1-one, also known as a chalconoid (Figure 2.18), is an α,β -unsaturated system with three carbon units connecting two aromatic rings. The scaffold can exist as *trans*- and *cis*-isomers, where the *trans*-isomer is thermodynamically more stable (Batovska & Todorova, 2010:1; Gao *et al.*, 2020:2050; Gomes *et al.*, 2017:1; Rajendran *et al.*, 2022:2; Zhuang *et al.*, 2017:7763). Chalcone-containing compounds have been reported to have various biological effects (Table 2.1) such as analgesic (Viana *et al.*, 2003:189), antiarthritic (Rojas *et al.*, 2003:183), anti-inflammatory (Hsieh *et al.*, 2010:163; Nowakowska, 2007:125), antibacterial (Abdullah *et al.*, 2014:31; Mustafa & Mostafa, 2020:417; Nielsen *et al.*, 2005:2667), antiviral (Ali *et al.*, 2007:702; Fu *et al.*, 2020:24483), anticancer (Constantinescu & Lungu, 2021:1; Zhou *et al.*, 2020:1), anti-Alzheimer (Cao *et al.*, 2020:736; Al Rahim *et al.*, 2008:10), anti-Parkinson (Królicka *et al.*, 2022:1), antidiabetic (Rammohan *et al.*, 2020:1) and antioxidant effects (Osipova *et al.*, 2020:504).

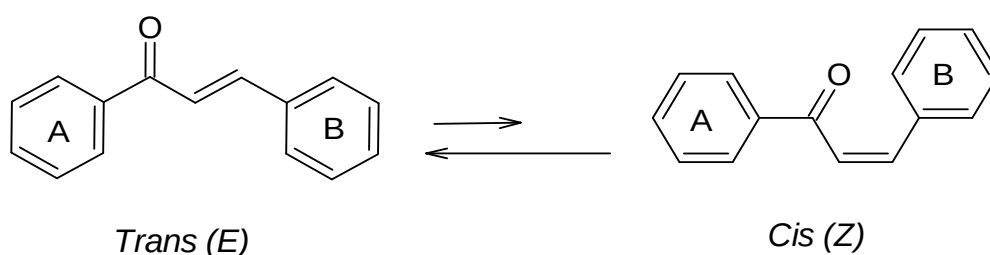
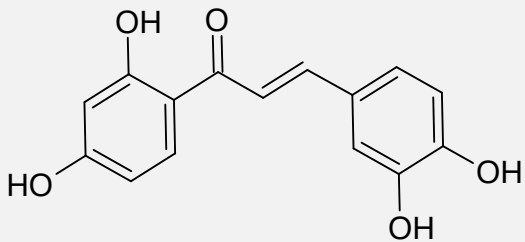
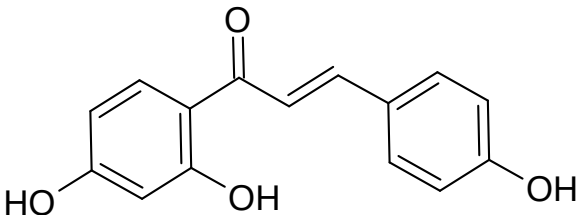
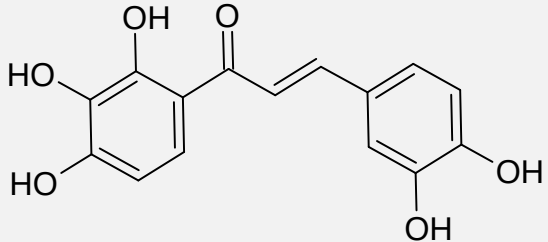
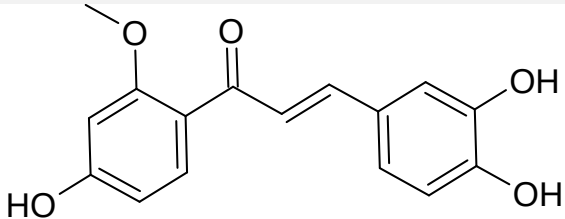
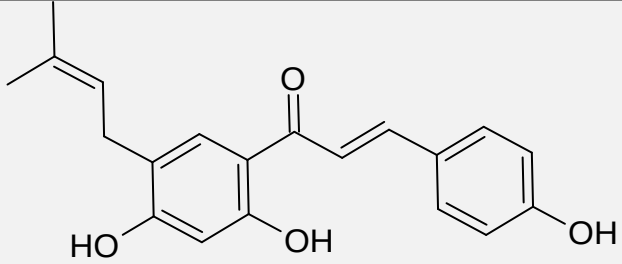
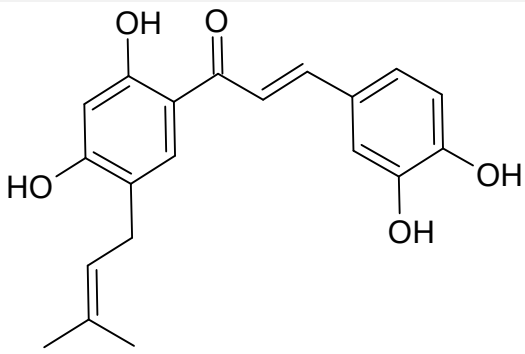
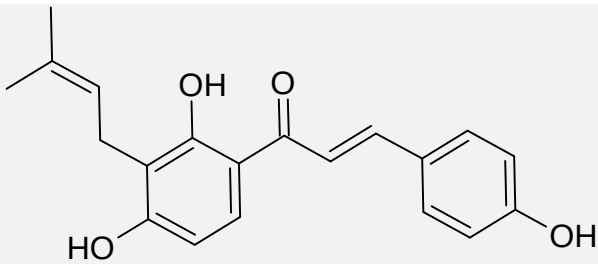
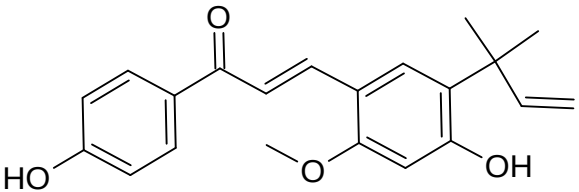
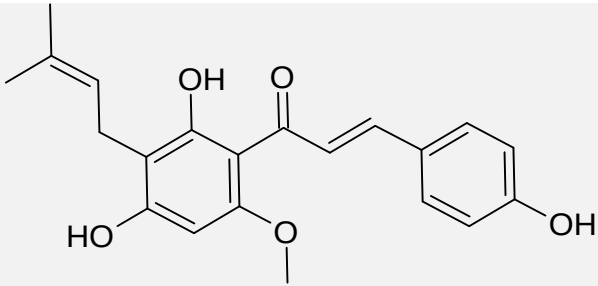


Figure 2.18: Structural representations of the chalcone scaffold as adapted from Gomes *et al.* (2017:2) and Zhuang *et al.* (2017:7763).

Table 2.1: Some naturally occurring chalcones and their biological activities

Name and Structure	Biological activity

 Butein	Antioxidant (Cai <i>et al.</i>, 2006:2884; Chen <i>et al.</i>, 2006:161; Cheng <i>et al.</i>, 1998:291), anticancer (Bai <i>et al.</i>, 2015:3085; Padmavathi <i>et al.</i>, 2015:1163), hypotensive (Kang <i>et al.</i>, 2003:1345), neuroprotective (Cho <i>et al.</i>, 2012:1940; Lee & Jeong, 2016:2894)
 Isoliquiritigenin	Anticancer (Kanazawa <i>et al.</i>, 2003:580; Yuan <i>et al.</i>, 2012:789), antioxidant (Zhang <i>et al.</i>, 2010:841), anti-inflammation (Lee <i>et al.</i>, 2009:257), antidiabetic (Li <i>et al.</i>, 2010:3205), antibacterial (Feldman <i>et al.</i>, 2011:1862; Qu <i>et al.</i>, 2019:1), neuroprotective (Shi <i>et al.</i>, 2020:207).
 Okanin	Anti-inflammation (Kil <i>et al.</i>, 2011:53), antioxidant (Dziedzic, 1983:205; Ma <i>et al.</i>, 2016:575), neuroprotective (Li <i>et al.</i>, 2015:839).
 Sappanchalcone	Anti-inflammation (Jung <i>et al.</i>, 2015:973), antiviral (Liu <i>et al.</i>, 2009:337), anticancer (Seo <i>et al.</i>, 2020:109185).

 Bavachalcone	Anticancer (Song <i>et al.</i>, 2018:37), antioxidant (Dang <i>et al.</i>, 2015:105), neuroprotective (Wu <i>et al.</i>, 2023:110552), antibacterial, antifungal (Baig, 2022:1).
 Brousochalcone A	Antioxidant (Cheng <i>et al.</i>, 2001:939), anticancer (Lee <i>et al.</i>, 2021:2316; Park <i>et al.</i>, 2018:199), antifungal (Sohn <i>et al.</i>, 2004:666)
 Isobavachalcone	Antifungal (Baig, 2022:1), antidiabetic (Oh <i>et al.</i>, 2010:940), antimicrobial (Assis <i>et al.</i>, 2022:1; Wang <i>et al.</i>, 2021:1), anticancer (Kuate & Sandjo, 2012:544), neuroprotective (Jing <i>et al.</i>, 2017:1).
 Licochalcone A	Anticancer (Li <i>et al.</i>, 2022:1; Xiao <i>et al.</i>, 2011:69), anti-inflammation (Song <i>et al.</i>, 2015:4453), antioxidant (Chen <i>et al.</i>, 2017:331), antibacterial (Tsukiyama <i>et al.</i>, 2002:1226), neuroprotective (Li <i>et al.</i>, 2022:1)
 Xanthohumol	Anticancer (Girisa <i>et al.</i>, 2021:1016), antioxidant (Harish <i>et al.</i>, 2021:1), anti-inflammation (Neumann <i>et al.</i>, 2022:1)

Chalcone derivatives generally have small polar surface areas that allow them to easily cross the BBB into the CNS, which is a crucial characteristic of PD targeting compounds. This feature is attributed to the hydrophobic nature of the two aromatic rings (ring A and ring B) (Mathew *et al.*, 2015:22). Synthetic chalcone compounds have been shown to have several PD-related biological targets: MAO-B, α -synuclein, adenosine A₁ and A_{2A}, oxidative stress and neuroinflammation (Dinkova-Kostova *et al.*, 2018:3577; Janse van Rensburg *et al.*, 2021:1585, 2019:300; Janse van Rensburg, Legoabe & Terre'Blanche, 2020:1; Janse van Rensburg, Legoabe, Terre'Blanche, *et al.*, 2020:243; Jiang *et al.*, 2022:1; Króllicka *et al.*, 2022:5; Magesh *et al.*, 2012:669; Tinku *et al.*, 2021:1; Woo *et al.*, 2014:1473).

The introduction of different substituents on both aromatic rings of chalcone derivatives was reported to have an important influence on the inhibitory activity and selectivity of chalcones against MAO (Figure 2.19). The introduction of heterocycles, nitro functional groups or lipophilic electron-donating groups and halogen substitution on rings A and/or B of the chalcone scaffold have been shown to influence their ability to bind to MAO (Guglielmi *et al.*, 2020:26; Iacovino *et al.*, 2021:1151). Studies have found that monosubstituted chalcones with a trifluoromethyl group in the *meta*-position of ring A acted as competitive reversible inhibitors of MAO-B (Iacovino *et al.*, 2021:1151). Furthermore, the choice of halogen also influenced the nature of MAO inhibition, as chloro-containing chalcone derivatives inhibited MAO-B competitively, whilst fluoro-containing chalcone derivatives inhibited the enzyme reversibly (Rehuman *et al.*, 2022:1). A structure-activity relationship (SAR) study of hydroxychalcones also suggested that methyl and fluorine substituents contributed to increasing both the inhibition potency and selectivity of the compounds to MAO-B (Oh *et al.*, 2022:940).

Chalcone derivatives have also been studied and developed to target both MAO and COMT in the treatment of PD. Nitrocatechol compounds are well-known inhibitors of COMT, and chalcone-related derivatives tend to act as MAO-B-specific inhibitors, as discussed. The nitrocatechol moiety is a standard pharmacophore for the development of potent inhibitors of COMT for the treatment of PD and is commonly found in COMT inhibitors like tolcapone and entacapone (Pinto *et al.*, 2024:1637). The nitrocatechol moiety allows a tight binding to the COMT enzyme, however, it has been reported to have potential toxicological liability (Silva *et al.*, 2020:1849). Studies found that nitrocatechol chalcone derivatives were potent COMT inhibitors but were relatively weak MAO inhibitors (De Beer *et al.*, 2021:5; Hitge *et*

al., 2020:127192) (Figure 2.20). Furthermore, substitution on ring B did not affect COMT inhibition, however, it did influence the potency of MAO-B inhibition (Engelbrecht *et al.*, 2018:115; Hitge *et al.*, 2020:5).

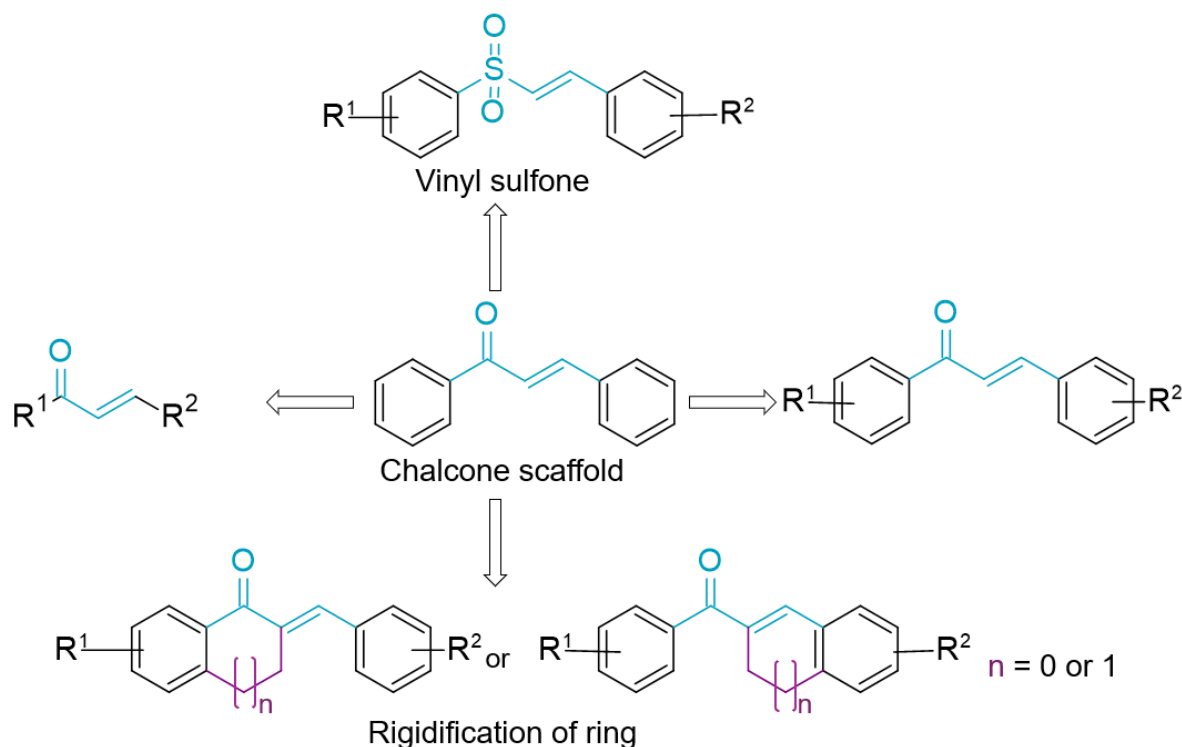
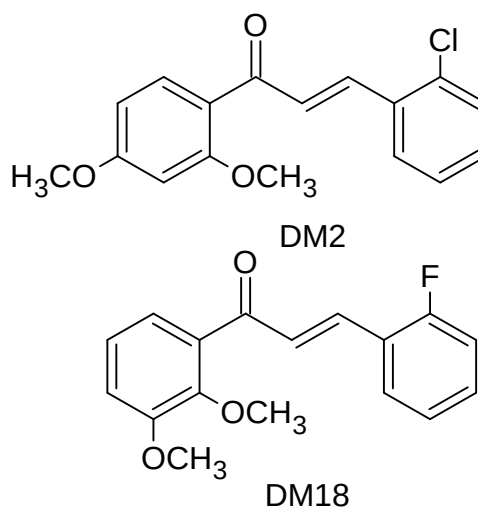
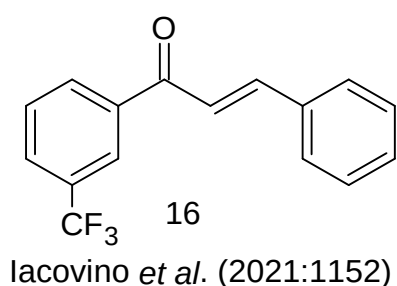


Figure 2.19: Summary of structural modifications observed in synthetic chalcones with PD-related activity as adapted from Królicka *et al.* (2022:5).

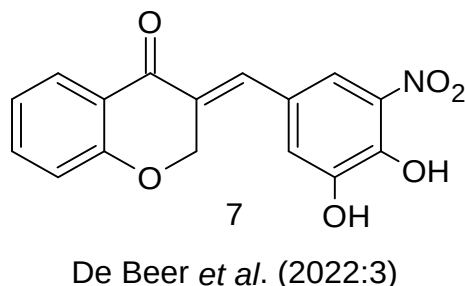
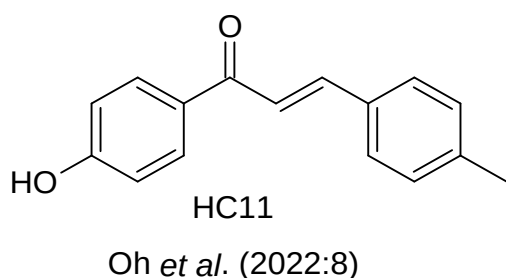
The quorum sensing (QS) system serves as a communication network among bacteria. It involves various transduction pathways that regulate key processes, including biofilm formation, virulence, antibiotic resistance, motility, and sporulation (Peng *et al.*, 2022:6; Sionov & Steinberg, 2022:1). Naturally occurring chalcones like echinatin and phloretin (Figure 2.21) have been shown to interfere with this system and SAR studies of phloretin reported that this antagonism can be attributed to the presence of two hydroxyl moieties on ring A (Bai *et al.*, 2022:13; Paczkowski *et al.*, 2017:4064; Wang *et al.*, 2023:10). A study done by Nijampatnam and colleagues has revealed that the antibiofilm activity of polyhydroxychalcones can be attributed to the stereochemistry of the double bond and the amount and regiochemistry of the hydroxyl groups. The chalcones that contained a hydroxyl group in the *meta*-position on ring A exhibited higher selectivity for biofilm inhibition (Nijampatnam *et al.*, 2016:3512). The cytoplasmic membranes of bacteria consist of equal

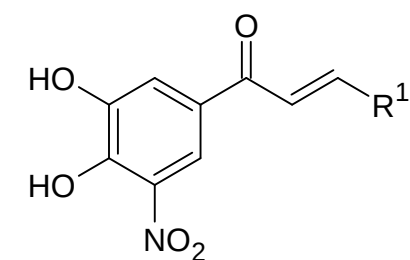
portions of proteins and lipids that serve as envelopes outlining the cell cytoplasm and regulate the transport and diffusion of small molecules in and out of the cell (Strahl & Errington, 2017:520). This helps to maintain vital survival chemicals and nutrients inside the cell. A break in the cell membrane can lead to cell death (Nourbakhsh *et al.*, 2022:35). Phloretin has the potential to cause damage to cell respiration and the cell membranes of bacteria (Wang *et al.*, 2023:12). Chalcones exhibit antibacterial activity by interfering with metabolic pathways, subsequently decreasing pathogenicity. Phloretin, licochalcone A to D, isoliquiritigenin, isobavachalcone and butein have been reported to target these metabolic pathways (Wang *et al.*, 2023:4).

Chalcones remain a popular scaffold for researchers due to their simplicity, straightforward synthesis and replaceable hydrogens to generate a variety of derivatives for possible therapeutic use (Xu *et al.*, 2019:2).



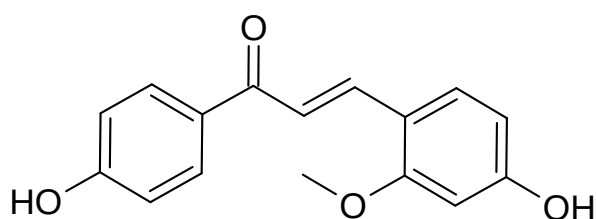
Rehuman *et al.* (2022:3)



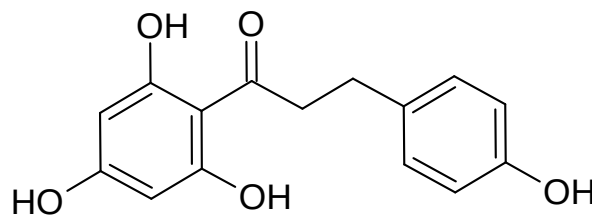


Hitge *et al.* (2020:127188)

Figure 2.20: Chalcone derivatives with activity against MAO-B.



Echinatin



Phloretin

Figure 2.21: Structures of natural chalcones echinatin and phloretin (Wang *et al.*, 2023:2).

2.4.2 Nitro-containing compounds

The nitro-group is a functional group consisting of nitrogen linked to two oxygens that has an electron-withdrawing effect since the nitrogen atom has no lone pair of electrons and bears a positive charge (Jezuita *et al.*, 2021:179; Noriega *et al.*, 2022:1). The electron-withdrawing effect is commonly observed within aromatic rings due to nitro-group resonance that will deactivate certain positions and cause changes in the molecular polarity of the compound. In some cases, this will favour the interaction of the molecule with certain nucleophilic sites in protein structures such as enzymes leading to the inhibition of the enzyme (Noriega *et al.*, 2022:1; Olender *et al.*, 2018:1; Squella *et al.*, 2005:566). Overall, the nitro-group can act as both a pharmacophore and a toxicophore. The nitro-group is not always directly involved in the activity of the molecule; however, it can influence the polarity and stereochemistry (Noriega *et al.*, 2022:2). As discussed, COMT-inhibitors such as entacapone and tolcapone contain a nitrocatechol moiety with a nitro-group that is responsible for their selectivity for COMT and its subsequent use in the treatment of PD (Rivest *et al.*, 1999:S35).

Nitro-containing antibiotics are some of the first-line treatments for infections caused by microorganisms, including metronidazole and chloramphenicol. The most common

mechanism of action of these compounds includes the reduction of the nitro-group to yield toxic intermediates that bind covalently to DNA, leading to nuclear damage and cell death (Müller *et al.*, 2018:271; Noriega *et al.*, 2022:2). Metronidazole, secnidazole, tinidazole and ornidazole (Figure 2.22) are prodrugs that contain the 5-nitroimidazole moiety that undergoes intercellular reduction to form a nitro-anion radical (NO_2^-) that plays an important role in its mechanism of antimicrobial action (Oliveira *et al.*, 2018:572).

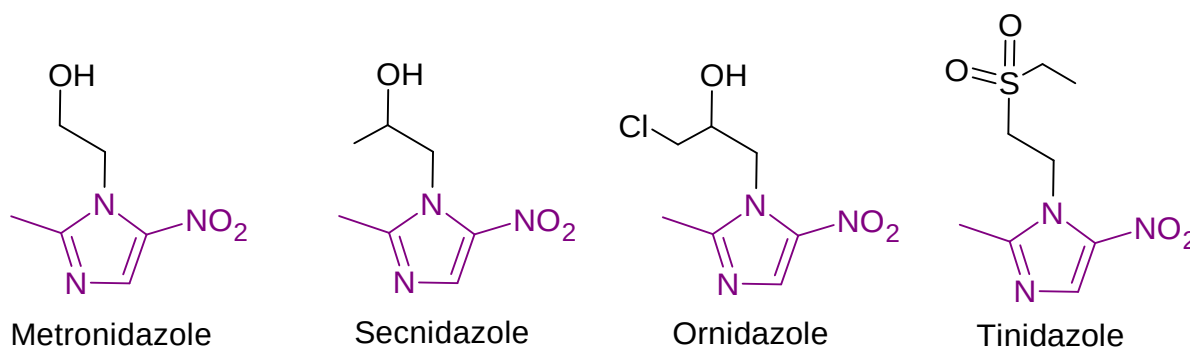


Figure 2.22: Chemical structures of 5-nitroimidazole prodrugs (Oliveira *et al.*, 2018:572).

2.4.3 Pyrazoline-containing compounds

Pyrazolines are five-membered heterocyclic rings (Figure 2.23) with two nitrogen atoms that serve as isosteres for other heterocyclic rings, *viz.*, imidazole, thiazole and oxazole. Pyrazoline derivatives have several benefits for neurodegenerative diseases (Ahsan *et al.*, 2022:38207). Pyrazoline-derivatives synthesised by Hitge *et al.* (2020:1) had both COMT and MAO-B inhibitory activity. The most active compound (figure 2.24) had activity against COMT with an IC_{50} -value of 0.048 μM and moderate activity against MAO-B with an IC_{50} -value of 83.2 μM . The compound was more active than entacapone. Furthermore, 2-pyrazoline derivatives synthesised by Evranos-Aksöz *et al.* (2014:3278) were shown to inhibit both MAO isoforms competitively and reversibly, except one compound (Figure 2.24) which was a selective MAO-B inhibitor.

Some pyrazoline derivatives have also been shown to have antimicrobial effects. Derivatives synthesised by Evranos Aksöz *et al.* (2020:500) had a wide range of antimicrobial activity against both Gram-negative and Gram-positive bacteria with minimum inhibitory concentration (MIC) values ranging from 32 – 512 $\mu\text{g/ml}$. The most active compound (figure 2.25) had activity against *E. faecalis* (Evranos Aksöz *et al.*, 2020:500). Furthermore, pyrazoline derivatives synthesised by Farooq & Ngaini (2020) yielded two compounds (Figure 2.25) with noteworthy activity against *S. aureus* (Farooq & Ngaini, 2020:9974).

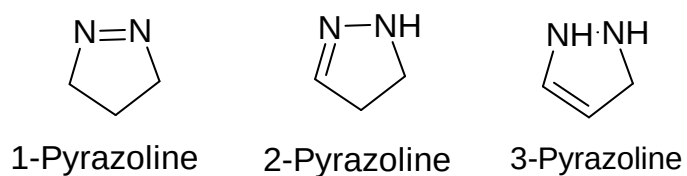


Figure 2.23: Different isomers of pyrazoline (Ahsan *et al.*, 2022:38207).

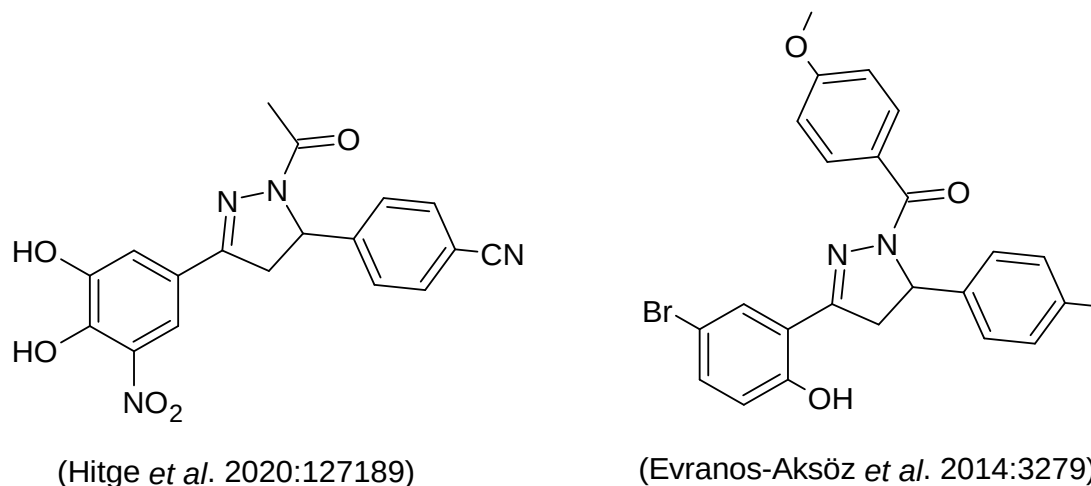


Figure 2.24: Active pyrazoline derivatives in the treatment of PD.

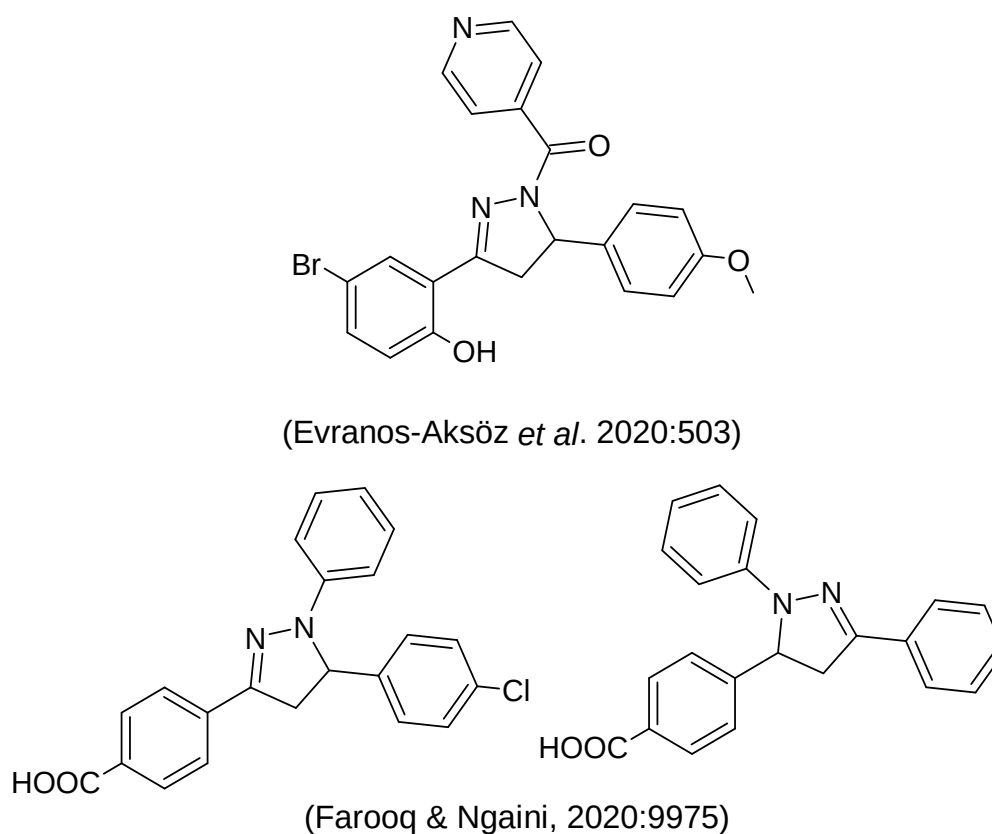


Figure 2.25: Antibacterial pyrazoline derivatives.

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CHAPTER 3: ARTICLE 1

In vitro and *in silico* antibacterial evaluation of nitrocatechol chalcone and pyrazoline derivatives

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Abstract

The aim of this study was to determine the *in vitro* antibacterial activity of nitrocatechol chalcone and pyrazoline derivatives previously synthesised by our research group against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Klebsiella aerogenes*, and to create and validate a pharmacophore model using this data. The enrichment factor (EF^{10%}) and the area under the receiver operating characteristic (ROC-AUC) curve were used to validate the pharmacophore model. Using the validated pharmacophore model novel derivatives were designed and synthesised, whereafter the *in vitro* antibacterial activity of these derivatives was also determined against the susceptible

bacteria. After the initial screening, the derivatives only had activity against *S. aureus*, with compound **2a**, **2b** and **1b** (1 - 2 µg/ml) having comparable activity to tetracycline (2 µg/ml). A common feature pharmacophore model (max. fit: 4, rank score: 84.02) was able to accurately identify active nitrocatechol chalcones and pyrazoline derivatives within a decoy test set. The best performing pharmacophore model, *i.e.*, hypothesis 9 (EF^{10%}: 6.7, ROC-AUC: 0.85) indicated that four hydrogen bond acceptors are important for antibacterial activity. This model was used to guide the design and synthesis of novel nitrocatechol chalcone and pyrazoline derivatives of which the *in vitro* antibacterial activity against both the drug susceptible and resistant *S. aureus* strains were determined. The most active compounds were **3i** (0.5 µg/ml) and **3c** (0.5 µg/ml) against the susceptible and resistant strain respectively, which were more active than tetracycline.

Keywords Drug repurposing, Nitrocatechol, Chalcone, Pyrazoline, Antibacterial activity, Common feature pharmacophore modelling

Introduction

Antimicrobial resistance has been a threat to human health for decades as exemplified by the development of penicillin resistant *Staphylococcus aureus* strains in the late 1950s [1]. The World Health Organization (WHO) has identified antimicrobial resistance as one of the top 10 threats to global health and launched a call-to-action against antimicrobial resistance stating that a one health approach will enhance national and global efforts to address the issue [2,3].

Novel drug development and approval is an expensive and time-consuming process that can take up to 15 years [4]. It also suffers from high attrition rates, with about 90% of experimental drugs failing the first phase of clinical trials [5]. Drug repurposing is a drug discovery strategy that entails identifying new indications for approved and investigational drugs that have been designed to target a different target [6]. This approach has been successful in developing new treatments for various diseases including cancer, tuberculosis, and Covid-19 just to name a few [7]. Drug repurposing offers a significant benefit in that all drugs approved for market use have already cleared the scientific, clinical, regulatory, and safety hurdles [8].

Computer-aided drug design (CADD) is another valuable tool in the drug discovery and development process. CADD has the potential to accelerate the drug discovery process and

has been found to minimise final-stage failures and reduce overall cost [9,10]. The aim of this approach is to simulate drug-target interactions by utilising computational tools [11]. CADD can be divided into two main approaches: structure-based drug design, based on the three-dimensional knowledge of the target, and ligand-based drug design, which correlates the observed biological activity of known ligands to their chemical features, and is utilised when the target is unknown [12].

Historically, natural products have been the primary source of new drugs [13]. One such class is chalcones, which contains an α,β -unsaturated carbonyl system [14]. Numerous studies have focused on the potential pharmaceutical uses of chalcones due to their low toxicity and known bioactivity e.g., antifungal, anti-inflammatory and anti-cancer properties [15,16]. Chalcone-based compounds such as metochalcone (choleretic drug) and sofalcone (anti-ulcer drug) have been approved for clinical use [13,17,18]. Both natural and synthetic chalcones have also shown antibacterial activity against various bacteria including Methicillin sensitive *S. aureus* (MSSA), Methicillin resistant *S. aureus* (MRSA) [13,19], *Bacillus subtilis*, *Staphylococcus epidermidis*, *Micrococcus luteus* [13,20], *Mycobacterium tuberculosis*, and *Bacillus anthracis* [21].

The nitrocatechol moiety is classified as a nitrophenol, consisting of a benzene ring bearing two hydroxyl groups and a nitro-group on different carbon atoms [22]. Nitrocatechol containing compounds are known to act as catechol O-methyl transferase (COMT) inhibitors, e.g., tolcapone, entacapone and opicapone, which are 3-nitrocatechol containing compounds approved for pharmaceutical use [23].

Pyrazolines are nitrogen-containing electron-rich heterocyclic compounds [24,25]. Pyrazoline containing bioactive compounds possess antibacterial, antifungal and antimalarial activity [26,27]. It has also been suggested that adding different substituents such as electron donors and electron acceptors to the pyrazoline nucleus can lead to structural stability and enhanced biological activity [24].

In this study, the *in vitro* antibacterial activity of previously synthesised nitrocatechol chalcone and pyrazoline derivatives [28] was determined against *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Klebsiella aerogenes* using the broth microdilution method. The minimum inhibitory concentration (MIC) of each active derivative was subsequently determined. Based on these results, a common feature pharmacophore model was created and validated to identify chemical entities essential for antibacterial activity. Based on the pharmacophore model, a novel series of nitrocatechol chalcone and

pyrazoline derivatives were designed and synthesised, and their *in vitro* antibacterial activity was determined against both drug susceptible and resistant *S. aureus* strains.

Materials and methods

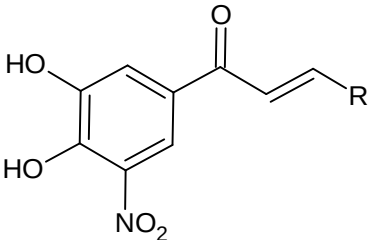
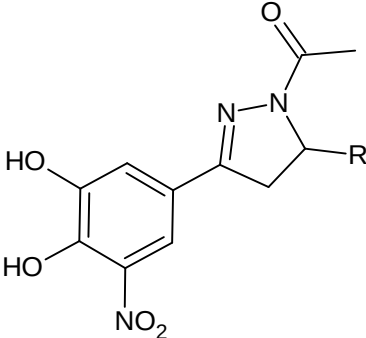
The *in vitro* antibacterial activity of the nitrocatechol chalcones and pyrazolines, were determined with the broth microdilution method against *S. aureus* (ATCC 29213), *S. aureus* (ATCC 43300), *K. pneumoniae* (ATCC 13883), *A. baumannii* (ATCC 19606) and *K. aerogenes* (ATCC 13048) (Virginia, United States). All non-sterile equipment were autoclaved at 121 °C for 15 min using an Equitron Medica autoclave (Equitron Medica Ltd) (United Scientific, South Africa). An ultraviolet/visible spectrophotometer (United Scientific, South Africa) was used to measure the optical density of the pathogens at a wavelength of 600 nm (OD₆₀₀). All pathogens were incubated in a 150 L digital shaking incubator (Labotech, South Africa).

Broth microdilution method

The MIC of the derivatives was determined according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method [29,30]. The previously synthesised nitrocatechol chalcone (**1a-i**) and pyrazoline (**2a-d**) derivatives [28] (Table 3.1) were firstly screened for antibacterial activity against all bacteria at only one concentration, *i.e.*, 256 µg/ml. Thereafter, the MIC of all derivatives was determined against the bacterial strains found to be susceptible. These data were used to develop and validate a common feature pharmacophore model (described below) which was used to design novel derivatives. The envisioned novel nitrocatechol chalcone (**3a-k**) and pyrazoline (**4a-j**) derivatives were synthesised, and their MIC was also determined against the relevant bacterial strains.

The derivatives were dissolved in dimethylsulfoxide (DMSO) (Merck, South Africa) and tetracycline (positive control) (Sigma Aldrich, South Africa) was dissolved in distilled water (dH₂O) whereafter they were filter sterilised (0.2 µm) (Lasec, South Africa). The derivative/DMSO stock solutions were subsequently dissolved in sterile dH₂O (512 µg/ml) resulting in a final concentration of 256 µg/ml. Positive control was dissolved in sterile dH₂O (64 µg/ml) resulting in a final concentration of 32 µg/ml. Each corning flat bottom 96-well plate (Lasec, South Africa) was primed with the positive control (200 µl, well B1), and two derivatives (200 µl, well C1, D1, E1 and F1, G1, H1).

Table 3.1: The nitrocatechol chalcone (**1a-i**) and pyrazoline (**2a-d**) derivatives previously synthesised by our group [28].

 1a-i		 2a-d	
Nitrocatechol chalcone		Pyrazoline derivatives	
R		R	
1a	3-CN-C ₆ H ₄ -	2a	3-CN-C ₆ H ₄ -
1b	4-CN-C ₆ H ₄ -	2b	4-CN-C ₆ H ₄ -
1c	2-thienyl-	2c	2-furyl-
1d	3-thienyl-	2d	3-OH-C ₆ H ₄ -
1e	2-furyl-		
1f	3-furyl-		
1g	2-thiazolyl-		
1h	2-pyrrole-		
1i	1-methyl-1 <i>H</i> -pyrazol-4-yl-		

A growth control, *i.e.*, sterile dH₂O (100 µl, wells A1 - 6), and sterility control *i.e.*, sterile Mueller Hinton broth (MHB) (Sigma Aldrich, South Africa) (200 µl, wells A7 - 12), were also added to each 96-well plate. The remaining empty wells were primed with 100 µl sterile dH₂O. Two-fold serial dilutions were made of the derivatives and positive control using a multichannel pipette. During screening the above-mentioned method was altered by screening all derivatives only at the highest concentration (256 µg/ml) in triplicate ($n = 3$) without making serial dilutions, allowing 24 derivatives to be screened on one 96 well plate.

The inoculum of each bacterium was prepared by suspending 2-3 colonies in sterile MHB (10 ml) and incubating the suspensions at 35 ± 2 °C for 2 - 5 h, shaking at 100 rpm. The inoculum was diluted with sterile MHB to a concentration of 1×10^6 CFU/ml. Lastly, 100 μ l inoculum was added to all wells (excluding the sterility control) resulting in a final volume of 200 μ l and a bacterial concentration of 5×10^5 CFU/ml in each well. To avoid changes in the bacterial cell count the inoculum was used within 30 min. To conduct the viability count, 10 μ l from one growth control well was suspended in 10 ml sterile MHB, then 100 μ l of this suspension was plated onto MuellerHinton agar (MHA) petri dishes ($n = 3$). The petri dishes were incubated at 35 ± 2 °C for 20 - 24 h, after which the colonies were counted. A colony count of 20 – 80 CFUs indicated that the correct bacterial concentration was used [29].

The plates were covered with their lids and incubated at 35 ± 2 °C for 20 - 24 h together with 60 μ l resazurin (150 μ g/ml) (Merck, South Africa). The bacterial growth was measured by the irreversible reduction of resazurin (blue) to resorufin (pink) by metabolically active bacteria. The colour changes were assessed visually, with any change from blue to pink considered positive for bacterial growth [31]. The MIC was defined as the lowest concentration where no metabolic activity was observed.

Common feature pharmacophore modelling

The docking procedures were conducted using Accelrys Discovery Studio (DS) 3.1 (San Diego, United States). The area under the receiver operating characteristic (ROC-AUC) curve calculations were conducted using SPSS Statistics for Windows, version 27 (SPSS Inc. Chicago, Ill., USA). The enrichment factor (EF) was calculated with Microsoft Excel for Microsoft 365 (Microsoft, United States) using equation 1.

All the structures were drawn in DS, then typed with the CHARMM force field before initiating the *Prepare ligands* protocol. The pyrazoline derivatives (**2a-d** and **4a-j**) are chiral molecules and both the R and S enantiomers of each derivative were included in the docking experiments. The five derivatives with the highest activity against *S. aureus* (**1a**, **1b**, **1d**, **S-2a**, **R-2a**, **S-2b**, **R-2b**) were selected for the training set and assigned a principal value of 2. A maximum omitted feature (MaxOmitFeat) of 0 was used for each of the derivatives in this set. Four common chemical groups, *i.e.*, hydrogen bond acceptors (A) and hydrogen bond donors (D), were identified by the *Feature Mapping Protocol*. During the *Common Feature Pharmacophore Generation* protocol, a diverse set of derivatives were generated by considering the BEST 200 conformations (20 kcal/ml threshold; 2 Å maximum interference

distance). The generated pharmacophore models were ranked according to their rank scores.

The remaining 8 derivatives and their enantiomers (**1c**, **1e**, **1f**, **1g**, **1h**, **1i**, *S*-**2c**, *R*-**2c**, *S*-**2d**, *R*-**2d**) were allocated to the test set and lastly, 1028 decoy compounds were downloaded from the Schrödinger database which contains druglike decoys with an average molecular weight of 400 Dalton [32,33]. The test set was docked into each of the 10 generated pharmacophore models with the *Ligand Profiler* protocol and a MaxOmitFeat of 1 assigned to all compounds which specify that all features except one should be mapped [34,35]. Using this data the EF^{10%} and the ROC-AUC which are common metrics used to validate the ability of the pharmacophore model to differentiate between active derivatives and decoy compounds were calculated [36–39]. The EF can be calculated by equation 1 below [37,40].

$$EF = (Hits_{sel} / Hits_{tot}) \times (NC_{tot} / NC_{sel}) \dots\dots\dots \text{Equation 1}$$

For this study the EF was determined at 10% of the test set. The equation components are the active derivatives correctly identified at 10% ($Hits_{sel}$), the total number of active derivatives in the entire test set ($Hits_{tot}$), the total number of compounds in the entire test set (NC_{tot}), and the number of compounds within 10% of the test set (NC_{sel}). A major disadvantage of the EF, however, is the fact that it depends on the ratio between the active and inactive compounds [40,41].

The ROC-AUC is another tool which can estimate the ability of a pharmacophore model to discriminate between active and decoy compounds [36,42,43]. The ROC-AUC values can be interpreted as follow: $0.9 \leq AUC \leq 1$ is excellent; $0.8 \leq AUC < 0.9$ is good; $0.7 \leq AUC < 0.8$ is fair; $0.5 \leq AUC < 0.7$ is poor; and $AUC < 0.5$ is a failure [40,44].

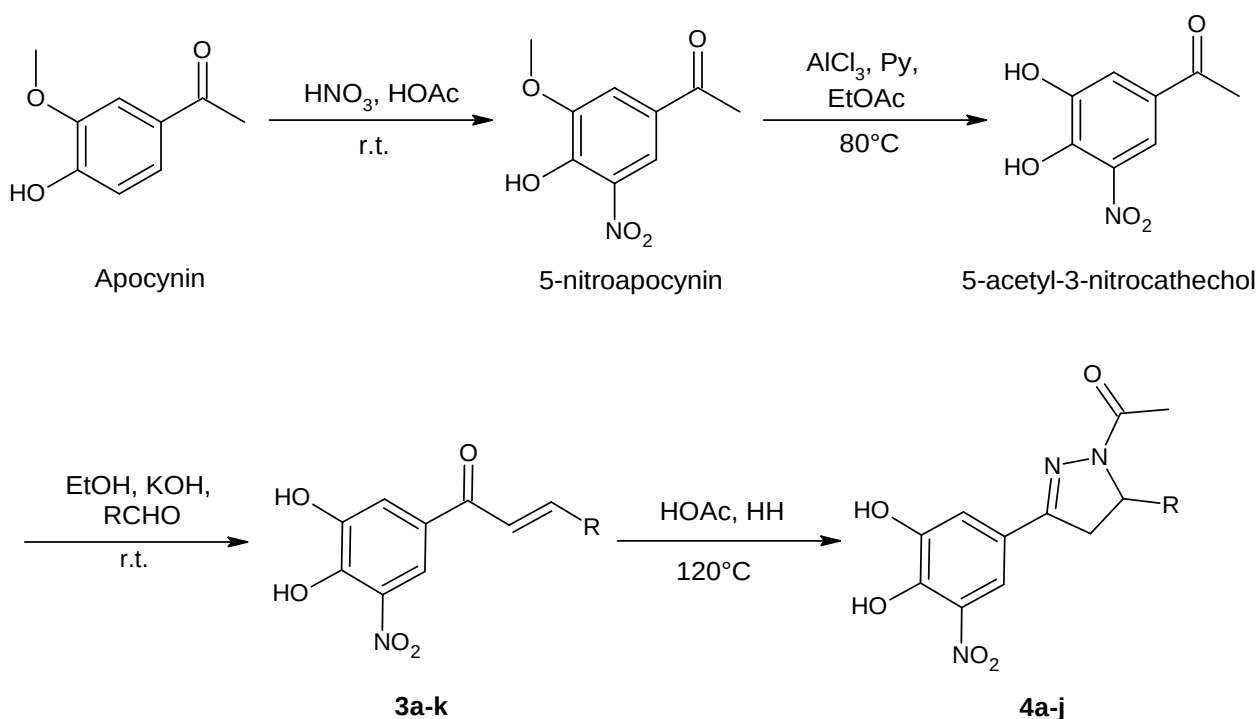
Synthesis of novel nitrocatechol chalcone and pyrazoline derivatives

All reagents were used without further purification and were obtained from Merck (South Africa). The structures were confirmed by nuclear magnetic resonance (NMR) (Bruker Advance III 600) by recording the one-dimensional proton (¹H) (600 MHz) and carbon (¹³C) (150 MHz) spectra as well as the two-dimensional heteronuclear single quantum coherence (HSQC) spectrum of each derivative. The NMR data were processed using MestReNova 14.1.2 and the information reported is the integration, multiplicity and coupling constants (J) given in hertz (Hz). Chemical shifts (δ) were given in parts per million (ppm) and referenced to the DMSO-d₆ (Merck, South Africa) signal at 2.5 ppm for ¹H NMR and 40.45 ppm for ¹³C NMR. Multiplicities are given as either a singlet (s), doublet (d), doublet of doublets (dd),

doublet of doublet of doublets (ddd), triplet (t) or multiplet (m). High-resolution mass spectrometry (HRMS) was conducted in atmospheric pressure chemical ionization (APCI) mode (Bruker MicroTOF Q II). Melting points were measured by means of differential scanning calorimetry (DSC) with a Mettler DSC 3 Star System (Mettler Toledo, Greifensee, Switzerland). Thin layer chromatography (TLC) was conducted using silica gel 60 plates impregnated with an UV254 fluorescent indicator (Merck, South Africa).

The purity of each compound was determined by high-performance liquid chromatography (HPLC) (Shimadzu Nexera-I LC 2040 C 3D Plus HPLC system) using a Shimadzu diode array detector. Milli-Q water (A) and HPLC grade acetonitrile (Merck, South Africa) (B) containing 0.1% formic acid were used as mobile phase at a starting ratio of 40:60, with a flow rate of 0.35 mL/min. A Venusil XBP C18 column (2.1 × 150 mm, 5 µm, 100 Å) was used for the separation. The concentration of B was maintained at 40% for 0.5 min and increased to 100% over a period of 3 min. The concentration of B was then maintained at 100% until 5 min and returned to 40% B at 5.1 min whereafter the system was allowed to equilibrate until 8 min, giving a total run-time of 8 min per sample. Each test compound solution (5 µl) was injected, and the eluent was monitored at a wavelength of 310 nm.

The reaction method used to synthesise the novel nitrocatechol chalcones (**3a-k**) and pyrazoline (**4a-j**) derivatives is described elsewhere and depicted in scheme 1 [28]. In short, the three-step synthetic route involved the nitration of apocynin to yield 5-nitroapocynin, followed by the demethylation of 5-nitroapocynin to give 5-acetyl-3-nitrocatechol [23,28,45]. The Claisen-Schmidt condensation reaction was used to conjugate 5-acetyl-3-nitrocatechol to the relevant aromatic aldehyde to yield the target nitrocatechol chalcone derivatives, *i.e.*, **3a-k** [28,46,47]. The corresponding pyrazoline derivatives, *i.e.*, **4a-j**, were obtained by reacting the relevant chalcone with hydrazine hydrate [46,48,49]. The derivatives were purified by crystallisation from a mixture of acetonitrile and methanol.



Scheme 1: The synthesis of the novel nitrocatechol chalcone (**3a-k**) and pyrazoline (**4a-j**) derivatives [28]. Reagents; nitric acid (HNO_3), acetic acid (HOAc), aluminium chloride (AlCl_3), Py (pyridine), ethyl acetate (EtOAc), ethanol (EtOH), potassium hydroxide (KOH), relevant aromatic aldehyde (RCHO), hydrazine hydrate (HH).

Results and discussion

Minimum inhibitory concentration and pharmacophore development

The *in vitro* antibacterial activity of the nitrocatechol chalcone (**1a-i**) and pyrazoline (**2a-d**) derivatives previously synthesised by our group (Table 3.1) were determined, whereafter the data were used to create and validate a common feature pharmacophore model. Each pyrazoline derivative is chiral and the MIC of the racemate was determined.

During initial screening all derivatives showed activity against *S. aureus* (Gram-positive), but none against the Gram-negative bacteria, *i.e.*, *K. pneumoniae*, *A. baumannii* and *K. aerogenes* (Table 3.2). The dearth of activity against the Gram-negative bacteria is because of the presence of an outer membrane which is absent in Gram-positive bacteria. This important layer prevents the entry of drugs into the bacterial cell rendering them less effective or inactive [50–53].

Table 3.2: Screening and MIC results of previously synthesised compounds [28].

Derivatives	Screening				MIC (µg/ml)
	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>A. baumannii</i>	<i>K. aerogenes</i>	<i>S. aureus</i>
1a	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	4
1b	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	2
1c	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	8
1d	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	4
1e	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	8
1f	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	16
1g	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	8
1h	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	64
1i	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	8
2a	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	2
2b	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	1
2c	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	16
2d	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	>256
Tetracycline	2 µg/ml	8 µg/ml	2 µg/ml	2 µg/ml	2

^a Active: A derivative had an MIC of ≤ 256 µg/ml

^b Inactive: A derivative had an MIC >256 µg/ml

After screening, the precise MIC of all derivatives (Table 3.2) were determined against *S. aureus*. All derivatives showed activity against *S. aureus* except **2d**. With regards to the nitrocatechol chalcones, **1b** (2 µg/ml) had the same activity compared to tetracycline (2 µg/ml), whilst **1a** (4 µg/ml) and **1d** (4 µg/ml) had similar activity. The other nitrocatechol chalcones, *i.e.*, **1c**, **1e-i**, had weaker antibacterial activity (8 - 64 µg/ml) compared to tetracycline. With regards to the pyrazoline derivatives, **2b** (1 µg/ml) and **2a** (2 µg/ml) were the most active, whereas the remaining two derivatives exhibited either low antibacterial activity, *i.e.*, **2c** (16 µg/ml), or no activity *i.e.*, **2d** (>256 µg/ml).

Common feature pharmacophore modelling

Based on the MIC data the derivatives were divided into either a training set, used to create the common feature pharmacophore models, or a test set (including the decoy compounds), which was used to validate them. Ten pharmacophore hypotheses (hypos.) were created by DS and the rank score (Table 3.3), EF^{10%} and ROC-AUC (Table 3.4) of each were considered when choosing the best hypo.

A high rank score indicates a low chance that the training set derivatives mapped the relevant hypo. by chance correlation [35,54]. Hypo. 1 – 4, hypo. 5 – 7 and hypo. 8 - 10 had acceptable rank scores, *i.e.*, 85.42, 84.54 and 84.02, respectively (Table 3.3).

Hypo 9. had the best EF^{10%} and ROC-AUC values (Table 3.4). Considering hypo. 9 it appears that four hydrogen acceptors are important for antibacterial activity (Figure 3.1). By mapping **R-2b** with hypo.9 (Figure 3.1) it is evident that one of the oxygen atoms of the nitro-group, one of the hydroxy-groups on ring A, the carbonyl-moiety in the pyrazoline ring as well as the nitrile substituent on ring B acts as important hydrogen bond acceptors. A hydrogen bond takes place between an electron rich heteroatom and an electron deficient hydrogen atom [55]. The pharmacophore model identified the nitrile group of derivative **1a**, **1b**, **2a** & **2b** (training set) as an important hydrogen bond acceptor. For the design of novel derivatives this hydrogen bond acceptor was important because different groups could easily be substituted on the various positions of ring B [56]. With this in mind, nitrocatechol chalcone (**3a-k**) and pyrazoline (**4a-j**) derivatives (Table 3.5) with different electron withdrawing substituents on ring B was synthesised, whereafter their *in vitro* MIC activity was determined against both the drug susceptible (ATCC 29213) and methicillin resistant (ATCC 43300) *S. aureus* strains (Table 3.6).

Table 3.3: Summary of the pharmacophore hypotheses created for *S. aureus*.

Hypo.	Feature ^a	Rank score	Direct hit ^b	Partial hit ^c	Max fit
01	DAAA	85.42	1111111	0000000	4
02	DAAA	85.42	1111111	0000000	4
03	DAAA	85.42	1111111	0000000	4
04	DAAA	85.42	1111111	0000000	4
05	DAAA	84.54	1111111	0000000	4
06	DAAA	84.54	1111111	0000000	4
07	DAAA	84.54	1111111	0000000	4
08	AAAA	84.02	1111111	0000000	4
09	AAAA	84.02	1111111	0000000	4
10	AAAA	84.02	1111111	0000000	4

^a D = Hydrogen bond donor, A = Hydrogen bond acceptor

^b Indicates whether (1) or not (0) a molecule is mapped

^c Indicates whether (1) or not (0) a molecule mapped all but one feature

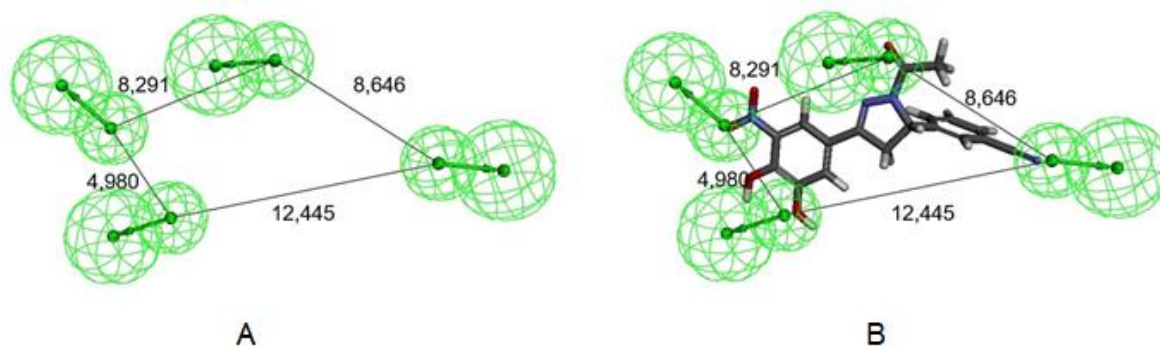
**Figure 3.1:** Hypothesis 9 (A) with inter-feature distance constraints and (B) with *R-2b* mapped.

Table 3.4: EF^{10%} and ROC-AUC of the pharmacophore hypotheses (hypo.).

Hypo.	EF ^{10%}	ROC-AUC
1	4.5	0.67±0.07
2	4.4	0.70±0.04*
3	5.6	0.84±0.00**
4	5.5	0.76±0.07
5	5.8	0.65±0.12
6	5.6	0.82±0.00**
7	4.5	0.75±0.01**
8	5.5	0.74±0.02*
9	6.7	0.85±0.00**
10	4.5	0.66±0.09

* Statistically significant ($p \leq 0.05$).

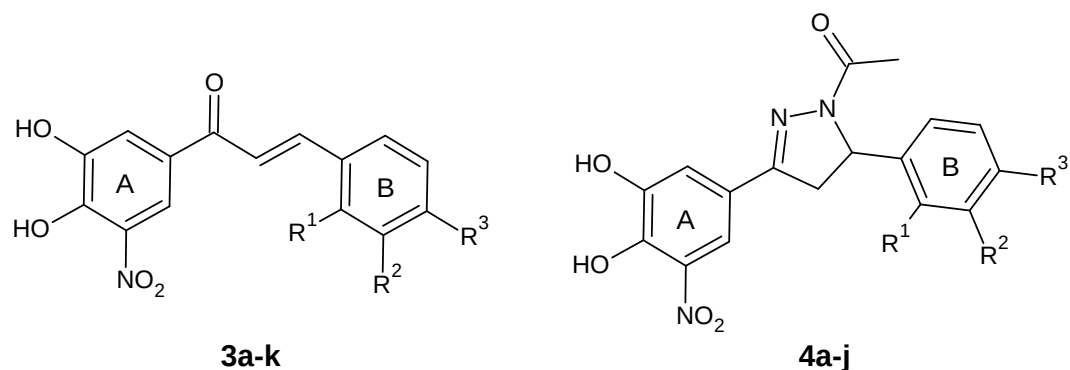
** Statistically significant ($p \leq 0.01$).

Synthesis of the nitrocatechol chalcone (3a-k) and pyrazoline (4a-j) target compounds

Both the nitrocatechol chalcone derivatives (**3a-k**) as well as the corresponding pyrazoline derivatives (**4a-j**) were synthesised successfully (Table 3.5).

(2E)-3-(4-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (**3a**)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 4-chlorobenzaldehyde (1 mmol) to a yield of 79.5% as a yellow-coloured powder, mp: 213 - 213.57°C. ¹H NMR (600 Hz, DMSO) δ 8.31 (d, J = 2.1 Hz, 1H, H-2), 7.97 (d, J = 8.5 Hz, 2H, H-19,21), 7.95 (d, J = 15.3 Hz, 1H, H-8), 7.74 (d, J = 2.1 Hz, 1H, H-3), 7.71 (d, J = 15.3 Hz, 1H, H-15), 7.53 (d, J = 8.5 Hz, 2H, H-17,20). ¹³C NMR (151 Hz, DMSO) δ 186.64 (C-7), 148.31 (C-5), 146.29 (C-6), 143.00 (C-15), 138.01 (C-1), 135.58 (C-18), 134.2 (C-16), 131.16 (C-19, C-21), 129.39 (C-17, C-20), 128.28 (C-4), 122.54 (C-8), 117.87 (C-3), 117.27 (C-2). APCI-HRMS m/z : calculated for C₁₅H₁₁ClNO₅ (MH⁺), 320.0320, found 320.0320. Purity (HPLC-210 nm): 99.6%.

Table 3.5: The synthesised nitrocatechol chalcone and pyrazoline derivatives.

Nitrocatechol chalcone derivatives				Pyrazoline derivatives			
	R ¹	R ²	R ³		R ¹	R ²	R ³
3a	H	H	Cl	4a	H	H	Cl
3b	Cl	H	Cl	4b	Cl	H	Cl
3c	H	Cl	Cl	4c	H	Cl	Cl
3d	H	Cl	H	4d	H	Cl	H
3e	H	H	OCH ₂ CH ₃	4e	H	H	OCH ₂ CH ₃
3f	H	H	I	4f	H	H	I
3g	H	H	SCH ₃	4g	H	H	SCH ₃
3h	F	H	Br	4h	F	H	Br
3i	H	CF ₃	H	4i	H	F	H
3j	H	F	H	4j	H	H	F
3k	H	H	F				

(2E)-3-(2,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (**3b**)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 2,4-dichlorobenzaldehyde. Yield: 262 mg (12.7%) as a yellow-coloured powder, mp: 202.4 - 204.0°C. ¹H NMR (600 Hz, DMSO) δ 8.32 (d, *J* = 2.1 Hz, 1H, H-2), 8.28, (d, *J* = 8.5 Hz, 1H, H-19), 8.01 (d, *J* = 15.5 Hz, 1H, H-15), 7.95 (d, *J* = 15.5 Hz, 1H, H-8), 7.75 (d, *J* = 2.2 Hz, 1H, H-20), 7.72 (d, *J* = 2.1 Hz, 1H, H-3), 7.55 (dd, *J* = 8.5 Hz, 2.1, 1H, H-17). ¹³C NMR (151

Hz, DMSO) δ 186.43 (C-7), 148.42 (C-5), 146.68 (C-6), 137.93 (C-1), 137.67 (C-15), 136.12 (C-21), 135.63 (C-18), 131.76 (C-19), 130.41 (C-16), 129.95 (C-17), 128.37 (C-20), 127.90 (C-4), 125.00 (C-8), 117.72 (C-3), 117.55 (C-2). APCI-HRMS m/z : calculated for $C_{15}H_{10}Cl_2NO_5$ (MH^+), 353.9931, found 353.9932. Purity (HPLC-210 nm): 98.3%.

(2E)-3-(3,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3c)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 3,4-dichlorobenzaldehyde. Yield: 416 mg (19.0%) as a yellow-coloured powder, mp: 222.9 - 223.8°C. ^1H NMR (600 Hz, DMSO) δ 8.33 (d, J = 2.1 Hz, 1H, H-2), 8.29 (d, J = 2.0 Hz, 1H, H-18), 8.04 (d, J = 15.6 Hz, 1H, H-13), 7.90 (dd, J = 8.4 Hz, 1H, H-17), 7.74 (d, J = 2.1 Hz, 1H, H-3), 7.72 (d, J = 8.4 Hz, 1H, H-19), 7.70 (d, J = 15.6 Hz, 1H, H-15). ^{13}C NMR (151 Hz, DMSO) δ 186.53 (C-12), 148.34 (C-5), 146.42 (C-6), 141.67 (C-15), 138.05 (C-1), 136.04 (C-21), 133.20 (C-16), 132.27 (C-19), 131.44 (C-20), 130.68 (C-18), 129.74 (C-17), 128.13 (C-4), 123.82 (C-13), 117.85 (C-3), 117.40 (C-2). APCI-HRMS m/z : calculated for $C_{15}H_{10}Cl_2NO_5$ (MH^+), 353.9931, found 353.9931. Purity (HPLC-210 nm): 92.4%.

(2E)-3-(3-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3d)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 3-chlorobenzaldehyde. Yield: 481 mg (27.0%) as a yellow-coloured powder, mp: 191.67 - 192.67°C. ^1H NMR (600 Hz, DMSO) δ 8.33 (d, J = 2.1 Hz, 1H, H-2), 8.18 (m, 1H, H-18), 8.02 (d, J = 15.6 Hz, 1H, H-13), 7.84 (d, J = 4.8 Hz, 1H, H-17), 7.74 (d, J = 2.1 Hz, 1H, H-3), 7.71 (d, J = 15.6 Hz, 1H, H-15), 7.53-7.46 (m, 2H, H-19, H-21). ^{13}C NMR (151 Hz, DMSO) δ 186.64 (C-12), 148.31 (C-5), 146.33 (C-6), 142.77 (C-15), 138.08 (C-16), 137.40 (C-20), 134.26 (C-1), 131.14 (C-21), 130.64 (C-19), 128.52 (C-17), 128.45 (C-18), 128.19 (C-4), 123.32 (C-13), 117.85 (C-3), 117.35 (C-2). APCI-HRMS m/z : calculated for $C_{15}H_{11}ClNO_5$ (MH^+), 320.0320, found 320.0322. Purity (HPLC-210 nm): 97.5%.

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-ethoxyphenyl)prop-2-en-1-one (3e)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 4-ethoxybenzaldehyde. Yield: 931 mg (47.3%) as a yellow-coloured powder, mp: 177.53 - 179.37°C. ^1H NMR (600 Hz, DMSO) δ 8.25 (d, J = 2.1 Hz, 1H, H-2), 7.85 (d, J = 8.5 Hz, 2H, H-19, H-21), 7.77 (d, J = 15.5 Hz, 1H, H-8), 7.72 (d, J = 2.1 Hz, 1H, H-3), 7.70 (d, J = 15.5 Hz, 1H, H-15), 7.00 (d, J = 8.5 Hz, 2H, H-17, H-20), 4.10 (q, J = 7.0 Hz, 2H, H-23), 1.35 (t, J

= 7.0 Hz, 3H, H-24). ^{13}C NMR (151 Hz, DMSO) δ 186.63 (C-7), 161.19 (C-18), 148.22 (C-5), 146.02 (C-6), 144.58 (C-15), 137.93 (C-4), 131.40 (C-19, C-21), 128.64 (C-1), 127.60 (C-16), 119.11 (C-8), 117.90 (C-3), 116.90 (C-2), 115.25 (C-17, C-20), 63.84 (C-23), 15.00 (C-24). APCI-HRMS m/z : calculated for $\text{C}_{17}\text{H}_{16}\text{NO}_6$ (MH^+), 330.0972, found 330.0969. Purity (HPLC-210 nm): 97.0%.

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-iodophenyl)prop-2-en-1-one (3f)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 4-iodobenzaldehyde. Yield: 326 mg (11.9%) as a yellow-coloured powder, mp: 220.37 - 223.57°C. ^1H NMR (600 Hz, DMSO) δ 8.30 (d, J = 2.1 Hz, 1H, H-2), 7.97 (d, J = 15.5 Hz, 1H, H-8), 7.85 (d, J = 8.5 Hz, 2H, H-17, H-20), 7.73 (d, J = 2.1 Hz, 1H, H-3), 7.73 (d, J = 8.5 Hz, 2H, H-19, H-21), 7.67 (d, J = 15.5 Hz, 1H, H-15). ^{13}C NMR (151 Hz, DMSO) δ 186.64 (C-7), 148.33 (C-5), 146.36 (C-6), 143.37 (C-15), 138.19 (C-17,20), 137.99 (C-4), 134.70 (C-16), 131.27 (C-19,21), 128.23 (C-1), 122.46 (C-8), 117.80 (C-3), 117.28 (C-2), 98.23 (C-18). APCI-HRMS m/z : calculated for $\text{C}_{15}\text{H}_{11}\text{INO}_5$ (MH^+), 411.9676, found 411.966. Purity (HPLC-210 nm): 98.3%.

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-[4-(methylsulfanyl)phenyl]prop-2-en-1-one (3g)

The title compound was prepared from 3,4-dihydroxy-5-nitroacetophenone (1 mmol) and 4-(methylsulfanyl)benzaldehyde. Yield: 1.312 g (63.4%) as a yellow-coloured powder, mp: 186.45 - 188.45 °C. ^1H NMR (600 Hz, DMSO) δ 8.28 (d, J = 2.1 Hz, 1H, H-2), 7.89 (d, J = 15.5 Hz, 1H, H-8), 7.85 (d, J = 8.5 Hz, 2H, H-19, H-21), 7.73 (d, J = 2.1 Hz, 1H, H-3), 7.71 (d, J = 15.5 Hz, 1H, H-15), 7.32 (d, J = 8.5 Hz, 2H, H-17, H-20), 2.51 (p, J = 19 Hz, 3H, H-23). ^{13}C NMR (151 Hz, DMSO) δ 186.62 (C-7), 148.26 (C-5), 146.11 (C-6), 144.13 (C-15), 142.53 (C-18), 138.00 (C-1), 131.52 (C-16), 129.95 (C-19, C-21), 128.51 (C-4), 125.98 (C-17, C-20), 120.61 (C-8), 117.90 (C-3), 117.05 (C-2), 14.64 (C-23). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{14}\text{NO}_5\text{S}$ (MH^+), 332.0587, found 332.0580. Purity (HPLC-210 nm): 97.5%.

(2E)-3-(4-bromo-2-fluorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3h)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 4-bromo-2-fluorobenzaldehyde. Yield: 350 mg (18.1%) as a yellow-coloured powder, mp: 172.13 - 183.47°C. ^1H NMR (600 Hz, DMSO) δ 8.29 (d, J = 2.1 Hz, 1H, H-2), 8.14 (t, J = 8.3 Hz, 1H,

H-19), 8.02 (d, $J = 15.6$ Hz, 1H, H-8), 7.75 (d, $J = 15.6$ Hz, 1H, H-15), 7.73 (d, $J = 2.1$ Hz, 1H, H-3), 7.70 (dd, $J = 10.2, 2.0$ Hz, 1H, H-20), 7.55 (dd, $J = 8.3, 2.0$ Hz, 1H, H-17). ^{13}C NMR (151 Hz, DMSO) δ 186.41 (C-7), 161.89 (C-18), 160.19 (C-18), 148.39 (C-5), 146.55 (C-6), 137.95 (C-1), 134.34 (C-15), 130.96 (C-19), 128.62 (C-17), 127.97 (C-4), 124.81 (C-21), 124.75 (C-21), 124.39 (C-8), 122.30 (C-16), 120.07 (C-20), 199.90 (C-20), 117.80 (C-3), 117.39 (C-2). APCI-HRMS m/z : calculated for $\text{C}_{15}\text{H}_{10}\text{BrFNO}_5$ (MH^+), 381.9721, found 381.9717. Purity (HPLC-210 nm): 98.6%.

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (3i)

The title compound was prepared from 3,4-dihydroxy-5-nitroacetophenone (1 mmol) and 3-(trifluoromethyl)benzaldehyde. Yield: 331 mg (18.5%) as a yellow-coloured powder, mp: 170.97-172.73°C. ^1H NMR (600 Hz, DMSO) δ 8.33-8.32 (m, 2H, H-2, H-21), 8.20 (d, $J = 7.8$ Hz, 1H, H-17), 8.09 (d, $J = 15.6$ Hz, 1H, H-13), 7.81 (d, $J = 15.4$ Hz, 1H, H-15), 7.80 (d, $J = 8.4$ Hz, 1H, H-19), 7.76 (d, $J = 2.1$ Hz, 1H, H-3), 7.69 (t, $J = 7.8$ Hz, 1H, H-18). ^{13}C NMR (150 MHz, DMSO) δ 186.71 (C-12), 148.29 (C-5), 146.30 (C-6), 142.66 (C-15), 138.06 (C-16), 136.30 (C-4), 133.38 (C-17), 130.59 (C-18), 130.37 (C-1), 130.17 (C-20), 129.96 (C-22), 128.20 (C-4), 127.18 (C-19), 125.72 (C-21), 123.75 (C-13), 117.96 (C-3), 117.35 (C-2). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{11}\text{F}_3\text{NO}_5$ (MH^+), 354.0584, found 354.0582. Purity (HPLC-210 nm): 99.2%.

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(3-fluorophenyl)prop-2-en-1-one (3j)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 3-fluorobenzaldehyde. Yield: 527 mg (69.4%) as a yellow-coloured powder, mp: 214.87 - 215.67°C. ^1H NMR (600 Hz, DMSO) δ 8.32 (d, $J = 2.2$ Hz, 1H, H-2), 7.99 (d, $J = 15.5$ Hz, 1H, H-8), 7.88 (d, $J = 8$ Hz, 1H, H-17), 7.74 (d, $J = 2.2$ Hz, 1H, H-3), 7.72 (d, $J = 15.9$ Hz, 1H, H-15), 7.74 – 7.68 (m, $J = 9.1$ Hz, 1H, H-19), 7.50 (m, 1H, H-21), 7.29 (m, 1H, H-18). ^{13}C NMR (150 MHz, DMSO) δ 186.68 (C-7), 163.78 (C-20), 162.17 (C-20), 148.31 (C-5), 146.32 (C-6), 143.01 (C-15), 138.03 (C-4), 137.66 (C-16), 131.33 (C-21), 128.21 (C-1), 126.21 (C-19), 123.21 (C-8), 117.86 (C-3), 117.69 (C-18), 117.32 (C-2), 115.20 (C-17). APCI-HRMS m/z : calculated for $\text{C}_{15}\text{H}_{11}\text{FNO}_5$ (MH^+), 304.0616, found 304.0615. Purity (HPLC-210 nm): 97.5%.

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-fluorophenyl)prop-2-en-1-one (3k)

The title compound was prepared from 5-acetyl-3-nitrocatechol (1 mmol) and 4-fluorobenzaldehyde. Yield: 660 mg (86.9%) as a yellow-coloured powder, mp: 216.50 - 217.80°C. ¹H NMR (600 Hz, DMSO) δ 8.30 (d, *J* = 2.0 Hz, 1H, H-2), 8.01 (m, 2H, H-17, H-20), 7.90 (d, *J* = 15.5 Hz, 1H, H-8), 7.74 (d, *J* = 2.1 Hz, 1H, H-3), 7.74 (d, *J* = 15.5 Hz, 1H, H-15), 7.30, (m, 2H, H-19, H-21). ¹³C NMR (150 MHz, DMSO) δ 186.67 (C-7), 164.73 (C-18), 163.08 (C-18), 148.26 (C-5), 146.16 (C-6), 143.27 (C-15), 137.99 (C-4), 131.83 (C-16), 131.86 (C-17, C-20), 128.40 (C-1), 121.66 (C-8), 117.93 (C-3), 117.17 (C-2), 116.30 (C-19, C-21). APCI-HRMS *m/z*: calculated for C₁₅H₁₁FO₅ (MH⁺), 304.0616, found 304.0620. Purity (HPLC-210 nm): 99.0%.

1-[5-(4-chlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4a)

The title compound was prepared from (2E)-3-(4-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (**3a**). Yield: 217 mg (61.5%) as a yellow-coloured powder, mp: 240.57 - 243.1 ± 2.08°C. ¹H NMR (600 Hz, DMSO) δ 7.59 (d, *J* = 1.9 Hz, 1H, H-3), 7.57 (d, *J* = 2.0 Hz, 1H, H-1), 7.38 (d, *J* = 8.5 Hz, 2H, H-21, H-25), 7.22 (d, *J* = 8.5 Hz, 2H, H-22, H-24), 5.33 (dd, *J* = 11.9, 4.7 Hz, 1H, H-15), 3.85-3.79 (m, 1H, H-16'), 3.12-3.15 (m, 1H, H-16''), 2.29 (s, 3H, C-18). ¹³C NMR (151 Hz, DMSO) δ 167.82 (C-17), 153.34 (C-12), 148.36 (C-6), 144.16 (C-5), 141.62 (C-20), 137.84 (C-4), 132.20 (C-23), 129.02 (C-21, C-25), 128.02 (C-22, C-24), 122.02 (C-2), 116.25 (C-1), 114.47 (C-3), 59.49 (C-15), 42.07 (C-16), 22.17 (C-18). APCI-HRMS *m/z*: calculated for C₁₇H₁₅ClN₃O₅ (MH⁺), 376.0695, found 376.0684. Purity (HPLC-210 nm): 99.2%.

1-[5-(2,4-dichlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4b)

The title compound was prepared from (2E)-3-(2,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (**3b**). Yield: 98 mg (42.4%) as a yellow-coloured powder, mp: 240.03 - 244.6 ± 2.49°C. ¹H NMR (600 Hz, DMSO) δ 7.67 (d, *J* = 2.1 Hz, 1H, H-24), 7.59 (d, *J* = 2.1 Hz, 1H, H-3), 7.56 (d, *J* = 2.1 Hz, 1H, H-1), 7.38 (dd, *J* = 8.5, 2.2 Hz, 1H, H-22), 7.09 (d, *J* = 8.4 Hz, 1H, H-21), 5.71 (dd, *J* = 12.0, 5.1 Hz, 1H, H-15), 3.92-3.87 (m, 1H, H-16'). 3.12-3.09 (m, 1H, H-16''), 2.34 (s, 3H, H-18). ¹³C NMR (150 MHz, DMSO) δ 167.95 (C-17),

153.58 (C-12), 148.36 (C-6), 144.23 (C-5), 138.44 (C-23), 137.82 (C-20), 132.99 (C-4), 132.28 (C-25), 129.58 (C-24), 128.41 (C-21), 128.20 (C-22), 121.81 (C-2), 116.21 (C-1), 114.56 (C-3), 57.57 (C-15), 40.96 (C-16), 22.09 (C-18). APCI-HRMS m/z : calculated for $C_{17}H_{14}Cl_2N_3O_5$ (MH^+), 410.0305, found 410.0304. Purity (HPLC-210 nm): 97.5%.

1-[5-(3,4-dichlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4c)

The title compound was prepared from (2E)-3-(3,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (**3c**). Yield: 113 mg (49.2%) as a yellow-coloured powder, mp: 197.07 - 199.00°C. ^1H NMR (600 Hz, DMSO) δ 7.59 (d, J = 2.1 Hz, 1H, H-3), 7.58 (d, J = 8.1 Hz, 1H, H-22), 7.57 (d, J = 2.1 Hz, 1H, H-1), 7.48 (d, J = 2.1 Hz, 1H, H-25), 7.18 (dd, J = 8.4, 2.1 Hz, 1H, H-21), 5.54 (dd, J = 12.0, 5.0 Hz, 1H, H-15), 3.80-3.78 (m, 1H, H-16'), 3.24-3.19 (m, 1H, H-16''), 2.30 (s, 3H, C-18). ^{13}C NMR (151 Hz, DMSO) δ 168.02 (C-17), 153.46 (C-12), 148.36 (C-6), 144.25 (C-5), 143.61 (C-20), 137.79 (C-4), 131.57 (C-24), 131.32 (C-22), 130.24 (C-23), 128.50 (C-25), 126.52 (C-21), 121.90 (C-2), 116.28 (C-1), 114.58 (C-3), 59.14 (C-15), 41.87 (C-16), 22.16 (C-18). APCI-HRMS m/z : calculated for $C_{17}H_{14}Cl_2N_3O_5$ (MH^+), 410.0305, found 410.0298. Purity (HPLC-210 nm): 97.1%.

1-[5-(3-chlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4d)

The title compound was prepared from (2E)-3-(3-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (**3d**). Yield: 159 mg (67.6%) as a yellow-coloured powder, mp: 208.50 - 211.40°C. ^1H NMR (600 Hz, DMSO) δ 7.60 (d, J = 2.1 Hz, 1H, H-3), 7.58 (d, J = 2.1 Hz, 1H, H-1), 7.37 (t, J = 7.8 Hz, 1H, H-23), 7.33-7.32 (m, 1H, H-22), 7.25 (t, J = 1.9 Hz, 1H, H-25), 7.16-7.14 (m, 1H, H-21), 5.54 (dd, J = 11.9, 4.9 Hz, 1H, H-15), 3.84-3.78 (m, 1H, H-16'), 3.19-3.17 (m, 1H, H-16''), 2.30 (s, 3H, C-18). ^{13}C NMR (150 MHz, DMSO) δ 167.93 (C-17), 153.42 (C-12), 148.36 (C-6), 145.08 (C-5), 144.21 (C-20), 137.82 (C-4), 133.62 (C-24), 131.08 (C-22), 127.70 (C-23), 126.09 (C-25), 124.71 (C-21), 121.95 (C-2), 116.24 (C-1), 114.56 (C-3), 59.58 (C-15), 42.08 (C-16), 22.17 (C-18). APCI-HRMS m/z : calculated for $C_{17}H_{15}ClN_3O_5$ (MH^+), 376.0695, found 376.0691. Purity (HPLC-210 nm): 96.1%.

1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-ethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4e)

The title compound was prepared from (2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-ethoxyphenyl)prop-2-en-1-one (**3e**). Yield: 201 mg (42.9%) as a yellow-coloured powder, mp: 193.80 - 193.90°C. ¹H NMR (600 Hz, DMSO) δ 7.59 (d, *J* = 2.1 Hz, 1H, H-3), 7.57 (d, *J* = 2.1 Hz, 1H, H-1), 7.08 (d, *J* = 8.6 Hz, 2H, H-21, 25), 6.85 (d, *J* = 8.6 Hz, 2H, H-22, 24), 5.47 (dd, *J* = 11.7, 4.4 Hz, 1H, H-15), 3.98 (q, *J* = 7.0 Hz, 1H, H-27), 3.80-3.74 (m, 1H, H-16'), 3.11-3.07 (m, 1H, H-16''), 2.27 (s, 3H, H-18), 1.30 (t, *J* = 7.0 Hz, 3H, H-28). ¹³C NMR (150 MHz, DMSO) δ 167.67 (C-17), 158.14 (C-23), 153.31 (C-12), 148.35 (C-6), 144.07 (C-5), 137.84 (C-4), 134.60 (C-20), 127.29 (C-21, C-25), 122.20 (C-2), 116.23 (C-1), 114.87 (C-22, C-24), 114.35 (C-3), 63.46 (C-27), 59.52 (C-15), 42.21 (C-16), 22.21 (C-18), 15.10 (C-28). Purity: APCI-HRMS *m/z*: calculated for C₁₉H₂₀N₃O₆ (MH⁺), 386.1347, found 386.1337. Purity (HPLC-210 nm): 99.2%.

1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-iodophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4f)

The title compound was prepared from (2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-iodophenyl)prop-2-en-1-one (**3f**). Yield: 121 mg (53.3%) as a yellow-coloured powder, mp: 207.57 - 213.40°C. ¹H NMR (600 Hz, DMSO) δ 7.69 (d, *J* = 8.4 Hz, 2H, H-22, H-24), 7.59 (d, *J* = 2.1 Hz, 1H, H-3), 7.57 (d, *J* = 2.1 Hz, 1H, H-1), 7.01 (d, *J* = 8.4 Hz, 2H, H-21, H-25), 5.49 (dd, *J* = 11.9, 4.7 Hz, 1H, H-15), 3.83-3.77 (m, 1H, H-16'), 3.14-3.10 (m, 1H, H-16''), 2.28 (s, 3H, H-18). ¹³C NMR (151 Hz, DMSO) δ 167.79 (C-17), 153.33 (C-12), 148.34 (C-6), 144.16 (C-5), 142.47 (C-4), 137.81 (C-20, C-22, C-24), 128.51 (C-21, C-25), 122.00 (C-2), 116.23 (C-1), 114.46 (C-3), 93.53 (C-23), 59.67 (C-15), 42.01 (C-16), 22.16 (C-18). APCI-HRMS *m/z*: calculated for C₁₇H₁₅IN₃O₅ (MH⁺), 468.0051, found 468.0038. Purity (HPLC-210 nm): 98.9%.

1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-[4-(methylsulfanyl)phenyl]-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4g)

The title compound was prepared from (2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-[4-(methylsulfanyl)phenyl]prop-2-en-1-one (**3g**). Yield: 662 mg (70.8%) as a yellow-coloured powder, mp: 221.17 - 222.03°C. ¹H NMR (600 Hz, DMSO) δ 7.59 (d, *J* = 2.1 Hz, 1H, H-3), 7.58 (d, *J* = 2.1 Hz, 1H, H-1), 7.22 (d, *J* = 8.4 Hz, 2H, H-22, H-24), 7.13 (d, *J* = 8.4 Hz, 2H,

H-21,H-25), 5.49 (dd, $J = 11.8, 4.6$ Hz, 1H, H-15), 3.82-3.77 (m, 1H, H-16'), 3.13-3.08 (m, 1H, H-16''), 2.45 (s, 3H, C-28), 2.28 (s, 3H, C-18). ^{13}C NMR (151 Hz, DMSO) δ 167.71 (C-17), 153.31 (C-12), 148.33 (C-6), 144.07 (C-5), 139.41 (C-20), 137.85 (C-23), 137.38 (C-4), 126.74 (C21, C22, C24, C25), 122.15 (C-2), 116.26 (C-1), 114.26, 59.68 (C-15), 42.13 (C-16), 22.19 (C-18), 15.29 (C-28). APCI-HRMS m/z : calculated for $\text{C}_{18}\text{H}_{18}\text{N}_3\text{O}_5\text{S}$ (MH^+), 388.0962, found 388.0946. Purity (HPLC-210 nm): 98.8%.

1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-bromo-2-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4h)

The title compound was prepared from (2E)-3-(4-bromo-2-fluorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (**3h**). Yield: 61 mg (26.6%) as a yellow-coloured powder, mp: 239.53 - 242.17°C. ^1H NMR (600 Hz, DMSO) δ 7.59 (d, $J = 2.0$ Hz, 1H, H-3), δ 7.57 (d, $J = 1.8$ Hz, 1H, H-1), δ 7.56-7.55 (m, 1H, H-24), 7.37 (dd, $J = 8.3, 2.0$ Hz, 1H, H-22), 7.12 (t, $J = 8.3$ Hz, 1H, H-21), 5.62 (dd, $J = 12.2, 5.2$ Hz, 1H, H-15), 3.85-3.80 (m, 1H, H-16'), 3.22-3.17 (m, 1H, H-16''), 2.28 (s, 1H, H-18). ^{13}C NMR (150 MHz, DMSO) δ 167.91 (C-17), 160.54 (C-25), 158.89 (C-25), 153.63 (C-12), 148.41 (C-5), 144.42 (C-6), 137.73 (C-4), 129.91 (C-20), 128.79 (C-21), 128.70 (C-22), 121.73 (C-2), 121.09 (C-23), 119.38 (C-24), 116.07 (C-1), 114.50 (C-3), 54.66 (C-15), 40.85 (C-16), 22.07 (C-18). APCI-HRMS m/z : calculated for $\text{C}_{17}\text{H}_{14}\text{BrFN}_3\text{O}_5$ (MH^+), 438.0095, found 438.0077. Purity (HPLC-210 nm): 97.4%.

1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(3-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4i)

The title compound was prepared from (2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(3-fluorophenyl)prop-2-en-1-one (**3j**). Yield: 107 mg (45.5%) as a yellow-coloured powder, mp: 228.23 - 229.93°C. ^1H NMR (600 Hz, DMSO) δ 7.59 (d, $J = 2.1$ Hz, 1H, H-3), 7.58 (d, $J = 2.1$ Hz, 1H, H-1), 7.40-7.36 (m, 1H, H-22), 7.10-7.07 (m, 1H, H-23), 7.05-6.99 (m, 2H, H-21, H-25), 5.55 (dd, $J = 11.9, 4.7$ Hz, 1H, H-15), 3.85-3.78 (m, 1H, H-16'), 3.18-3.14 (m, 1H, H-16''), 2.31 (s, 3H, H-18). ^{13}C NMR (150 MHz, DMSO) δ 167.92 (C-17), 163.54 (C-24), 161.93 (C-24), 153.37 (C-12), 148.33 (C-6), 145.50 (C-20), 144.16 (C-5), 137.80 (C-4), 131.19 (C-22), 121.98 (C-2, C-21), 116.21 (C-1), 114.53 (C-23), 114.42 (C-3), 113.05 (C-25), 59.59 (C-15), 42.11 (C-16), 22.17 (C-18). APCI-HRMS m/z : calculated for $\text{C}_{17}\text{H}_{15}\text{FN}_3\text{O}_5$ (MH^+), 360.0990, found 360.0983. Purity (HPLC-210 nm): 98.5%.

1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (**4j**)

The title compound was prepared from (2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-fluorophenyl)prop-2-en-1-one (**3k**). Yield: 107 mg (45.5%) as a yellow-coloured powder, mp: 244.80 - 245.60°C. ¹H NMR (600 Hz, DMSO) δ 7.59 (d, *J* = 2.1 Hz, 1H, H-3), 7.57 (d, *J* = 2.1 Hz, 1H, H-1), 7.24-7.21 (m, 2H, H21, H25), 7.16-7.13 (m, 2H, H22, H24), 5.54 (dd, *J* = 11.8, 4.6 Hz, 1H, H-15), 3.84-3.79 (m, 1H, H-16'), 3.15-3.10 (m, 1H, H-16''), 2.29 (s, 3H, H-18). ¹³C NMR (150 MHz, DMSO) δ 167.80 (C-17), 162.54 (C-23), 160.93 (C-23), 153.34 (C-12), 148.36 (C-6), 144.23 (C-5), 138.87 (C-20), 137.79 (C-4), 128.17 (C-21, C-25), 121.98 (C-2), 116.14 (C-1), 115.86 (C-22, C-24), 114.49 (C-3), 59.39 (C-15), 42.18 (C-16), 22.19 (C-18). APCI-HRMS *m/z*: calculated for C₁₇H₁₅FN₃O₅ (MH⁺), 360.0990, found 360.0991. Purity (HPLC-210 nm): 97.1%.

Minimum inhibitory concentration of novel nitrocatechol chalcone and pyrazoline derivatives

Comparing the antibacterial activity of the nitrocatechol chalcone (**3a-k**) vs. pyrazoline derivatives (**4a-j**) all pyrazoline derivatives had weaker antibacterial activity compared to their nitrocatechol chalcone counterparts (Table 3.6). This is in contrast to **1a-i** vs. **2a-d** [28]. All the synthesised derivatives (**3a-k** & **4a-j**) had comparable antibacterial activity against both *S. aureus* strains. The four most active nitrocatechol chalcone derivatives were **3i** (0.5 µg/ml) and **3b, c, d** (1 µg/ml) which were more active than the positive control, tetracycline (2 µg/ml). Derivative **3h** (2 µg/ml) and **3a, j, k** (4 µg/ml) have comparable antibacterial activities to tetracycline, whilst the remaining derivatives, *i.e.*, **3e, f** (8 µg/ml) and **3g** (16 µg/ml), have considerably weaker activity. The two most active pyrazoline derivatives were **4a** and **4c** (16 µg/ml) which have considerably weaker antibacterial activity compared to tetracycline. The remaining pyrazoline derivatives have either weak antibacterial activity, *i.e.*, **4f** (32 µg/ml) & **4b, g & h** (128 µg/ml), or lost all activity, *i.e.*, **4d, e, i & j** (>256 µg/ml).

Table 3.6: MIC of novel nitrocatechol chalcones (**3a-k**) and pyrazoline derivatives (**4a-j**).

Derivative	MIC (µg/ml)	
	<i>S. aureus</i> (ATCC 29213)*	<i>S. aureus</i> (ATCC 43300)†
3a	4	1
3b	1	1
3c	1	0.5
3d	1	2
3e	8	16
3f	8	4
3g	16	16
3h	2	2
3i	0.5	2
3j	4	8
3k	4	8
4a	16	32
4b	128	>256
4c	16	32
4d	>256	>256
4e	>256	>256
4f	32	64
4g	128	>256
4h	128	128
4i	>256	>256
4j	>256	>256
Tetracycline	2	2

* Susceptible *S. aureus* strain

† Methicillin resistant *S. aureus* strain

The most active derivative (**3i**) has a trifluoromethyl substituent on the *meta*-position of ring B, which is interesting because fluorine is the most electronegative halogen. The other active derivatives (**3b**, **c**, **d**) have either one or two chlorine substituents on various positions on ring B however, derivative **3a**, was less active than **3b**, **c** and **d** and only has one chlorine on the *para*-position. This trend was also observed for derivatives **3e**, **3f**, **3g** and **3k** which have an ethoxy, iodine, methyl sulphide and fluorine on the *para*-position, respectively. It appears that the electron-withdrawing effect on the aromatic ring B increases the antibacterial activity unless the substituent is solely on the *para*-position, except compound **3i**.

For this study, the choice of substituents on ring B was based on the pharmacophore model that highlighted the importance of a hydrogen bond acceptor as well as other studies that demonstrated the importance of electron withdrawing substituents on the *para*-position of ring B [56]. The majority of derivatives had halide substituents which are considered bio-isosteres of nitrile, which were found to be important during the initial screening [57]. Sulphur and halides are strong electron-withdrawing groups, however, both moieties will likely only form weak hydrogen bonds. The good antibacterial activity observed by derivatives **3b** - **d** and **i** against the susceptible *S. aureus* strain, might be a result of the weak hydrogen bonds forming with the active site or because of the electron-withdrawing effect on ring B. Chlorine and bromine substituents are also hydrophobic and might interact with hydrophobic pockets in the target binding site [55].

Other studies have also shown that chalcones and pyrazoline derivatives have antibacterial activity against *S. aureus* [58–60]. The presence of the reactive α , β -unsaturated carbonyl-group in chalcones and the N-N bond in pyrazolines are key factors responsible for their biological activity [61,62]. An oxygenated chalcone, licochalcone A (Figure 3.2), was shown to possess antibacterial activity against Gram-positive bacteria including *S. aureus* [63]. The hydroxyl group at position 4 on ring A, preferably in the presence of lipophilic substituents on ring B, is essential for antibacterial activity [64,65]. The nitrocatechol chalcones and pyrazoline derivatives tested in the current study, all contain a hydroxyl group in the same position on ring A as seen in licochalcone A.

The antibacterial action of chalcones might be a result of their reaction with thiol groups in essential bacterial enzymes, which is influenced by the addition of electron-withdrawing substituents on ring B. This could explain the potent antibacterial activity of **1b**, **3b**, **3c**, **3d**,

3h and **3i** against *S. aureus* [64]. The antibacterial mechanism of chalcones is not yet well understood, however, studies suggest that they exert their antibacterial properties against an array of targets including efflux pumps, the type II fatty acid biosynthetic pathway, DNA replication, virulence factor, protein tyrosine phosphatase and the filamentous temperature-sensitive mutant Z protein [13,16,66–73]. With regards to the pyrazoline derivatives, it is hypothesised that glucosamine-6-phosphate synthase (GlcN-6-P) and D-alanine-D-alanine ligase (Ddl) might be their antibacterial target [74,75].

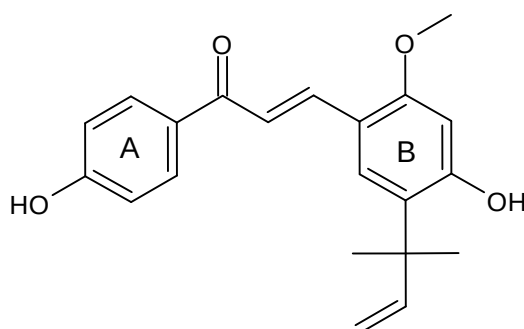


Figure 3.2: Licochalcone A [64].

Conclusion

In this study, nitrocatechol chalcone and pyrazoline derivatives that have been synthesised previously by our research group were firstly screened for antibacterial activity against *S. aureus*, *K. pneumoniae*, *A. baumannii* and *K. aerogenes*. The derivatives only showed activity against *S. aureus* and subsequently, the MIC was determined for each derivative. The derivatives containing a nitrile group (**1a**, **1b**, **2a**, **2b**) were the most active with MIC values ranging from 1 - 4 µg/ml. The *in vitro* activity of each derivative was used to generate common feature pharmacophore models for *S. aureus*, which were evaluated by considering their rank scores, EF^{10%} and ROC-AUC values. Hypo. 9 was the most promising pharmacophore model and indicated that four hydrogen bond acceptors are important for antibacterial activity. With this in mind a novel series of nitrocatechol chalcone and pyrazoline derivatives were synthesised containing bio-isosteres of the functional nitrile group. The MIC values of each derivative were determined against both the susceptible and resistant *S. aureus* strains. Derivatives **3i** and **3c** were the most active against the susceptible and resistant *S. aureus* strains, respectively. Other derivatives also had good activity against both strains, *i.e.*, **3b**, **c** and **d** (susceptible) and **3a** and **b** (resistant) and were

more active than tetracycline. Our findings correspond with the literature that an electron-withdrawing substituent on ring B increases the antibacterial activity of the nitrocatechol chalcone derivatives. The pharmacophore model created in this study suggests that halides might form hydrogen bonds with the intended target, albeit weak.

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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Data availability

Data will be made available on request.

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CHAPTER 4: ARTICLE 2

Guaiacol-chalcone and nitroguaiacol-chalcone derivatives: *In vitro* and *in silico* antibacterial evaluation

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Abstract

Antimicrobial resistance is a pandemic that has already caused several antibiotics to lose their clinical relevance. The speed at which novel antibiotics are discovered cannot keep up with the increase in antimicrobial resistance. This study aimed to design and synthesise novel guaiacol-chalcone and nitroguaiacol-chalcone derivatives and determine their possible *in vitro* antibacterial activity against four pathogens *viz.* *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella aerogenes*. The checkerboard assay was used to explore potential synergism with existing antibiotics to shed light on a possible antibacterial mechanism of action. A pharmacophore model was constructed to identify key chemical entities present within the active derivatives necessary for activity. The pharmacophore model was validated utilising the enrichment factor (EF^{10%}) and the area under the receiver operating characteristic (ROC-AUC) curve metrics. Most guaiacol-chalcone derivatives had no activity whereas the nitroguaiacol-chalcone derivatives were active against *S. aureus*. The most active derivative was the nitroguaiacol-chalcone derivative, **2c** (8 µg/ml), with activity against a susceptible and

methicillin-resistant strain of *S. aureus*. The checkerboard assay showed that one nitroguaiacol-chalcone derivative (**2a**) had a synergistic effect when combined with chloramphenicol (FIC_{min}: 0.45 ± 0.09), implying its antibacterial mechanism of action might also target bacterial protein synthesis. Active guaiacol-chalcone and nitroguaiacol-chalcone derivatives were accurately identified by the common feature pharmacophore model (max. fit: 4, rank score: 52.34) within a test set. The leading pharmacophore model, *i.e.*, hypothesis 1 (EF^{10%}: 8, ROC-AUC: 0.95 ± 0.02) indicated that four features are important for antibacterial activity *i.e.*, ring aromatic, a hydrophobic moiety and two hydrogen bond acceptors.

Introduction

The discovery of antibiotics saved countless lives, however because of antimicrobial resistance (AMR) these once-potent drugs are sometimes no longer effective against infections (Golkar *et al.*, 2014:129). As a result of AMR thousands of people die annually which resulted in the World Health Organization declaring AMR a global health threat (Bengtsson-Palme *et al.*, 2018:68; Urban-Chmiel *et al.*, 2022:1). The need for new antibiotics is great; however, the discovery of new antibiotics is slow and does not keep up with the rising threat of AMR (Iskandar *et al.*, 2022:1).

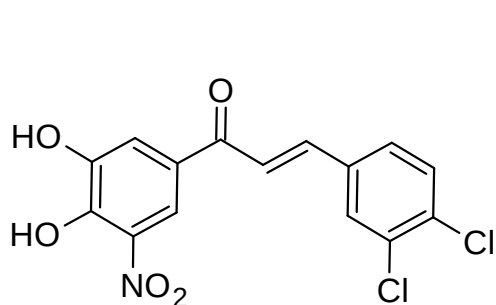
Computer-aided drug design (CADD) is a comprehensive term used to describe the use of computers in the drug discovery process and can greatly accelerate the drug discovery process (Song *et al.*, 2009:580; Vemula *et al.*, 2023:1). Ligand-based drug design (LBDD) utilises the structure of active ligands to create molecular descriptors that are desired for biological activity against an unknown target (Macalino *et al.*, 2015:1692). These molecular descriptors are depicted in a pharmacophore model (Giordano *et al.*, 2022:5). If properly validated, a pharmacophore model can accurately identify potential bioactive compounds and be used to design new antibiotics (Ye *et al.*, 2022:2).

As part of the rational drug design approach, it is beneficial to identify the mode of action of bioactive compounds (Adam, 2005:514). The checkerboard assay is a two-dimensional, two-compound matrix assay which aims to determine if two drugs, *i.e.*, a novel and known antibiotic, act synergistic, indifferent or antagonistic. If a synergistic or antagonistic effect is observed, it indicates that the two drugs target the same bacterial metabolic pathway,

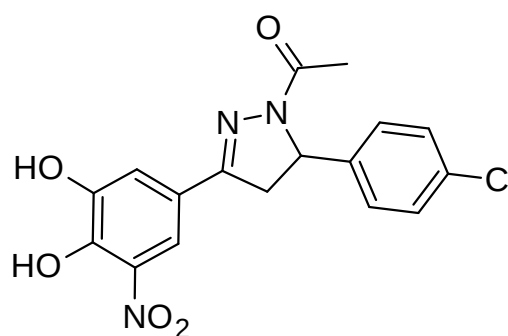
providing insight into a possible mechanism of action (Bellio *et al.*, 2021:2; Farha & Brown, 2010:853; Farha *et al.*, 2015:11048).

In a previous study, our research team synthesised nitrocatechol-chalcone and nitrocatechol-pyrazoline derivatives, which demonstrated significant antibacterial activity against both *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) (Figure 4.1) (Hoepfner *et al.*, 2023:1). Chalcones are recognised for their non-toxic nature and possess anti-inflammatory, antimicrobial and antifungal properties (Figure 4.2) (Badreddin Musatat *et al.*, 2023:1; Dhaliwal *et al.*, 2022:1). Nitrocatechol-containing drugs such as entacapone and tolcapone are registered to treat Parkinson's' disease and have also been identified as potential treatments for multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis (Kinnings *et al.*, 2009:1). Studies have also shown that tolcapone acts synergistically with vancomycin against MRSA (Moore *et al.*, 2020:283). Compounds containing a pyrazoline moiety, including other chalcone-pyrazoline derivatives, have also been found to have antibacterial activity (Figure 4.2) (Mishra *et al.*, 2017:1949; Munirajasekhar *et al.*, 2014:459; Tok *et al.*, 2022:1453).

The chalcone derivatives, synthesised by our group, generally had better antibacterial activity against both *S. aureus* strains compared to the pyrazoline derivatives (Figure 4.1). A pharmacophore model showed that the nitro- and the alcohol groups on the nitrocatechol moiety were important for antibacterial activity and acted as hydrogen bond acceptors (Hoepfner *et al.*, 2023:1).



S. aureus: 1 µg/ml
MRSA: 0.5 µg/ml



S. aureus: 16 µg/ml
MRSA: 32 µg/ml

Figure 4.1: Most active nitrocatechol-chalcone and nitrocatechol-pyrazoline derivatives previously synthesised (Hoepfner *et al.*, 2023:1).

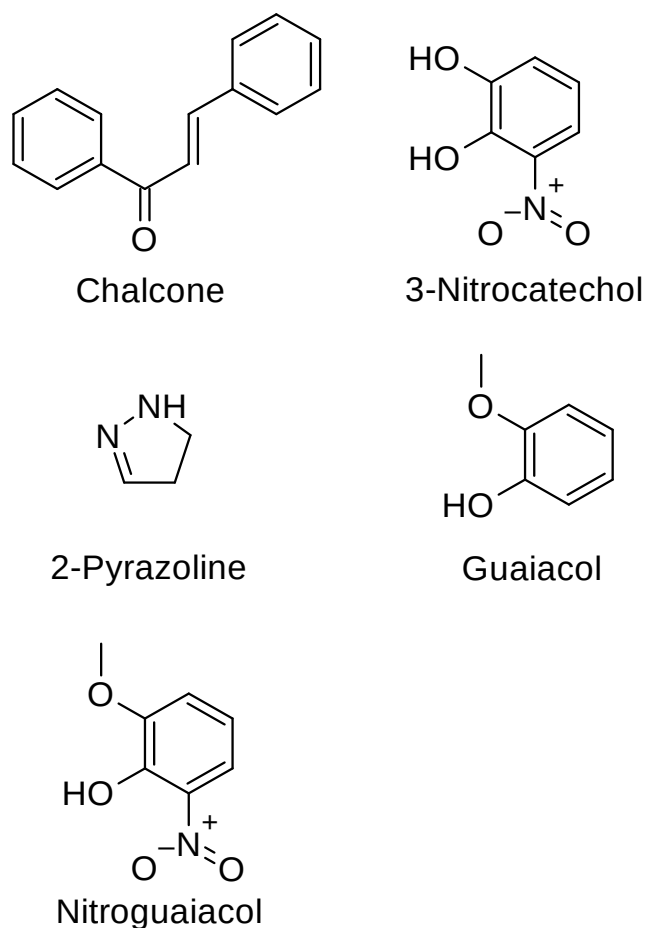


Figure 4.2: Important structures discussed in the text.

In this current study, novel derivatives were synthesised and the nitrocatechol moiety was replaced with either a guaiacol (series 1) or a nitroguaiacol moiety (series 2) (Figure 4.2). This was done to confirm the importance of the nitro- and alcohol groups for antibacterial activity. On their own, guaiacol derivatives have also been found to exhibit various biological activities, including antimicrobial properties (Liu *et al.*, 2011:1908). Using the broth microdilution method, the novel derivatives were screened against *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Klebsiella aerogenes*. Subsequently, the minimum inhibitory concentration (MIC) of each active derivative was determined against the relevant bacteria. To elucidate a possible antibacterial mode of action, a checkerboard assay was conducted incorporating both the novel derivatives and known antibiotics. Finally, a pharmacophore model was developed to identify the essential functional groups required for antibacterial activity.

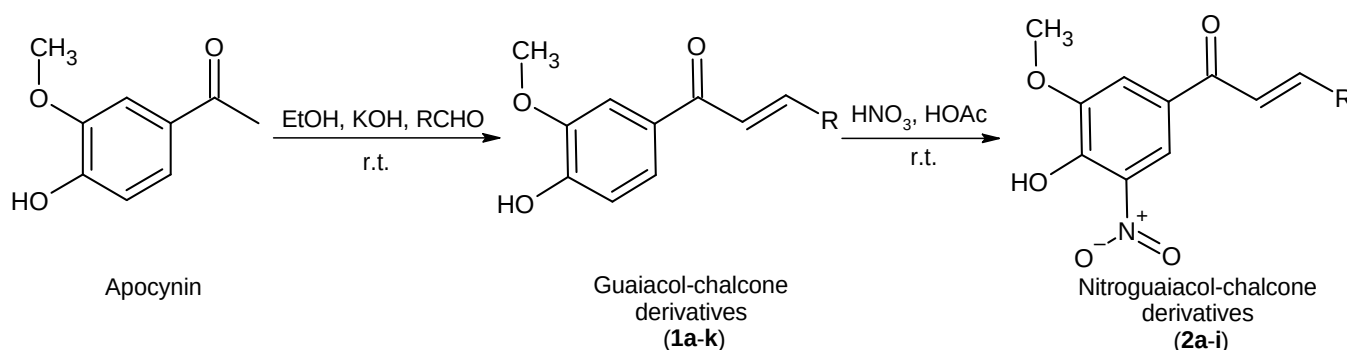
Materials and methods

Synthesis of guaiacol- and nitroguaiacol-chalcone derivatives

The reagents used in this study were sourced from Merck (South Africa) and utilised without additional purification. Nuclear magnetic resonance (NMR) analysis (Bruker Advance Africa) was used to confirm the structures, and subsequently recorded as one-dimensional proton (^1H) (600 MHz) and carbon (^{13}C) (150 MHz) and two-dimensional HSQC spectra. MestRenova 14.1.2 was utilised to identify and report chemical shifts (δ) *i.e.*, peaks, and determine the integration, multiplicity, and coupling constants (J) of observed peaks. Each chemical shift was reported in parts per million (ppm) in deuterated dimethylsulfoxide (DMSO- d_6) and deuterated chloroform (CDCl_3) (Merck, South Africa), and all multiplicities were reported as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), or multiplet (m). During chemical synthesis, the reactions were closely monitored through thin-layer chromatography (TLC). This was achieved by dissolving the reagents and resulting products in methanol, which were then applied to Silica gel 60 aluminium-coated TLC sheets (Sigma Aldrich, South Africa). Following this, the samples were introduced to the relevant mobile phase and subsequently scrutinised under UV light. High-performance liquid chromatography (HPLC) (Shimadzu Nexera-I LC 2040 C 3D Plus HPLC system) was utilised to determine the purity of each compound using a Venusil XBP C18 column (2.1×150 mm, $3 \mu\text{m}$, $100\text{\AA}$) for separation. Milli-Q water (A) and HPLC grade acetonitrile (Merck, South Africa) (B) containing 0.1% formic acid were used as the mobile phase at a ratio of 55:45 for series 1 and 95:5 for series 2. The flow rate was set at 0.4 ml/min. The total run-time for series 1 and 2 was 7 min per sample at an oven temperature of 40°C and 30°C respectively. During the run-time of series 1, the concentration of B remained at 45% for 1.5 min before increasing to 100% over 2 min where it remained for 1 min. The concentration returned to 45% at the 4.7 min mark and was allowed to equilibrate. In the case of series 2, the concentration of B remained at 5% for 1.5 min before increasing to 100% over 2 min where it remained for 1 min. The concentration returned to 5% at the 4.7 min mark and was allowed to equilibrate. The eluent was monitored at the lambda-max (λ_{max}) of the compounds *i.e.*, 300 nm. The melting points of each novel derivative were determined with a Mettler DSC 3 Star System (Mettler Toledo, Greifensee, Switzerland) by differential scanning calorimetry (DSC).

The synthesis of novel guaiacol-chalcones (series 1) and nitroguaiacol-chalcones (series 2) (scheme 1) involved the reaction of apocynin and the relevant aromatic aldehyde *via*

Claisen-Schmidt condensation to yield the target guaiacol-chalcone derivatives (**1a-k**). The nitration of the relevant guaiacol-chalcones yielded the corresponding nitroguaiacol-chalcones derivatives (**2a-i**). The yields range from 13.12% to 83.4%.



Scheme 1: Synthesis of the guaiacol-chalcone (**1a-k**)–and nitroguaiacol-chalcone (**2a-i**) derivatives.

To synthesise series 1, apocynin (1 mmol) was dissolved in ethanol (1.5 ml) and heated, whereafter the relevant aromatic aldehyde (1 mmol) was added at room-temperature and stirred until the aldehyde was dissolved. Potassium hydroxide (60%, 0.5 ml) was added dropwise, and the reaction was stirred at room-temperature for 24 h. To stop the reaction, ice-cold hydrochloric acid (2N, 20 ml) was added and stirred for 15 min, after which the reaction was placed in an ice bath and stirred for another 15 min forming a precipitate. The mixture was filtered, and the resulting residue was dissolved in either acetonitrile or methanol and allowed to recrystallise. The crystals were filtered and dried under a vacuum (Engelbrecht *et al.*, 2018:119; Hitge *et al.*, 2020:127190; Klinke & Gibian, 1961:28). To synthesise series 2, the relevant guaiacol-chalcone (1 mmol) was dissolved in acetic acid (30 mmol, 13 ml) and heated. Nitric acid (0.8 mmol, 3 ml) was added dropwise to the reaction mixture and stirred for 1 h. For most compounds this was done at room-temperature, however for compound **2b** the guaiacol-chalcone was firstly heated to ensure it dissolved before adding nitric acid. To stop the reaction, ice-cold distilled water (32 ml) was added to the reaction and stirred for 10 minutes (Engelbrecht *et al.*, 2018:119; Hitge *et al.*, 2020:127189; Kiss *et al.*, 2010:3397). The reaction mixture was filtered, with the resulting residue being dissolved in either acetonitrile or ethyl acetate and allowed to recrystallise. With regards to **2b**, brown impurities formed in addition to yellow crystals during recrystallisation. The particles were removed *via* filtration, after which the crystals were recrystallised. All compounds were dried under vacuum.

Screening and minimum inhibitory concentration

The antibacterial activity of guaiacol- and nitroguaiacol-chalcones was assessed *in vitro* using the broth microdilution method against various bacterial strains: *S. aureus* (ATCC 29213), *S. aureus* (ATCC 43300) (MRSA), *K. pneumoniae* (ATCC 13883), *A. baumannii* (ATCC 19606), *P. aeruginosa* (ATCC 27853), and *K. aerogenes* (ATCC 13048) (Virginia, United States). All pathogens were incubated in a 150 L shaking incubator (Labotec, South Africa). Equipment was sterilised by autoclave at 121 °C for 30 minutes using an Equitron Medica autoclave (Equitron Medica Ltd) (United Scientific, South Africa).

The MIC of each derivative was determined using an adapted Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2012; Cockerill *et al.*, 2012:16; Wiegand *et al.*, 2008:167) broth microdilution method as described elsewhere (Hoepfner *et al.*, 2023:2). The novel derivatives were firstly screened for antibacterial activity at 256 µg/ml, against all bacteria in triplicate ($n = 3$). Two-fold serial dilutions starting at the highest concentration (256 µg/ml) were subsequently conducted to determine the MIC of derivatives against the susceptible bacterial strains.

All derivatives were dissolved in dimethyl sulfoxide (DMSO) (Merck, South Africa), filter sterilised (0.2 µm) (Lasec, South Africa) and dissolved in distilled water (dH₂O) (512 µg/ml). Tetracycline (Sigma Aldrich, South Africa) served as positive control and was dissolved in distilled water, filter sterilised and further diluted with sterile dH₂O (64 µg/ml). Each corning flat bottom 96-well plate (Lasec, South Africa) was primed with 200 µl tetracycline (well B1), sterility control *i.e.*, sterile MuellerHinton broth (MHB) (Sigma Aldrich, South Africa) (200 µl, wells A7 - 12) and two novel derivatives (well C1, D1, E1 and F1, G1, H1). The growth control, *i.e.*, sterile dH₂O (100 µl, wells A1 - 6) was also added to each 96-well plate. The remaining empty wells were primed with 100 µl sterile dH₂O. A multichannel pipette was utilised to create serial dilutions (Cockerill *et al.*, 2012:16).

To prepare the bacterial inoculum, 2-3 colonies were suspended in sterile MHB and incubated at 35 ± 2 °C, shaking at 100 rpm, for 4-5 hours. The inoculum was then diluted to a concentration of 1×10^6 CFU/ml, and 100 µl was added to each well, except the sterility control, resulting in a final well volume of 200 µl, a bacterial concentration of 5×10^5 CFU/ml and a final derivative and tetracycline concentration of 256 µg/ml and 32 µg/ml respectively. Each experiment required a viability count that involved taking 10 µl from one of the bacterial growth control wells and diluting it in 10 ml sterile MHB. From this suspension, 100 µl was plated in triplicate onto Mueller Hinton Agar (MHA) Petri dishes. The 96-well plates and petri

dishes were incubated for 20 hours at 35 ± 2 °C. After incubation the colonies were counted and a colony count of 20-80 CFUs confirmed that the correct bacterial concentration was used (Cockerill *et al.*, 2012:30; EUCAST, 2003:4). Resazurin (150 µg/ml) (Merck, South Africa) was added to the 96-well plate wells (60 µl) and further incubated at 35 ± 2 °C for 4 h. The reduction of the resazurin (blue) to resorufin (pink) was assessed visually as an indication of bacterial growth (Elshikh *et al.*, 2016:1016; Präbst *et al.*, 2017:7; Sarker *et al.*, 2007:322).

Checkerboard assay

To elucidate a possible antibacterial mode of action a novel nitroguaiacol-chalcone derivative synthesised during this study, *i.e.*, **2a**, was screened with the checkerboard assay. The derivative (**2a**) was in turn combined with nine known antibiotics (one antibiotic at a time), after which the antibacterial activity of the relevant combination was determined against *S. aureus* (ATCC 29213). The different antibiotics used were chloramphenicol and clindamycin (protein synthesis inhibitors) (Biswas *et al.*, 2008:212; Smieja, 1998:23), D-cycloserine, ampicillin and fosfomycin (cell-wall synthesis inhibitors) (Batson *et al.*, 2017:1; Falagas *et al.*, 2016:323; Rafailidis *et al.*, 2007:1829), norfloxacin, sulfamethoxazole and trimethoprim (bacterial DNA-synthesis inhibitors) (Masters *et al.*, 2003:402; Mazuel, 1991:559) and triclosan (RNA and protein synthesis inhibitor) (Dann & Hontela, 2011:285). Before conducting the main checkerboard assay, screening was conducted using a simplified checkerboard method described elsewhere (Farha & Brown, 2010:854). During screening, each derivative was combined with a known antibiotic at a quarter and an eighth of their MIC, *i.e.*, $\frac{1}{4}$ x MIC and an $\frac{1}{8}$ x MIC. If synergism was observed at an $\frac{1}{8}$ x MIC, the derivative-antibiotic combination was considered highly synergistic, and at a $\frac{1}{4}$ x MIC, only synergistic (Farha & Brown, 2010:854). If any synergism was observed, a full checkerboard assay was conducted on the relevant combination.

With regards to the screening each derivative was dissolved in DMSO (12 600 µg/ml) and the antibiotics (including the positive control) in distilled water (dH₂O) and filter sterilised (0.2 µm). The DMSO and antibiotic stock solutions were further diluted in sterile dH₂O to yield two stock solutions, *i.e.*, 1 x MIC and $\frac{1}{2}$ x MIC. A corning flat bottom 96-well plate was primed with sterile dH₂O (100 µl, wells A1 - A6 and B2 - B12) and the positive control (200 µl, well B1). A sterility control, *i.e.*, sterile MHB (200 µl, wells A7 - A12) was also included. Two-fold serial dilutions were made of the positive control. For each derivative, the 1 x MIC stock solution was firstly dispensed into three separate wells (100 µl, $n = 3$) and the

relevant antibiotic stock solution ($1 \times \text{MIC}$) was added ($100 \mu\text{l}$, $n = 3$) to the same wells, creating a $\frac{1}{2} \times \text{MIC}$ solution of both compounds in each of the three wells. The mixture was thoroughly mixed and $100 \mu\text{l}$ of each well was discarded resulting in a final volume of $100 \mu\text{l}$. This was repeated with the $\frac{1}{2} \times \text{MIC}$ stock solutions, creating a $\frac{1}{4} \times \text{MIC}$ ($100 \mu\text{l}$) solution of both compounds in three adjacent wells. Finally, $100 \mu\text{l}$ of the *S. aureus* inoculum ($1 \times 10^6 \text{ CFU/ml}$) was added to all the wells (except the sterility control), resulting in a bacterial concentration of $5 \times 10^5 \text{ CFU/ml}$, and a derivative-antibiotic concentration of either $\frac{1}{4} \times \text{MIC}$ or $\frac{1}{8} \times \text{MIC}$ in each well. Using this method **2a** could be screened in combination with all nine antibiotics at both the $\frac{1}{4} \times \text{MIC}$ and the $\frac{1}{8} \times \text{MIC}$ concentrations using two 96-well plates.

The full checkerboard assay is described by others (Farha & Brown, 2010:854; Leber, 2016). In short, the above-mentioned DMSO and dH_2O stock solutions were used to create a $2 \times$ maximum concentration ($2 \times [\text{Max.}]$) and a $4 \times$ maximum concentration ($4 \times [\text{Max.}]$) of the derivative as well as the antibiotic. To ensure the MIC of each compound was well positioned on the 96-well plate, the $2 \times [\text{Max.}]$ was twice the relevant MIC and the $4 \times [\text{Max.}]$ four times. A 96-well plate was primed with the $2 \times [\text{Max.}]$ derivative solution ($200 \mu\text{l}$, wells A1-A9), as well as the $4 \times [\text{Max.}]$ ($200 \mu\text{l}$, well A10). Sterile dH_2O ($100 \mu\text{l}$) was dispensed into row B (B1 – B10) to row H (H1 – H10). With a multichannel pipette a series dilution was created vertically by transferring $100 \mu\text{l}$ from row A (A1 – A10) to row B (B1 – B10) and repeating this until row G was reached. The positive control (tetracycline) ($200 \mu\text{l}$) was added to well A11 ($64 \mu\text{g/ml}$) and a serial dilution was made by transferring $100 \mu\text{l}$ from A11 to B11, and repeating this step until G11 was reached. The antibiotic stock solution ($4 \times [\text{Max.}]$) was added to the 96-well plate ($100 \mu\text{l}$, wells A10-H10) and serial dilutions were made horizontally by transferring $100 \mu\text{l}$ from column 10 to 9 and repeating this until column 2 was reached, after which the remaining $100 \mu\text{l}$ was discarded. For the bacterial growth control $100 \mu\text{l}$ sterile dH_2O was added to wells A12 - D12 and $200 \mu\text{l}$ sterile MHB was added as a sterility control to wells E12 - H12. The inoculum was prepared as described above, after which $100 \mu\text{l}$ inoculum ($1 \times 10^6 \text{ CFU/ml}$) was added to all the wells (except the sterility control) resulting in a final bacterial concentration of $5 \times 10^5 \text{ CFU/ml}$. Every assay was performed in duplicate (Bellio *et al.*, 2021:4).

Bacterial growth was measured as described above using resazurin. The assay is designed to produce wells with respective MIC data of single antimicrobials as well as combinations of the two (Hiller *et al.*, 2023:2). The effect of the combination was assessed by calculating

the Σ FIC index (FICI) (Equation 1) (Berenbaum, 1978:122; Dawis *et al.*, 2003:1204; Hiller *et al.*, 2023:2).

$$\Sigma \text{FIC index} = (\text{MIC}_{\text{A Combination A\&B}}/\text{MIC}_{\text{A alone}}) + (\text{MIC}_{\text{B Combination A\&B}}/\text{MIC}_{\text{B alone}}) \dots\dots\dots \text{Equation 1}$$

Agent A is the novel derivative being tested, and agent B is the known antibiotic. The $\text{MIC}_{\text{A Combination A\&B}}$ is the MIC of agent A in combination with agent B, and $\text{MIC}_{\text{A alone}}$ is the MIC of agent A alone. The $\text{MIC}_{\text{B Combination A\&B}}$ is the MIC of agent B in combination with agent A, and $\text{MIC}_{\text{B alone}}$ is the MIC of agent B alone. The FICI is obtained by adding these two values (Bremmer *et al.*, 2016:6892; Noel *et al.*, 2021:10). The FICI was calculated for each repeat and the average of their minimum FICI value (FICI_{min}) was reported together with the standard deviation (Meletiadis *et al.*, 2010:602; Noel *et al.*, 2021:10).

The FICI values can be interpreted as follows: $\text{FICI} \leq 0.5$ is synergistic; $0.5 < \text{FICI} \leq 1.0$ is additive; $1.0 < \text{FICI} \leq 4$ is indifferent, and $\text{FICI} > 4$ is antagonistic (Doern, 2014:4125). Various thresholds and cut-off values have been implemented in previous publications to include partial synergism ($0.5 < \text{FICI} \leq 1$) (Kheshti *et al.*, 2018:131; Noel *et al.*, 2021:10). The inclusion of partial synergism is widely debated, with some arguing the inherent variability of the checkerboard assay restricts fine-scale interpretations leading to false positive findings. Therefore, it is more acceptable to only accept a $\text{FICI} \leq 0.5$ as synergistic (Odds, 2003:1).

Common feature pharmacophore modelling

Accelrys Discovery Studio (DS) 3.1 (San Diego, USA) was used for docking procedures, while SPSS Statistics for Windows, version 27 (SPSS Inc., Chicago, IL, USA) was employed for calculating the receiver operating characteristics (ROC-AUC) curve. Additionally, the enrichment factor (EF) was determined using Microsoft Excel for Microsoft 365 (Microsoft, USA) with equation 2.

Structures were drawn in DS and typed with a CHARMM force field. Nine inactive guaiacol-chalcones (**1a-1i**), one active guaiacol-chalcone (**1j**), and four active nitroguaiacol-chalcone derivatives (**2c**, **2d**, **2f**, and **2i**) were chosen for the training set, with principal values of 0 and 2 assigned to inactive and active derivatives, respectively. A maximum omitted feature (MaxOmitFeat) of 0 was assigned to all derivatives before initiating the *Prepare Ligands* protocol. The Feature Mapping protocol identified four features: hydrogen bond acceptor (A), hydrogen bond donor (D), hydrophobic (H), and ring aromatic (R). In the process of generating the Common Feature Pharmacophore, a diverse range of derivatives was

created by analysing the top 200 conformations (with a 20 kcal/mol threshold and a maximum interference distance of 2 Å). The pharmacophore models were ranked based on their rank scores.

The remaining 5 derivatives (**2a**, **2b**, **2e**, **2g** and **2h**) were included in the test set with 500 decoy compounds obtained from the Schrodinger database (Halgren *et al.*, 2004:1750; Hevener *et al.*, 2009:445). To validate the 10 generated pharmacophore models, the test set of compounds was docked into all the models with the *Ligand Profiler* protocol and a MaxOmitFeat of 1. The generated data was used to calculate the EF^{10%} and the ROC-AUC. The EF was calculated by utilising equation 2 (Hamza *et al.*, 2012:969; Halgren *et al.*, 2004:1751).

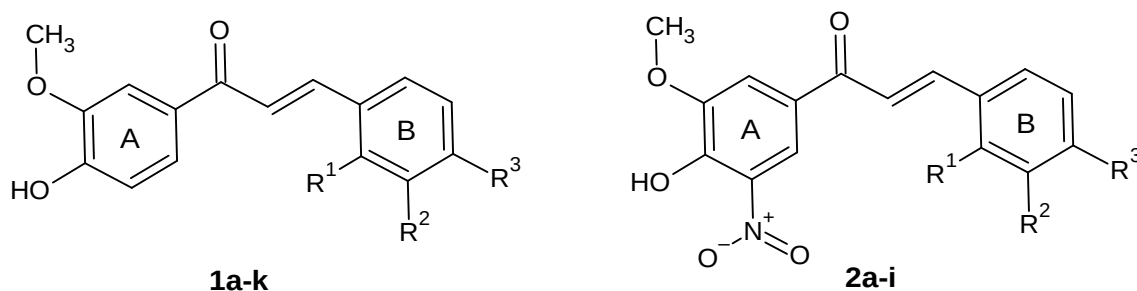
$$\text{EF} = (\text{Hits}_{\text{sel}}/\text{Hit}_{\text{tot}}) \times (\text{NC}_{\text{tot}}/\text{NC}_{\text{sel}}) \dots\dots\dots \text{Equation 2}$$

In this study, the EF was determined at 10% of the test set. The Hits_{sel} represents the correctly identified active derivatives at 10% of the test set, while Hits_{tot} refers to the total number of active derivatives in the test set. NC_{tot} represents the total number of compounds in the test set, and NC_{sel} is the number of compounds within 10% of the test set. Finally, the ROC-AUC was calculated and interpreted as follows: an AUC between 0.9 and 1 indicates excellent performance; between 0.8 and 0.9 was considered good; between 0.7 and 0.8 was fair; between 0.5 and 0.7 was poor; and an AUC below 0.5 signified failure (Hamza *et al.*, 2012:970; Opo *et al.*, 2021:6).

Results and discussion

Synthesis of guaiacol- and nitroguaiacol-chalcone derivatives

Both guaiacol- and corresponding nitroguaiacol-chalcone derivatives were synthesised successfully (Table 4.1). The formation of an *E* double bond between the two vinylic protons, from the Claisen-Schmidt condensation of apocynin and an aromatic aldehyde, was identified by the coupling constant of ±15.6 Hz present in both the guaiacol- and nitroguaiacol-chalcones. The successful nitration of the relevant guaiacol chalcone was evident by the absence of the proton splitting into a doublet at 6.99 ppm in CDCl₃ and 6.93 ppm in DMSO-d₆. In some cases the alcohol present on the guaiacol moiety did not appear on the relevant proton spectrum, resulting in a discrepancy with the calculated molecular weight.

Table 4.1: The synthesised target guaiacol- and nitroguaiacol-chalcone derivatives

Guaiacol-chalcone derivatives				Nitroguaiacol-chalcone derivatives			
	R ¹	R ²	R ³		R ¹	R ²	R ³
1a	H	H	Cl	2a	H	H	Cl
1b	Cl	H	Cl	2b	Cl	H	Cl
1c	H	Cl	Cl	2c	H	Cl	Cl
1d	H	Cl	H	2d	H	Cl	H
1e	H	H	SCH ₃	2e	H	H	SCH ₃
1f	H	CF ₃	H	2f	H	CF ₃	H
1g	H	H	F	2g	H	H	F
1h	H	F	H	2h	H	F	H
1i	F	H	Br	2i	H	H	NO ₂
1j	H	H	OCH ₂ CH ₃				

(2E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1a)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 4-chlorobenzaldehyde (1 mmol). Yield: 432 mg (49.73%), mp: 133.1 - 134.1°C. ¹H NMR (600 MHz, CDCl₃) δ 7.77 (d, *J* = 15.5 Hz, 1H, H-13), 7.66 (m, 2H, H-2, H-3), 7.60 (d, *J* = 8.5 Hz, 2H, H-17, H-18), 7.54 (d, *J* = 15.5 Hz, 1H, H-11), 7.41 (d, *J* = 8.5 Hz, 2H, H-15, H-16), 7.02 (d, *J* = 8.2 Hz, 1H, H-4), 6.32 (s, 1H, OH), 4.01 (s, 3H, H-8). ¹³C NMR (150 MHz, CDCl₃)

δ 188.24 (C-10), 150.61 (C-6), 147.01 (C-5), 142.41 (C-13), 136.20 (C-19), 133.63 (C-14), 130.87 (C-1), 129.49 (C-15, C-16), 129.20 (C-17, C-18), 128.70 (C-2), 123.73 (C-11), 122.17 (C-4), 113.88 (C-3), 56.16 (C-8). APCI-HRMS m/z : calculated for $C_{16}H_{14}ClO_3$ (MH^+), 289.0626, found 289.0618. Purity (HPLC-300 nm): 96.8 %.

(2E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1b)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 2,4-dichlorobenzaldehyde (1 mmol). Yield: 1 500 mg (75%), mp: 189.7 - 191.5°C. ^1H NMR (600 MHz, $CDCl_3$) δ 8.06 (d, J = 15.5 Hz, 1H, H-13), 7.68 (d, J = 8.5 Hz, 1H, H-16), 7.60 (m, 2H, H-2, H-3), 7.48 (d, J = 15.6 Hz, 1H, H-11), 7.46 (m, 1H, H-17), 7.29 (d, J = 8.2 Hz, 1H, H-18), 6.98 (d, J = 8.2 Hz, 1H, H-4), 3.97 (s, 3H, H-8). ^{13}C NMR (150 MHz, $CDCl_3$) δ 188.12 (C-10), 151.21 (C-6), 147.39 (C-5), 138.23 (C-13), 136.14 (C-19), 135.85 (C-15), 132.13 (C-14), 130.33 (C-1), 130.04 (C-17), 128.51 (C-16), 127.50 (C-18), 124.96 (C-11), 123.83 (C-2), 114.26 (C-4), 110.91 (C-3), 56.11 (C-8). APCI-HRMS m/z : calculated for $C_{16}H_{12}Cl_2O_3$ (MH^+), 323.0236, found 323.0235. Purity (HPLC-300 nm): 99.9 %.

(2E)-3-(3,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1c)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 3,4-dichlorobenzaldehyde (1 mmol). Yield: 233 mg (23.97%), mp: 161.8 - 166.4°C. ^1H NMR (600 MHz, $CDCl_3$) δ 7.75 (d, J = 2.0 Hz, 1H, H-15), 7.71 (d, J = 15.6 Hz, 1H, H-13), 7.66 (d, J = 1.9 Hz, 1H, H-3), 7.65 (m, 1H, H-2), 7.55 (d, J = 15.6 Hz, 1H, H-11), 7.51 (d, J = 8.3 Hz, 1H, H-18), 7.48 (dd, J = 8.3, 2.0 Hz, 1H, H-16), 7.03 (d, J = 8.2 Hz, 1H, H-4), 6.18 (s, 1H, OH-9), 4.00 (s, 3H, H-8). ^{13}C NMR (150 MHz, $CDCl_3$) δ 187.82 (C-10), 150.71 (C-6), 147.01 (C-5), 141.01 (C-13), 135.21 (C-19), 134.16 (C-14), 133.30 (C-17), 130.92 (C-15), 130.70 (C-1), 129.66 (C-16), 127.44 (C-18), 123.84 (C-2), 123.24 (C-11), 113.87 (C-4), 110.48 (C-3), 56.19 (C-8). APCI-HRMS m/z : calculated for $C_{16}H_{13}Cl_2O_3$ (MH^+), 323.0236, found 323.0230. Purity (HPLC-300 nm): 100%.

(2E)-3-(3-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1d)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 3-chlorobenzaldehyde (1 mmol). Yield: 1 571 mg (45.21%), mp: 135.3 - 139.4°C. ^1H

NMR (600 MHz, DMSO) δ 8.05-8.04 (m, 1H, H-16), 8.00 (d, J = 15.6 Hz, 1H, H-11), 7.85-7.79 (m, 2H, H-2, H-15), 7.66 (d, J = 15.6 Hz, 1H, H-13), 7.64 (d, J = 2.1 Hz, 1H, H-3), 7.49-7.48 (m, 2H, H-17, H-19), 6.93 (d, J = 8.3 Hz, 1H, H-4), 3.89 (s, 3H, H-8). ^{13}C NMR (150 MHz, DMSO) δ 187.48 (C-10), 152.65 (C-6), 148.33 (C-5), 141.40 (C-13), 137.70 (C-14), 134.26 (C-18), 131.09 (C-17), 130.28 (C-19), 129.84 (C-1), 128.31 (C-15), 128.10 (C-16), 124.45 (C-11), 124.26 (C-2), 115.55 (C-4), 112.44 (C-3), 56.33 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{14}\text{ClO}_3$ (MH^+), 289.0626, found 289.0622. Purity (HPLC-300 nm): 99.5%.

(2E)-1-(4-hydroxy-3-methoxyphenyl)-3-[4-(methylsulfonyl)phenyl]prop-2-en-1-one (1e)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 4-methylthiobenzaldehyde (1 mmol). Yield: 310 mg (34.30%), mp: 121.7 -122.0°C. ^1H NMR (600 MHz, CDCl_3) δ 7.79 (d, J = 15.7 Hz, 1H, H-13), 7.67 (m, 2H, H-2, H-3), 7.58 (d, J = 8.4 Hz, 2H, H-15, H-16), 7.53 (d, J = 15.5 Hz, 1H, H-11), 7.28 (d, J = 8.3 Hz, 2H, H-17, H-18), 7.02 (d, J = 8.1 Hz, 1H, H-4), 4.01 (s, 3H, H-8), 2.54 (s, 3H, H-21). ^{13}C NMR (150 MHz, CDCl_3) δ 188.47 (C-10), 150.33 (C-6), 146.89 (C-5), 143.43 (C-13), 142.07 (C-19), 131.67 (C-14), 131.18 (C-1), 128.73 (C-15, C-16), 126.09 (C17, C-18), 123.61 (C-2), 120.68 (C-11), 113.78 (C-4), 110.53 (C-3), 56.16 (C-8), 15.22 (C-21). APCI-HRMS m/z : calculated for $\text{C}_{17}\text{H}_{17}\text{O}_3\text{S}$ (MH^+), 301.0893, found 301.0903. Purity (HPLC-300 nm): 99.1 %.

(2E)-1-(4-hydroxy-3-methoxyphenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (1f)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 3-trifluoromethylbenzaldehyde (1 mmol). Yield: 160 mg (16.5%), mp: 119.8 - 121.0°C. ^1H NMR (600 MHz, DMSO) δ 8.31-8.29 (m, 1H, H-15), 8.18 (d, J = 7.9 Hz, 1H, H-16), 8.09 (d, J = 15.6 Hz, 1H, H-11), 7.87 (dd, J = 8.3 Hz, 2.0 Hz, 1H, H-2), 7.79-7.78 (m, 1H, H-18), 7.77 (d, J = 15.6 Hz, 1H, H-13), 7.71-7.68 (m, 1H, H-19), 7.64 (d, J = 2.0 Hz, 1H, H-3), 6.93 (d, J = 8.3 Hz, 1H, H-4), 3.89 (s, 3H, H-8). ^{13}C NMR (150 MHz, DMSO) δ 187.48 (C-10), 152.72 (C-6), 148.37 (C-5), 141.28 (C-13), 136.61 (C-14), 133.05 (C-16), 130.35 (C-19), 129.77 (C-20), 129.77 (C-1), 126.84 (C-18), 125.54 (C-15), 124.66 (C-2), 124.59 (C-11), 123.66 (C-17), 115.50 (C-4), 112.34 (C-3), 56.30 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{17}\text{H}_{14}\text{F}_3\text{O}_3$ (MH^+), 323.0889, found 323.0898. Purity (HPLC-300 nm): 99.3 %.

(2E)-3-(4-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1g)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 4-fluorobenzaldehyde (1 mmol). Yield: 1 374 mg (41.93%), mp: 107.4 - 108.6°C. ¹H NMR (600 MHz, CDCl₃) δ 7.79 (d, *J* = 15.6 Hz, 1H, H-13), 7.66 (m, 4H, H-2, H-3, H-15, H-16), 7.50 (d, *J* = 15.5 Hz, 1H, H-11), 7.13 (m, 2H, H-17, H-18), 7.02 (d, *J* = 8.1 Hz, 1H, H-4), 6.24 (s, 1H, OH-9), 4.01 (s, 3H, H-8). ¹³C NMR (150 MHz, CDCl₃) δ 188.36 (C-10), 164.80 & 163.13 (C-19), 150.51 (C-6), 146.96 (C-5), 142.64 (C-13), 131.39 (C-14), 130.98 (C-1), 130.24 (C-15, C-16), 123.69 (C-2), 121.48 (C-11), 116.14 (C-17, C-18), 113.86 (C-4), 110.57 (C-3), 56.15 (C-8). APCI-HRMS *m/z*: calculated for C₁₆H₁₄FO₃ (MH⁺), 273.0921, found 273.0922. Purity (HPLC-300 nm): 99.2%.

(2E)-3-(3-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1h)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 3-fluorobenzaldehyde (1 mmol). Yield: 1 482 mg (45.22%), mp: 129.7 - 130.9°C. ¹H NMR (600 MHz, CDCl₃) δ 7.77 (d, *J* = 15.8 Hz, 1H, H-13), 7.67 (m, 2H, H-2, H-3), 7.56 (d, *J* = 15.4 Hz, 1H, H-11), 7.38 (m, 3H, H-15, H-16, H-17), 7.14 (m, 1H, H-19), 7.03 (d, *J* = 8.2 Hz, 1H, H-4), 6.18 (s, 1H, OH-9), 4.01 (s, 3H, H-8). ¹³C NMR (150 MHz, CDCl₃) δ 188.18 (C-10), 163.92 & 162.28 (C-18), 150.61 (C-6), 146.97 (C-5), 142.42 (C-13), 137.46 (C-14), 130.85 (C-1), 130.48 (C-17), 124.43 (C-15), 123.81 (C-2), 122.94 (C-11), 117.19 (C-19), 117.06 (C-19), 114.32 (C-16), 113.87 (C-4), 110.53 (C-3), 56.18 (C-8). APCI-HRMS *m/z*: calculated for C₁₆H₁₄FO₃ (MH⁺), 273.0921, found 273.0931. Purity (HPLC-300 nm): 99.7 %.

(2E)-3-(4-bromo-2-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1i)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 4-bromo-2-fluorobenzaldehyde (1 mmol). Yield: 565 mg (53.45%), mp: 170.6 - 172.3°C. ¹H NMR (600 MHz, CDCl₃) δ 7.82 (d, *J* = 15.8 Hz, 1H, H-13), 7.68 – 7.65 (m, 3H, H-2, H-3, H-11), 7.53 (t, *J* = 8.2 Hz, 1H, H-16), 7.36 (t, *J* = 7.9 Hz, 2H, H-17, H-18), 7.02 (d, *J* = 8.7 Hz, 1H, H-4), 6.20 (s, 1H, OH), 4.01 (s, 3H, H-8). ¹³C NMR (150 MHz, CDCl₃) δ 188.18 (C-10), 162.12 & 160.40 (C-15), 150.65 (C-6), 146.97 (C-5), 135.45 (C-13), 130.74 (C-1),

130.59 (C-16), 127.96 (C-18), 124.82 (C-11), 124.33 & 124.26 (C-19), 123.86 (C-2), 122.44 (C-14), 120.08 & 119.92 (C-17), 113.87 (C-4), 110.53 (C-3), 56.16 (C-8). APCI-HRMS m/z : calculated for $C_{16}H_{13}BrFO_3$ (MH^+), 351.0027, found 351.0018. Purity (HPLC-300 nm): 99.3%.

(2E)-3-(4-ethoxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1j)

The title compound was prepared by reacting 4-Hydroxy-3-methoxyacetophenone (1 mmol) and 4-ethoxybenzaldehyde (1 mmol). Yield: 1 482 mg (45.22%), mp: 129.7 - 130.9°C. ^1H NMR (600 MHz, DMSO) δ 10.10 7.82 (d, J = 8.8 Hz, 2H, H-15, H-16), 7.79-7.76 (m, 2H, H-2, H-11), 7.66 (d, J = 15.5 Hz, 1H, H-13), 7.62 (d, J = 2.0 Hz, 1H, H-3), 7.00 (d, J = 8.8 Hz, 2H, H-17, H-18), 6.92 (d, J = 8.3 Hz, 1H, H-4), 4.10 (q, J = 7.0 Hz, 2H, H-21), 3.88 (s, 3H, H-8), 1.35 (t, J = 7.0 Hz, 3H, H-22). ^{13}C NMR (150 MHz, DMSO) δ 187.52 (C-10), 160.90 (C-19), 152.20 (C-6), 148.22 (C-5), 143.13 (C-13), 131.03 (C-15, C-16), 130.23 (C-1), 127.89 (C-14), 123.93 (C-2), 120.04 (C-11), 115.50 (C-4), 115.26 (C-17, C-18), 112.26 (C-3), 63.80 (C-21), 56.25 (C-8), 15.01 (C-22). APCI-HRMS m/z : calculated for $C_{18}H_{19}O_4$ (MH^+), 299.1278, found 299.1286. Purity (HPLC-300 nm): 98.5 %.

(2E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2a)

The title compound was prepared by reacting (2E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 1 195 mg (59.76%), mp: 190.7 - 191.5°C. ^1H NMR (600 MHz, $CDCl_3$) δ 11.17 (s, 1H, OH), 8.42 (d, J = 1.9 Hz, 1H, H-2), 7.88-7.87 (m, 1H, H-3), 7.85 (d, J = 15.3 Hz, 1H, H-13), 7.64 (d, J = 8.4 Hz, 2H, H-17, H-18), 7.53 (d, J = 15.6 Hz, 1H, H-11), 7.45 (d, J = 8.5 Hz, 2H, H-15, H-16), 4.07 (s, 3H, H-8). ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.38 (C-10), 150.70 (C-6), 150.12 (C-5), 144.42 (C-13), 136.20 (C-19), 133.04 (C-4), 133.00 (C-14), 129.81 (C-15, C-16), 129.38 (C-17, C-18), 129.18 (C-1), 120.42 (C-11), 117.22 (C-2), 116.19 (C-3), 56.98 (C-8). APCI-HRMS m/z : calculated for $C_{16}H_{13}ClNO_5$ (MH^+), 334.0477, found 334.0460. Purity (HPLC-300 nm): 100 %.

(2E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2b)

The title compound was prepared by reacting (2E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 116 mg (13.12%), mp: 201 – 203.9°C. ^1H NMR (600 MHz, DMSO) δ 8.40 (d, J = 2.1 Hz, 1H, H-2),

8.31 (d, $J = 8.5$ Hz, 1H, H-16), 8.06 (d, $J = 15.4$ Hz, 1H, H-13), 7.99 (d, $J = 15.5$ Hz, 1H, H-11), 7.84 (d, $J = 2.0$ Hz, 1H, H-3), 7.75 (d, $J = 2.2$ Hz, 1H, H-17), 7.56 (dd, $J = 8.5$ Hz, 2.2 Hz 2H, H-18), 4.01 (s, 3H, H-8). ^{13}C NMR (150 MHz, DMSO) δ 186.40 (C-10), 150.19 (C-6), 147.13 (C-5), 137.89 (C-13), 136.16 (C-19), 135.66 (C-4), 131.86 (C-15), 130.49 (C-16), 129.96 (C-17), 128.35 (C-14, C-18), 127.98 (C-1), 125.18 (C-11), 118.68 (C-2), 114.63 (C-3), 57.40 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{12}\text{Cl}_2\text{NO}_5$ (MH^+) 368.0087, found 368.0096. Purity (HPLC-300 nm): 100 %.

(2E)-3-(3,4-dichlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2c)

The title compound was prepared by reacting (2E)-3-(3,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 116 mg (13.12%), mp: 201-203.9°C. ^1H NMR (600 MHz, CDCl_3) δ 11.19 (s, 1H, OH), 8.46 (d, $J = 1.9$ Hz, 1H, H-2), 7.88 (d, $J = 1.9$ Hz, 1H, H-3), 7.88-7.78 (m, 2H, H-13, H-15), 7.56-7.52 (m, 3H, H-11, H-16, H-18), 4.07 (s, 3H, H-8). ^{13}C NMR (150 MHz, CDCl_3) δ 186.11 (C-10), 150.77 (C-6), 150.28 (C-5), 143.04 (C-13), 134.94 (C-19), 134.53 (C-14), 133.52 (C-4), 133.04 (C-17), 131.08 (C-16), 130.06 (C-15), 128.96 (C-1), 127.61 (C-18), 121.50 (C-11), 117.32 (C-2), 116.11 (C-3), 57.01 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{12}\text{Cl}_2\text{NO}_5$ (MH^+), 368.0087, found 368.0082. Purity (HPLC-300 nm): 100%.

(2E)-3-(3-chlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2d)

The title compound was prepared by reacting (2E)-3-(3-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 578 mg (83.4%), mp: 178.4-182.1°C. ^1H NMR (600 MHz, DMSO) δ 8.41 (d, $J = 2.0$ Hz, 1H, H-2), 8.10 (s, 1H, H-16), 8.06 (d, $J = 15.5$ Hz, 1H, H-11), 7.68 (d, $J = 7.2$ Hz, 1H, H-15), 7.84 (d, $J = 2.0$ Hz, 1H, H-3), 7.75 (d, $J = 15.6$ Hz, 1H, H-13), 7.53 – 7.48 (m, 2H, H-17, H-19), 4.01 (s, 3H, H-8). ^{13}C NMR (150 MHz, DMSO) δ 186.59 (C-10), 150.10 (C-6), 146.85 (C-5), 143.00 (C-13), 137.98 (C-4), 137.38 (C-14), 134.27 (C-18), 131.11 (C-17), 130.68 (C-19), 128.52 (C-15, C-16), 128.22 (C-1), 123.33 (C-11), 118.52 (C-2), 114.57 (C-3), 57.38 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{13}\text{ClNO}_5$ (MH^+), 334.0477, found 334.0491. Purity (HPLC-300 nm): 100 %.

(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-[4-(methylsulfanyl)phenyl]prop-2-en-1-one (2e)

The title compound was prepared by reacting (2E)-1-(4-hydroxy-3-methoxyphenyl)-3-[4-(methylsulfanyl)phenyl]prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 429 mg (49.09%), mp: 182.7-183.9°C. ¹H NMR (600 MHz, DMSO) δ 8.37 (d, *J* = 2.0 Hz, 1H, H-2), 7.95 (d, *J* = 15.5 Hz, 1H, H-11), 7.87 (d, *J* = 8.5 Hz, 2H, H-15, H-16), 7.84 (d, *J* = 2.0 Hz, 1H, H-3), 7.75 (d, *J* = 15.5 Hz, 1H, H-13), 7.33 (d, *J* = 8.5 Hz, 2H, H-17, H-18), 4.01 (s, 3H, H-8), 2.51 (s, 3H, H-21). ¹³C NMR (150 MHz, DMSO) δ 186.55 (C-10), 150.06 (C-6), 146.65 (C-5), 144.35 (C-13), 142.61 (C-19), 137.92 (C-4), 131.55 (C-14), 130.01 (C-15,C-16), 128.54 (C-1), 126.05 (C-17,C-18), 120.66 (C-11), 118.22 (C-2), 114.60 (C-3), 57.36 (C-8), 14.71 (C-21). APCI-HRMS *m/z*: calculated for C₁₇H₁₆NO₅S (MH⁺), 346.0744, found 346.0765. Purity (HPLC-300 nm): 88.0 %.

(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (2f)

The title compound was prepared by reacting (2E)-1-(4-hydroxy-3-methoxyphenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 343 mg (64.4%), mp: 171.5-172.2°C. ¹H NMR (600 MHz, DMSO) δ 8.43 (d, *J* = 2.0 Hz, 1H, H-2), 8.34-8.30 (m, 1H, H-15), 8.23 (d, *J* = 7.8 Hz, 1H, H-16), 8.13 (d, *J* = 15.6 Hz, 1H, H-11), 7.83-7.80 (m, 2H, H-13, H-18), 7.84 (d, *J* = 1.9 Hz, 1H, H-3), 7.72-7.69 (t, *J* = 7.8 Hz, 1H, H-19), 4.01 (s, 3H, H-8). ¹³C NMR (150 MHz, DMSO) δ 186.58 (C-10), 150.12 (C-6), 147.01 (C-5), 142.85 (C-13), 137.93 (C-14), 136.28 (C-4), 133.32 (C-16), 130.36 (C-19), 130.17 (C-1), 128.02 (C-17), 127.24 (C-18), 125.90 (C-15), 128.88 (C-20), 125.44 (C-11), 118.67 (C-2), 114.34 (C-3), 57.32 (C-8). APCI-HRMS *m/z*: calculated for C₁₇H₁₃F₃NO₃ (MH⁺), 369.0740, found 368.0729. Purity (HPLC-300 nm): 100 %.

(2E)-3-(4-fluorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2g)

The title compound prepared by reacting (2E)-3-(4-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 646 mg (55.43%), mp: 208.3-209.4°C. ¹H NMR (600 MHz, DMSO) δ 8.38 (d, *J* = 2.0 Hz, 1H, H-2), 8.04-8.00 (m, 2H, H-15, H-16), 7.96 (d, *J* = 15.5 Hz, 1H, H-11), 7.84 (d, *J* = 2.0 Hz, 1H, H-

3), 7.78 (d, $J = 15.5$ Hz, 1H, H-13), 7.33-7.30 (m, 2H, H-17, H-18), 4.01 (s, 3H, H-8). ^{13}C NMR (150 MHz, DMSO) δ 186.59 (C-10), 164.78 & 163.13 (C-19), 150.10 (C-6), 146.79 (C-5), 143.48 (C-13), 137.92 (C-4), 131.92 (C-15, C-16), 131.82 (C-14), 128.35 (C-1), 121.71 (C-11), 118.35 (C-2), 116.43 (C-17, C-18), 114.59 (C-3), 57.36 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{13}\text{FNO}_5$ (MH^+), 318.0772, found 318.0780. Purity (HPLC-300 nm): 100%.

(2E)-3-(3-fluorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2h)

The title compound was prepared by reacting (2E)-3-(3-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 651 mg (55.89%), mp: 189.5-191.4°C. ^1H NMR (600 MHz, DMSO) δ 8.41 (d, $J = 2.0$ Hz, 1H, H-2), 8.05 (d, $J = 15.5$ Hz, 1H, H-11), 7.90 (d, $J = 10.2$ Hz, 1H, H-17), 7.84 (d, $J = 2.0$ Hz, 1H, H-3), 7.76 (d, $J = 15.5$ Hz, 1H, H-13), 7.73-7.72 (m, 1H, H-16), 7.53-7.19 (m, 1H, H-15), 7.33-7.27 (m, 1H, H-19), 4.01 (s, 3H, H-8). ^{13}C NMR (150 MHz, DMSO) δ 186.58 (C-10), 163.79 & 162.18 (C-18), 150.13 (C-6), 146.93 (C-5), 143.21 (C-13), 137.97 (C-4), 137.67 (C-14), 131.31 (C-15), 128.18 (C-1), 126.25 (C-16), 123.24 (C-11), 118.52 (C-2), 117.86 (C-19), 115.12 (C-3), 114.54 (C-17), 57.3 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{13}\text{FNO}_5$ (MH^+), 318.0772, found 318.0789. Purity (HPLC-300 nm): 99.5 %.

(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-(4-nitrophenyl)prop-2-en-1-one (2i)

The title compound was prepared by reacting (2E)-1-(4-hydroxy-3-methoxyphenyl)-3-(4-nitrophenyl)prop-2-en-1-one (1 mmol) and nitric acid (0.76 mmol). Yield: 225 mg (19.53%), mp: 229.4-230.5°C. ^1H NMR (600 MHz, DMSO) δ 8.43 (d, $J = 2.0$ Hz, 1H, H-2), 8.29 (d, $J = 8.8$ Hz, 2H, H-17, H-18), 8.20 (d, $J = 8.9$ Hz, 2H, H-15, H-16), 8.18 (d, $J = 15.6$ Hz, 1H, H-11), 7.58 (d, $J = 15.6$ Hz, 1H, H-13), 7.84 (d, $J = 2.0$ Hz, 1H, H-3), 4.01 (s, 3H, H-8). ^{13}C NMR (150 MHz, DMSO) δ 186.50 (C-10), 150.20 (C-6), 148.62 (C-5), 147.16 (C-19), 141.77 (C-13), 141.60 (C-14), 137.93 (C-4), 130.47 (C-15, C-16), 127.95 (C-1), 125.85 (C-11), 124.32 (C-17, C-18), 118.76 (C-2), 114.51 (C-3), 57.37 (C-8). APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{13}\text{N}_2\text{O}_7$ (MH^+), 345.0717, found 345.0728. Purity (HPLC-300 nm): 100%.

Screening and minimum inhibitory concentration

The *in vitro* antibacterial activity of the guaiacol- and nitroguaiacol-chalcone derivatives was initially determined at a single concentration (256 $\mu\text{g/ml}$) against all bacteria. Subsequently, the precise MIC of each derivative was established against the susceptible bacterial strains. In general, the guaiacol-chalcone derivatives had low solubility in water, forming a

precipitate in the wells at high concentrations, *i.e.*, >64 µg/ml, prohibiting the use of absorbance to measure bacterial growth. To circumvent this problem, resazurin was used as a colorimetric indication to indicate bacterial growth, and not absorbance.

During screening only one guaiacol-chalcone derivative (**1j**) and all nitroguaiacol-chalcone derivatives displayed activity against *S. aureus* (Gram-positive bacteria). However, none of the derivatives exhibited antibacterial activity against the Gram-negative bacteria (Table 4.2). This is consistent with a previous study in which all nitrocatechol-chalcone and nitrocatechol-pyrazoline derivatives only showed activity against *S. aureus*. This suggests that the guaiacol- and nitroguaiacol moieties did not broaden the antibacterial effectiveness of the chalcone derivatives (Hoepfner *et al.*, 2023:1). Comparing the activity of the guaiacol- vs. the nitroguaiacol-chalcone derivatives, the omission of the nitro-substituent led to the complete loss of antibacterial activity except for **1j**. It is worth noting that this might be a result of poor solubility in the test medium as mentioned above.

Studies have shown that altering the substituents on both rings A and B of chalcone derivatives can significantly alter their antibacterial activity. For example, some have found that chalcones with *para*-substituents on ring B tend to only have activity against Gram-positive bacteria. In contrast, 2,5-dichloro thiophene containing chalcones, *i.e.*, substituents in both the *ortho*- and *meta*-positions, have broad activity against both Gram-positive and Gram-negative bacteria (Ávila *et al.*, 2008:9790). The absence of antibacterial activity in the guaiacol-chalcone derivatives might be attributed to the lack of the nitro-group on aromatic ring A. Several antibiotics possess a nitro-moiety, *e.g.*, metronidazole, tinidazole and chloramphenicol, which is a strong electron-withdrawing moiety that creates electron-deficient sites in molecules that can interact with biological nucleophiles, *e.g.*, proteins, amino acids and enzymes (Jezuíta *et al.*, 2020:3; Nepali *et al.*, 2019:2851; Noriega *et al.*, 2022:1). Similarly, the antibacterial action of **1j** may be attributed to the electron-accepting nature of the ethoxy group located on ring B.

Table 4.2: Screening and MIC results of novel derivatives (**1a - j** and **2a - h**)

Derivatives	Screening					MIC (µg/ml)	
	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>A. baumannii</i>	<i>P. aeruginosa</i>	<i>K. aerogenes</i>	<i>S. aureus</i>	MRSA
1a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1c	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1d	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1e	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1f	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1g	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1h	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1i	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	ND	ND
1j	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	16	32
2a	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	16	32
2b	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	256	256
2c	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	8	8
2d	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	16	64
2e	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	128	>256
2f	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	32	64
2g	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	64	64
2h	Active ^a	Inactive ^b	Inactive ^b	Inactive ^b	Inactive ^b	32	64

ND: Not done. Only the MIC of compounds found to be active during screening was done.

^aActive: A derivative had an MIC of ≤ 256 $\mu\text{g/ml}$

^bInactive: A derivative had an MIC of > 256 $\mu\text{g/ml}$

After the screening concluded, the precise MIC of the active guaiacol-chalcone derivative, *i.e.*, **1j**, and the nitroguaiacol-chalcone derivatives were determined against *S. aureus* and MRSA (Table 4.2). Against *S. aureus* and MRSA, **2c** was the most active (8 $\mu\text{g/ml}$), then **2a** & **1j** (16 $\mu\text{g/ml}$ (*S. aureus*) & 32 $\mu\text{g/ml}$ (MRSA)) and then **2d** (16 $\mu\text{g/ml}$ (*S. aureus*) & 64 $\mu\text{g/ml}$ (MRSA)). The other derivatives had weaker antibacterial activity against *S. aureus* (32 - 256 $\mu\text{g/ml}$) and MRSA (64 – 256 $\mu\text{g/ml}$) while **2e** were inactive against MRSA. The most active (**2c**) and least active (**2b**) derivatives both contain two chlorine substituents on ring B, *i.e.*, **2c** (*m*-Cl & *p*-Cl) and **2b** (*o*-Cl & *p*-Cl). Substitution on the *ortho*-position thus led to a substantial reduction in antibacterial activity. Derivatives **2a** (*p*-Cl) and **2d** (*m*-Cl) had only one chlorine substituent and had similar antibacterial activity against *S. aureus* compared to **2c**, however, they were weaker against MRSA. This suggests that two chlorine substituents on both the *meta*- and *para*-positions (**2c**) increase antibacterial activity against MRSA. Replacing the chlorine substituent with fluorine, decreased the antibacterial activity against *S. aureus* in two instances, *viz.* **2a** (*p*-Cl) vs. **2g** (*p*-F) and **2d** (*m*-Cl) vs. **2h** (*m*-F), indicating that the chlorine atom is more important for antibacterial activity. Neither of the other derivatives had substantial antibacterial activity against either *S. aureus* or MRSA, *i.e.*, **2e** (*p*-SCH₃), **2f** (*m*-CF₃) and **2i** (*p*-NO₂).

Studies have shown that halogen-protein/ligand complexes can be a powerful tool to enhance binding selectivity (Hardegger *et al.*, 2011:318). Antibiotics like vancomycin and salinosporamid A, require one to two chlorine substituents to exert their antibacterial activity. The presence of these substituents plays a key role in the structural alignment of the molecules in the binding pocket, and their absence or relocation will consequently result in the loss of antibacterial activity (Groll *et al.*, 2006:5136; Harris *et al.*, 1985:6652; Molchanova *et al.*, 2020:1). The chlorine substituents on ring B of derivatives **2a**, **c** and **d**, might play a similar role. Halogens can act as hydrogen-bond acceptors or form complexes with hydrogen-bond acceptors. Incorporating halogens can also alter the lipophilicity of molecules, promoting their passive diffusion across biological membranes and *in vivo* transport (Adams *et al.*, 2004:657; Faleye *et al.*, 2024:92; Jeschke, 2010:12; Lin & MacKerell, 2017:6813; Priimagi *et al.*, 2013:2687).

Checkerboard assay

During screening, **2a** showed synergism with one antibiotic, *i.e.*, chloramphenicol, against *S. aureus*. The checkerboard assay was repeated five times ($n = 5$) and the FICI of each repeat is shown in table 4.5-4.9 in the supplementary data. The minimum FICI (FICI_{min}) for all five repeats ranged from 0.31 to 0.53 (some above and below the 0.5 threshold) and the average and standard deviation were 0.45 ± 0.09 , which indicates synergism.

Chloramphenicol (Figure 4.3) is a bacteriostatic broad-spectrum antibiotic that consists of a *p*-nitrobenzene ring, a dichloroacetyl tail and a 2-amino-1,3-propanediol moiety (Controulis *et al.*, 1949:2463; Pongs, 1979:26) and is one of few natural compounds with two strong electron-withdrawing groups (Tevyashova, 2021:1). Chloramphenicol is a competitive translation inhibitor and binds with the peptidyl transferase centre of 23S ribosomal ribonucleic acid (rRNA) found in the 50S subunit of bacterial ribosomes. The hydroxy-groups and carbonyl-group of chloramphenicol form hydrogen bonds with the 23S rRNA to inhibit peptide bond formation (Schlünzen *et al.*, 2001:814; Tevyashova, 2021:1). The synergism observed between **2a** and chloramphenicol would suggest that their antibacterial mode of action might be within the same metabolic pathway, *i.e.*, protein synthesis.

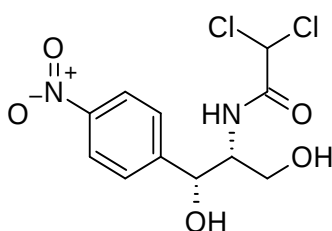


Figure 4.3: Structure of chloramphenicol.

Common feature pharmacophore modelling

The *in vitro* data was used as a metric to determine which derivatives should be included in the training and test sets. The sets were utilised to create and validate common feature pharmacophore models. In total ten pharmacophore hypotheses (hypos.) were developed, and the best hypothesis was chosen based on its rank score (Table 4.3), EF^{10%}, and ROC-AUC (Table 4.4).

The ten pharmacophore hypos. generated by the training set presented with rank scores ranging from 52.34 – 50.41 (Table 4.3). The first 8 hypos. contained four common features, *i.e.* a ring aromatic (R), a hydrophobic (H) and two hydrogen bond acceptors. Two of the

hypos. also contained four common features, *i.e.* two ring aromatics (R), a hydrophobic (H) and two hydrogen bond acceptors.

Of the ten generated hypotheses, hypo 1. had the best EF^{10%} and ROC-AUC values (Table 4.4). Hypo. 1 reports that a ring aromatic, a hydrophobic and two hydrogen bond accepting groups are important for antibacterial activity (Figure 4.4). When **2c** is mapped with hypo 1. (Figure 4.4) it is evident that two of the oxygen atoms, the methoxy-group on ring A and the carbonyl-group, act as hydrogen bond acceptors. Ring B and the chlorine on the *para*-position of ring B act as the ring aromatic and the hydrophobic moiety respectively.

Table 4.3: Pharmacophore hypotheses as created for *S. aureus*.

Hypo.	Feature ^a	Rank Score	Direct Hit ^b	Partial Hit ^c	Max fit
1	RHAA	52.34	11111	00000	4
2	RHAA	52.34	11111	00000	4
3	RHAA	51.59	11111	00000	4
4	RHAA	51.59	11111	00000	4
5	RHAA	51.08	11111	00000	4
6	RHAA	51.08	11111	00000	4
7	RHAA	50.84	11111	00000	4
8	RHAA	50.61	11111	00000	4
9	RRHA	50.45	11111	00000	4
10	RRHA	50.41	11111	00000	4

^a R = Ring aromatic, H = Hydrophobic, A = Hydrogen bond acceptor.

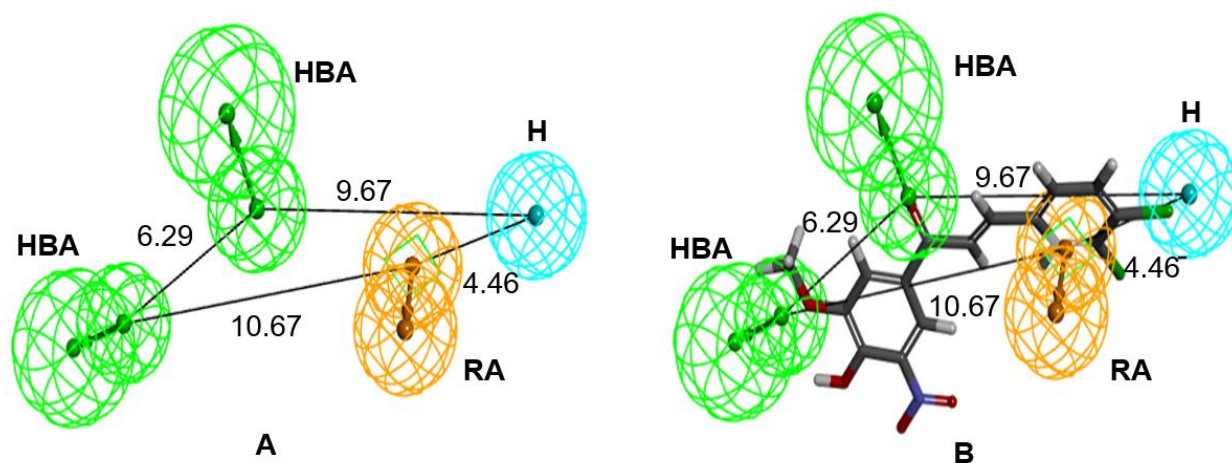


Figure 4.4: Hypothesis 1 (A) with inter-feature constraints and (B) with **2c** mapped. Hydrogen bond acceptor (HBA), Ring aromatic (RA), hydrophobic (H).

Table 4.4: EF^{10%} and ROC-AUC data of each pharmacophore hypothesis.

Hypo.	EF ^{10%}	ROC-AUC
1	8	0.95 ± 0.02*
2	8	0.95 ± 0.02*
3	2	0.27 ± 0.16
4	0	0.20 ± 0.15
5	8	0.95 ± 0.03*
6	4	0.89 ± 0.04*
7	2	0.27 ± 0.17
8	6	0.61 ± 0.20
9	6	0.73 ± 0.13
10	6	0.78 ± 0.09

*Statistically significant ($p \leq 0.05$).

Conclusion

For this study novel guaiacol-chalcone and nitroguaiacol-chalcone derivatives were synthesised, which were then tested against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Klebsiella aerogenes*. One guaiacol-chalcone derivative (**1j**) and all the nitroguaiacol-chalcone derivatives were active against *S. aureus*. The MIC was determined for each active derivative against *S. aureus* and MRSA with the most active compound (**2c**) having an MIC of 8 µg/ml against both *S. aureus* and MRSA. Both the most-and least active derivatives contained two chlorine substituents on ring B, *i.e.*, **2c** (*m*-Cl & *p*-Cl) and **2b** (*o*-Cl & *p*-Cl), however, the positions of the substituents had a noticeable effect on their *in vitro* activity. The substitution of the chloride on the *meta*-position was favoured over the *ortho*-position. The checkerboard assay showed that **2a** had a synergistic effect when combined with chloramphenicol (FIC_{min}: 0.45 ± 0.09), suggesting the nitroguaiacol-chalcone derivative may possess the same mechanism of antibacterial action as chloramphenicol or share the same metabolic pathway, *i.e.* peptide bond formation inhibition in bacteria. The *in vitro* activity of each derivative was utilised to generate common feature pharmacophore models for *S. aureus*, which were subsequently evaluated by calculating their rank scores, EF^{10%} and ROC-AUC values. The best pharmacophore model was hypo. 1 (rank score of 52.34; EF^{10%}:8; ROC-AUC: 0.95 ± 0.02). This hypothesis identified a ring aromatic, a hydrophobic and two hydrogen bond accepting groups as important for antibacterial activity.

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Supplementary data

Table 4.5: Checkerboard assay results, **2a** and chloramphenicol, repeat 1

No growth	Derivative 2a		Chloramphenicol (Chl)		Σ FIC (FIC of 2a + FIC of Chl)	Interpretation
	Conc.	FIC of	Conc.	FIC of Chl		
	($\mu\text{g/ml}$)	2a	($\mu\text{g/ml}$)			
B1	16	MIC _{2a}	0	MIC _{2a}	MIC _{2a}	MIC _{2a}
B2	16	1	0.125	0.03	1.03	ADD*
B3	16	1	0.25	0.06	1.06	ADD*
C4	8	0.5	0.5	0.13	0.63	ADD*
D5	4	0.25	1	0.25	0.50	SYN [†]
E5	2	0.13	1	0.25	0.38	SYN [†]
F5	1	0.06	1	0.25	0.31	SYN [†]
G6	0.5	0.03	2	0.50	0.53	ADD*
H7	0	MIC _{Chl}	4	MIC _{Chl}	MIC _{Chl}	MIC _{Chl}

*ADD is additive; [†]SYN is synergism.

Table 4.6: Checkerboard assay results, **2a** and chloramphenicol, repeat 2

No growth	Derivative 2a		Chloramphenicol (Chl)		Σ FIC (FIC of 2a + FIC of Chl)	Interpretation
	Conc. (μ g/ml)	FIC of 2a	Conc. (μ g/ml)	FIC of Chl		
B1	16	MIC _{2a}	0	MIC _{2a}	MIC _{2a}	MIC _{2a}
B2	16	1	0.125	0.06	1.06	ADD*
B3	16	1	0.25	0.13	1.13	ADD*
B4	16	1	0.5	0.25	1.25	ADD*
C5	8	0.5	1	0.5	1	ADD*
D5	4	0.25	1	0.5	0.75	ADD*
E5	2	0.13	1	0.5	0.63	ADD*
F5	1	0.06	1	0.5	0.56	ADD*
G5	0.5	0.03	1	0.5	0.53	ADD*
H6	0	MIC _{Chl}	2	MIC _{Chl}	MIC _{Chl}	MIC _{Chl}

*ADD is additive

Table 4.7: Checkerboard assay results, **2a** and chloramphenicol, repeat 3

No growth	Derivative 2a		Chloramphenicol (Chl)		Σ FIC (FIC of 2a + FIC of Chl)	Interpretation
	Conc. (μ g/ml)	FIC of 2a	Conc. (μ g/ml)	FIC of Chl		
B1	32	MIC _{2a}	0	MIC _{2a}	MIC _{2a}	MIC _{2a}
B2	16	0.5	0.125	0.03	0.53	ADD*
B3	16	0.5	0.25	0.06	0.56	ADD*
B4	16	0.5	0.5	0.13	0.63	ADD*
B5	16	0.5	1.0	0.25	0.75	ADD*
C6	8	0.25	1	0.5	0.75	ADD*
D6	4	0.13	2	0.5	0.63	ADD*
E6	2	0.06	2	0.5	0.56	ADD*
F6	1	0.03	2	0.5	0.53	ADD*
G6	0.5	0.02	2	0.5	0.52	ADD*
H6	0	MIC _{Chl}	4	MIC _{Chl}	MIC _{Chl}	MIC _{Chl}

*ADD is additive

Table 4.8: Checkerboard assay results, **2a** and chloramphenicol, repeat 4

No growth	Derivative 2a		Chloramphenicol (Chl)		Σ FIC (FIC of 2a + FIC of Chl)	Interpretation
	Conc. ($\mu\text{g/ml}$)	FIC of 2a	Conc. ($\mu\text{g/ml}$)	FIC of Chl		
B1	32	MIC _{2a}	0	MIC _{2a}	MIC _{2a}	MIC _{2a}
B2	16	0.5	0.125	0.03	0.53	ADD*
B3	16	0.5	0.25	0.06	0.56	ADD*
B4	16	0.5	0.5	0.13	0.63	ADD*
B5	16	0.5	1	0.25	0.75	ADD*
C6	8	0.25	1	0.5	0.75	ADD*
D6	4	0.13	2	0.5	0.63	ADD*
E6	2	0.06	2	0.5	0.56	ADD*
F6	1	0.03	2	0.5	0.53	ADD*
G6	0.5	0.02	2	0.5	0.52	ADD*
H6	0	MIC _{Chl}	4	MIC _{Chl}	MIC _{Chl}	MIC _{Chl}

*ADD is additive

Table 4.9: Checkerboard assay results, **2a** and chloramphenicol, repeat 5

No growth	Derivative 2a		Chloramphenicol (Chl)		Σ FIC (FIC of 2a + FIC of Chl)	Interpretation
	Conc. (μ g/ml)	FIC of 2a	Conc. (μ g/ml)	FIC of Chl		
B1	32	MIC _{2a}	0	MIC _{2a}	MIC _{2a}	MIC _{2a}
B2	16	0.5	0.125	0.03	0.53	ADD*
B3	16	0.5	0.25	0.06	0.56	ADD*
B4	16	0.5	0.5	0.13	0.63	ADD*
C5	8	0.25	1	0.25	0.5	SYN [†]
D5	4	0.13	1	0.25	0.38	SYN [†]
E6	2	0.06	2	0.5	0.56	ADD*
F6	1	0.03	2	0.5	0.53	ADD*
G6	0.5	0.02	2	0.5	0.52	ADD*
H6	0	MIC _{Chl}	4	MIC _{Chl}	MIC _{Chl}	MIC _{Chl}

*ADD is additive; [†]SYN is synergism.

CHAPTER 5: ARTICLE 3

Evaluation of nitrocatechol-, guaiacol-, nitroguaiacol- and pyrazoline-chalcone derivatives as inhibitors of monoamine oxidase

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Abstract

Chalcone and pyrazoline derivatives have been described as inhibitors of the monoamine oxidase (MAO) enzymes with specificity for the MAO-B isoform while nitrocatechol derivatives have been reported to possess potent catechol O-methyltransferases (COMT) inhibition activities. MAO and COMT are key enzymes for the metabolism of dopamine and levodopa, and are of importance for the treatment of Parkinson's disease (PD). This study attempted to enhance the MAO inhibitory activity of chalcone and pyrazoline derivatives by synthesising new nitrocatechol-chalcone and nitrocatechol-pyrazoline derivatives. Additionally, guaiacol- and nitroguaiacol-chalcone derivatives were synthesised to explore the importance of the nitrocatechol moiety for MAO inhibition. The novel derivatives were evaluated as human MAO inhibitors and the activities were reported as half-maximal

inhibitory concentration (IC_{50}) values. Lineweaver-Burk graphs were constructed to determine the mechanism of action of the most active MAO inhibitors. A common feature pharmacophore model was created to identify the chemical entities that are important for MAO inhibition. The pharmacophore model was validated utilising the ranking score, enrichment factor ($EF^{10\%}$) and the area under the receiver operating characteristic (AUC-ROC) curve metrics. The chalcone derivatives were more potent inhibitors of MAO-B than MAO-A and the pyrazoline derivatives had comparable inhibitory activity against both isoforms. The guaiacol-chalcone derivative, **3c**, was the most active compound against MAO-A ($IC_{50} = 0.49 \mu M$) and MAO-B ($IC_{50} = 0.06 \mu M$) and derivatives **3b** ($IC_{50} = 0.12 \mu M$) and **3f** ($IC_{50} = 0.12 \mu M$) inhibited MAO-B with activities comparable to the positive control, safinamide. The Lineweaver-Burk graphs indicated that **3c** was a competitive inhibitor of MAO-A and MAO-B. The leading pharmacophore model, *i.e.*, hypothesis 5 (rank score: 62.24, $EF^{10\%}$: 7.5, AUC-ROC: 1.00 ± 0.002) indicated that four features are important for activity *i.e.*, a hydrogen bond donor, a hydrogen bond acceptor and two hydrophobic features.

Keywords: *Chalcones; Pyrazoline; Monoamine oxidase; Parkinson's disease, Inhibition; Competitive.*

Introduction

After Alzheimer's disease, Parkinson's disease (PD) is the second most common neurodegenerative disorder, imposing an increasing social and economic burden as the population ages. Although the exact cause of this disease is not understood, previous research suggests that mitochondrial dysfunction, oxidative stress, misfolding of proteins and neuroinflammation are the main contributing factors (Gao *et al.*, 2017:1380; Greenamyre & Hastings, 2004:1120; De Lau & Breteler, 2006:525). The neuropathological indicators of PD are dopaminergic neuron degeneration in the substantia nigra (SN) in the midbrain and intracellular accumulation of the α -synuclein protein, also known as Lewy bodies (Poewe *et al.*, 2017:2; Simon *et al.*, 2020:2).

Levodopa is the most effective symptomatic treatment for PD; however, long-term use can induce motor complications. Dopamine agonists, catechol-O-methyl transferase (COMT) inhibitors, which inhibit levodopa degradation in the periphery, and monoamine oxidase (MAO) B inhibitors, which reduce dopamine degradation, are frequently co-administered

with levodopa to counteract these complications (Gershanik, 2015:103; Saura *et al.*, 1996:756; Tan *et al.*, 2022:478). The MAOs are mitochondrial, flavin adenine dinucleotide (FAD) enzymes that catalyse the oxidative deamination of monoamine neurotransmitters and exist in two different isoforms, MAO-A and MAO-B (Iacovino *et al.*, 2021:1151; Mathew, 2019:120). MAO-B metabolises dopamine to produce hydrogen peroxide as by-product, and thus is a significant contributor to oxidative stress and damage in PD (Dezsi & Vecsei, 2017:426). The inhibition of MAO has been shown to reduce oxidative stress and subsequently might have a neuroprotective effect (Edmondson & Binda, 2018:117).

Chalcones (*trans*-1,3-diphenyl-2-propen-1-ones) are α,β -unsaturated ketones that serve as a privileged scaffold for developing compounds with central nervous system activity (Guglielmi *et al.*, 2020:3). Previous structure-activity relationship (SAR) studies have investigated the inhibitory activity and selectivity of chalcones towards the two MAO isoforms by examining the impact of various substituents on two aromatic rings, commonly known as ring A and B, of chalcone derivatives. These studies suggested that the addition of heterocycles, nitro-groups, or lipophilic electron-donating groups, as well as halogen substituents on one or both aromatic rings, could enhance the activity of chalcone derivatives (Guglielmi *et al.*, 2020:26; Iacovino *et al.*, 2021:1151). Furthermore, it has been observed that chalcone derivatives with higher selectivity towards the MAO-B isoform possess hydroxyl and methoxyl substituents on the *ortho*- and *para*-positions of ring A, respectively, along with a chlorine on the *para*-position of ring B (Figure 5.1) (Chimenti *et al.*, 2009:2819; Mathew, 2019:120; Mathew *et al.*, 2016:1).

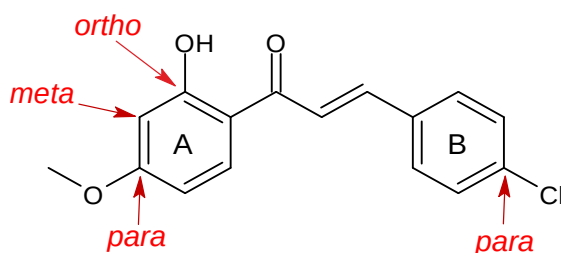


Figure 5.1: Common structure of a chalcone and suggested structural alterations (Chimenti *et al.*, 2009:2818; Mathew, 2019:6).

Previous studies by our group determined that nitrocatechol-chalcone and pyrazoline derivatives had weak MAO-B inhibitory activity, although ring B has been identified as a future site for structural modification to improve the potency of these derivatives (Engelbrecht *et al.*, 2018:125; Hitge *et al.*, 2020:5). In this study, previously synthesised nitrocatechol-, guaiacol-, nitroguaiacol-chalcone and pyrazoline derivatives (Hoepfner *et al.*,

2023:6) (Table 5.1) were evaluated as potential MAO-A and MAO-B inhibitors using the recombinant human MAO enzymes. The inhibitory activities were expressed as half-maximal inhibitory concentration (IC₅₀) values while the mechanism of inhibition of the most active derivatives was investigated by constructing sets of Lineweaver-Burk graphs.

Materials and methods

MAO-A and MAO-B inhibition studies

Previously synthesised nitrocatechol (**1a-k**), pyrazoline (**2a-j**), guaiacol (**3a-j**) and nitroguaiacol (**4a-i**) derivatives were evaluated for potential *in vitro* human MAO-A and MAO-B inhibition using a literature protocol. The recombinant human MAO enzymes were used for these studies and kynuramine was used as substrate for both MAO isoforms. MAO converts kynuramine to 4-hydroxyquinoline which was subsequently measured by fluorescence spectrophotometry at excitation and emission wavelengths of 310 nm and 400 nm, respectively. Harmine and safinamide served as reference inhibitors for MAO-A and MAO-B, respectively (Mostert *et al.*, 2015:862).

The enzymatic reactions were carried out in white polypropylene 96-well microtiter plates to a final volume of 200 µl. Firstly, the chalcone and pyrazoline derivatives were screened for inhibitory activity at 10 µM and 100 µM. For the active derivatives, full evaluation was conducted at a concentration range of 0.003-100 µM, with experiments carried out in triplicate. The enzyme reactions contained potassium phosphate buffer (92 µl; 100 mM, pH 7.4, made isotonic with 20.1 mM KCl), kynuramine (50 µl), test compound (8 µl) and MAO enzyme (50 µl). The stock solutions of test compounds were prepared in dimethyl sulfoxide (DMSO) and introduced to the reaction to yield a final concentration of 4% (v/v) DMSO. Reactions performed in the absence of inhibitors also included 4% (v/v) DMSO. The final concentration of kynuramine in the incubations was 50 µM. The reactions were initiated with the addition of MAO-A and MAO-B to yield a final enzyme concentration of 0.0075 and 0.015 mg protein/ml for MAO-A and MAO-B, respectively, and were subsequently incubated at 37 °C for 20 min. At endpoint, the reactions were terminated with the addition of NaOH (80 µl, 2 N). Fluorescence spectrophotometry was used to measure the concentration of 4-hydroxyquinoline generated by MAO. To calculate the enzyme catalytic rates, a linear calibration curve was constructed with authentic 4-hydroxyquinoline (0.047-1.50 µM). Sigmoidal plots of rate versus the logarithm of the concentration of the test inhibitor were constructed and fitted to the one-site competition model in the Prism 5 Software Package

(GraphPad). These plots enabled the estimation of the IC₅₀ values of the test compounds (Engelbrecht *et al.*, 2018:121; Hitge *et al.*, 2020:3; Mostert *et al.*, 2015:863).

Mode of MAO-A and MAO-B inhibition

As the guaiacol-chalcone derivatives were the most potent MAO inhibitors, their inhibitory properties were further evaluated by constructing Lineweaver-Burk plots for the inhibition of MAO-A and MAO-B. Compound **3c** was selected for further evaluation. The Lineweaver-Burk graphs were constructed by measuring the activity of MAO in the absence and the presence of the following concentration of the selected inhibitor: $\frac{1}{4} \times \text{IC}_{50}$, $\frac{1}{2} \times \text{IC}_{50}$, $\frac{3}{4} \times \text{IC}_{50}$, $1 \times \text{IC}_{50}$ and $1\frac{1}{4} \times \text{IC}_{50}$. The MAO catalytic rates were recorded at kynuramine concentrations ranging from 15 to 250 μM (Engelbrecht *et al.*, 2018:123; Mostert *et al.*, 2015:872).

Common feature pharmacophore modelling

Pharmacophore modelling was carried out with Accelrys Discovery Studio (DS) 3.1 (San Diego, USA) and the area under the receiver operating characteristics (AUC-ROC) curves were calculated in SPSS Statistics for Windows, version 27 (SPSS Inc., Chicago, IL, USA). Microsoft Excel for Microsoft 365 (Microsoft, USA) and equation 1 was utilised to determine the enrichment factor (EF).

The structures of the 40 derivatives were drawn in DS. The derivatives were randomly allocated into a training ($n = 23$ compounds) and test ($n = 27$ compounds) set. For the training set, principal values of 0 and 2 were assigned to the inactive ($\text{IC}_{50} > 1 \mu\text{M}$) and active ($\text{IC}_{50} < 1 \mu\text{M}$; **3b**, **3d**, **3e**, **3f**, **3g**, **3i**) derivatives, respectively, and the maximum omitted feature (MaxOmitFeat) was set to 0. The derivatives were prepared with the *prepare ligands* protocol and four features, *i.e.*, hydrogen bond acceptor (A), hydrogen bond donor (D), hydrophobic (H) and ring aromatic (R) were identified with the *Feature Mapping* protocol. Using the *Common Feature Pharmacophore* protocol, 10 hypotheses were generated with the maximum conformations set to 255, the energy threshold at 20 kcal/mol and the inter-feature distance to 2.97 Å. The generated pharmacophores were then ranked based on the rank scores.

Decoy compounds from the Schrodinger dataset (999 compounds) were added to the derivatives of the test set ($n = 27$ compounds) to yield a new test set of 1026 compounds. The test set was prepared with the *prepare ligands* protocol, the MaxOmitFeat value was set to 1 for all compounds, and the test set was fitted to the 10 hypotheses using *Ligand Profiler*. The *Fit Values* were utilised to calculate EF and the AUC-ROC. The EF was

calculated using equation 1 (Hamza *et al.*, 2012:969). The EF was determined at 10% of the test set.

$$EF = (\text{Hits}_{\text{sel}}/\text{Hits}_{\text{tot}}) \times (\text{NC}_{\text{tot}}/\text{NC}_{\text{sel}}) \dots\dots\dots \text{Equation 1}$$

Results and discussion

MAO-A and MAO-B inhibition studies

The MAO inhibitory properties of the novel nitrocatechol (**1a-k**), pyrazoline (**2a-j**), guaiacol (**3a-j**) and nitroguaiacol (**4a-i**) derivatives were determined against MAO-A and MAO-B with the recombinant human MAOs as enzyme sources. Sigmoidal dose-response curves of activity versus logarithm of inhibitor concentration (log[I]) (Figure 5.2) were constructed. The curves were used to estimate IC₅₀-values which are reported as mean ± standard deviation of triplicate determinations (Table 5.1) (Prinsloo *et al.*, 2023:1069; Stear *et al.*, 2024:166).

Comparing the inhibition activities of the derivatives for the two isoforms, *i.e.*, MAO-A vs. MAO-B, the nitrocatechol-, guaiacol- and nitroguaiacol-chalcone derivatives were in general more potent inhibitors of MAO-B. The pyrazoline derivatives had comparable MAO-A and MAO-B inhibition potencies.

For series 1, the most potent MAO-A inhibitor was nitrocatechol-chalcone derivative **1b** (IC₅₀ = 22.7 µM) which was substituted with two chloro-substituents on the *ortho*- and *para*-positions of the B-ring. This, however, was less potent than the reference inhibitor *i.e.*, harmine (IC₅₀ = 0.01 µM). The following structure-activity relationships were evident for MAO-A inhibition: (a) For the chloro-substituted derivatives, disubstitution on the *ortho*- and *para*- positions (**1b**) is more favourable for MAO-A inhibition compared to disubstitution on the *meta*- and *para*- positions (**1c**) and monosubstitution in the *meta*- (**1d**) and *para*-positions (**1a**) with IC₅₀ ≥ 30.0 µM. (b) Considering the *meta*-substituted derivatives, the derivative with the fluoro-substituent (**1j**; IC₅₀ = 26.3 µM) was overall the second most potent inhibitor in the series and more potent than the chloro- (**1d**; IC₅₀ = 30.0 µM) and trifluoromethyl-substituted (**1i**; IC₅₀ = 39.0 µM) derivatives. (c) Considering the *para*-substituted derivatives, the derivative substituted with the methoxy-group (**1e**; IC₅₀ = 28.7 µM) was the most active followed by the chloro- (**1a**; IC₅₀ = 31.8 µM), methylthio- (**1g**; IC₅₀ = 33.2 µM), fluoro- (**1k**; IC₅₀ = 42.2 µM) and iodo-substituted (**1f**; IC₅₀ = 47.3 µM) derivatives.

The most potent MAO-B inhibitor was derivative **1i** (IC₅₀ = 4.2 µM), which was substituted with a trifluoromethyl-substituent on the *meta*-position of the B-ring, however, it was less potent than the reference inhibitor safinamide (IC₅₀ = 0.12 µM). The following structure-

activity relationships were evident for MAO-B inhibition: (a) Both *meta*- and *para*-substitution yielded good potency MAO-B inhibitors and in certain instances, the *meta*-substituted compound was more potent than the *para*-substituted compound and *vice versa* (e.g., **1d** vs. **1a** and **1j** vs. **1k**). Considering the *meta*-substituted derivatives, the trifluoromethyl-substituted derivative (**1i**) was the most potent followed by the chloro- (**1d**; IC₅₀ = 7.1 μM) and the fluoro-substituted (**1j**; IC₅₀ = 18.5 μM) derivatives. (b) For the *para*-substituted derivatives, the fluoro-substituted derivative was the most potent (**1k**; IC₅₀ = 12.6 μM) and the methoxy-derivative was the least potent (**1e**; IC₅₀ = 26.1 μM).

For series 2, the most potent inhibitor of MAO-A was pyrazoline derivative **2b** (IC₅₀ = 29.4 μM), which was disubstituted with chloro-substituents on the *ortho*- and *para*-positions of the B-ring. The second most active compound was the *para*-substituted fluoro-derivative (**2j**; IC₅₀ = 37.2 μM). The following structure-activity relationships for MAO-A inhibition were noted: (a) The pyrazoline derivatives were less potent than their nitro-catechol counterparts, this may be attributed to the absence of the α,β-unsaturated carbonyl system of the chalcone or the presence of the pyrazoline moiety. (b) Overall, neither the *meta*- nor the *para*-substitution on the B-ring were favoured for inhibitory action. Considering the chloro-substitution, disubstitution on the *ortho*- and *para*-position (**2b**; IC₅₀ = 29.4 μM) was favoured over disubstitution on the *meta*- and *para*-positions (**2c**; IC₅₀ = 49.3 μM). Monosubstitution on the *para*-position (**2a**; IC₅₀ = 39.9 μM) was also more potent than the *meta*-substitution (**2d**; IC₅₀ = 45.3 μM).

The most potent MAO-B inhibitors were derivatives **2c** (IC₅₀ = 34.1 μM) and **2f** (IC₅₀ = 34.2 μM), which were disubstituted with chloro-substituents on the *meta*- and *para*-positions and monosubstituted with an iodo-group on the *para*-position of the B-ring, respectively. The following structure-activity relationships were evident for MAO-B inhibition: (a) The pyrazoline derivatives were less potent than their nitrocatechol counterparts, and as mentioned above this may be attributed to the absence of the α,β-unsaturated carbonyl system of the chalcone or the presence of the pyrazoline moiety. (b) Neither the *meta*- nor the *para*-substitution on the B-ring were favoured for inhibitory activity.

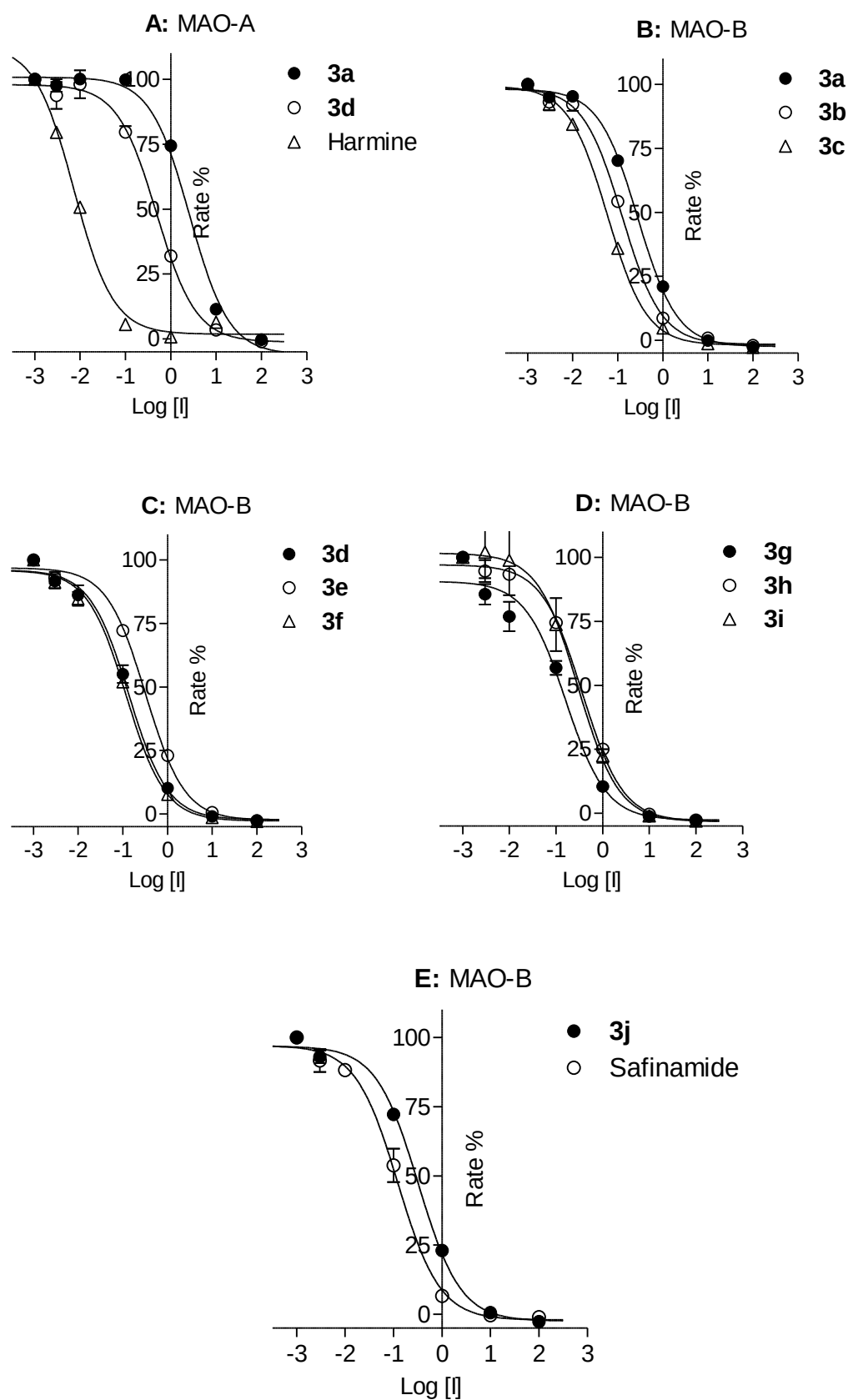
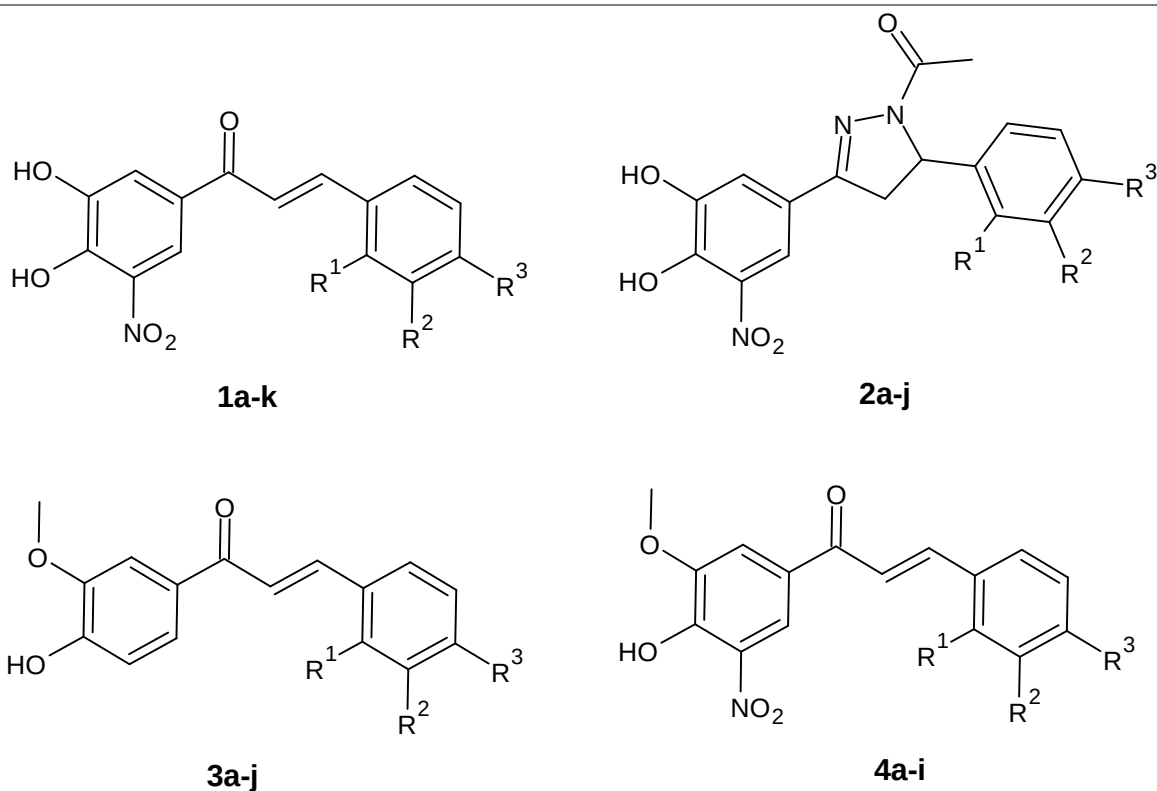


Figure 5.2: Sigmoidal plots for the inhibition of MAO-A and MAO-B by selected novel derivatives.

Table 5.1: The potencies (IC₅₀ values) of human MAO-A and MAO-B inhibition by the nitrocatechol-, guaiacol-, nitroguaiacol-chalcone and pyrazoline derivatives.



Compound	R			IC ₅₀ (μM) ± SD	
	R ¹	R ²	R ³	MAO-A	MAO-B
1a			Cl	31.8 ± 0.38	16.3 ± 1.82
1b	Cl		Cl	22.7 ± 0.23	12.7 ± 0.30
1c		Cl	Cl	32.0 ± 1.08	7.6 ± 0.42
1d		Cl		30.0 ± 1.90	7.1 ± 0.11
1e			OCH ₃	28.7 ± 1.24	26.1 ± 1.93
1f			I	47.3 ± 5.49	16.5 ± 2.16
1g			SCH ₃	33.2 ± 1.68	25.9 ± 1.32
1h	F		Br	31.1 ± 2.56	11.0 ± 0.72
1i		CF ₃		39.0 ± 0.09	4.2 ± 0.16
1j		F		26.3 ± 0.01	18.5 ± 0.33
1k			F	42.2 ± 0.67	12.6 ± 0.50

2a			Cl	39.9 ± 2.29	57.3 ± 2.81
2b	Cl		Cl	29.4 ± 2.21	55.9 ± 5.89
2c		Cl	Cl	49.3 ± 1.86	34.1 ± 2.01
2d		Cl		45.3 ± 0.67	47.6 ± 0.33
2e			OCH ₃	54.3 ± 4.58	56.0 ± 0.16
2f			I	47.1 ± 1.78	34.2 ± 5.48
2g			SCH ₃	49.5 ± 3.54	57.7 ± 0.28
2h	F		Br	60.0 ± 3.20	57.7 ± 2.11
2i		F		59.1 ± 6.07	62.0 ± 1.53
2j			F	37.2 ± 1.60	56.7 ± 1.58
3a			Cl	2.85 ± 0.09	0.28 ± 0.02
3b	Cl		Cl	4.58 ± 0.47	0.12 ± 0.01
3c		Cl	Cl	0.49 ± 0.03	0.06 ± 0.01
3d		Cl		2.62 ± 0.09	0.14 ± 0.02
3e			SCH ₃	10.0 ± 0.56	0.35 ± 0.06
3f		CF ₃		7.45 ± 0.81	0.12 ± 0.01
3g			F	2.45 ± 0.07	0.16 ± 0.01
3h		F		1.57 ± 0.10	0.37 ± 0.01
3i	F		Br	2.29 ± 0.11	0.30 ± 0.06
3j			OCH ₃	21.3 ± 8.14	0.78 ± 0.03
4a			Cl	46.0 ± 1.75	16.4 ± 1.68
4b	Cl		Cl	23.1 ± 1.66	19.3 ± 1.16
4c		Cl	Cl	38.3 ± 3.71	7.81 ± 0.56
4d		Cl		65.8 ± 13.1	13.2 ± 0.09
4e			SCH ₃	110 ± 4.53	13.3 ± 2.33
4f		CF ₃		28.8 ± 2.40	7.28 ± 0.94

4g	F	27.4 ± 2.45	21.5 ± 4.97
4h	F	48.1 ± 5.93	33.5 ± 1.98
4i	NO ₂	33.6 ± 0.13	11.3 ± 0.18
Safinamide			0.12 ± 0.02
Harmine		0.01 ± 0.0002	

For series 3, the most potent inhibition of MAO-A was observed for the guaiacol-chalcone derivatives **3a-d**, **3g-i** with IC₅₀ < 5 µM. However, the most potent MAO-A inhibitor, **3c** (IC₅₀ = 0.49 µM), was less potent than the reference inhibitor. The least active derivative was the *para*-substituted methoxy-containing derivative (**3j**; IC₅₀ = 21.3 µM). The following structure-activity relationships were evident for MAO-A inhibition: (a) The guaiacol-chalcone derivatives were more potent than the pyrazoline, nitrocatechol- and nitroguaiacol-chalcone homologues. This may be attributed to the absence of the nitro-group on the *meta*-position; (b) the chloro-substituted derivatives all exhibited IC₅₀ < 5 µM, with the 3,4-dichloro-substituted derivative (**3c**) being the most active. (c) Interestingly, the presence of fluoro-substituents (*i.e.*, **3g**, **3h** and **3i**) led to more potent MAO-A inhibition compared to the lower potency observed for the trifluoromethyl-substituted derivative (**3f**).

Similar to their activities against MAO-A, the guaiacol-chalcone derivatives were also the most potent inhibitors of MAO-B. Among the test compounds, all ten derivatives (**3a-j**) demonstrated IC₅₀ values < 1 µM against MAO-B. The most potent inhibition was reported for compound **3c** showing an IC₅₀ value of 0.06 µM. This is more potent than the reference inhibitor. Furthermore, **3b** and **3f** possessed potencies comparable to safinamide, with an IC₅₀-value of 0.12 µM. The least active derivative was the *para*-substituted methoxy-containing derivative (**3j**; IC₅₀ = 0.78 µM). The following structure-activity relationships were noted for the inhibition of MAO-B: (a) The guaiacol-chalcone derivatives were, as for MAO-A, more potent than their pyrazoline, nitrocatechol- and nitroguaiacol-chalcone homologues. This may be attributed to the absence of the nitro-group on the *meta*-position; (b) considering the *meta*-substituted derivatives, the derivative substituted with the trifluoromethyl (**3f**; IC₅₀ = 0.12 µM) was most potent followed by the chloro- (**3d**; IC₅₀ = 0.14 µM) and lastly fluoro-substituted (**3h**; IC₅₀ = 0.37 µM) derivatives. (c) For the *para*-substituted derivatives, the derivatives substituted with the chloro-group (**3a**; IC₅₀ = 0.28 µM) and the

thiomethyl-group (**3e**; $IC_{50} = 0.35 \mu M$) were less potent than the fluoro-substituted derivative (**3g**; $IC_{50} = 0.16 \mu M$).

For series 4, the most potent MAO-A inhibitor was nitroguaiacol-chalcone derivative **4b** ($IC_{50} = 23.1 \mu M$) which was disubstituted with chloro-substituents on the *ortho*- and *para*-positions of the B-ring. The least active derivative was the *para*-substituted thiomethyl derivative (**4e**; $IC_{50} = 110 \mu M$). The following structure-activity relationships for MAO-A inhibition were noted: (a) Considering chloro-substitution, disubstitution on the *ortho*- and *para*-positions was favoured over disubstitution on the *meta*- and *para*-positions (**4c**; $IC_{50} = 38.3 \mu M$). Monosubstitution on the *para*-position (**4a**; $IC_{50} = 46.0 \mu M$) was also more potent than *meta*-substitution (**4d**; $IC_{50} = 65.8 \mu M$). (b) Considering the *meta*-substituted derivatives, the derivative substituted with the trifluoromethyl (**4f**; $IC_{50} = 28.8 \mu M$) was most potent followed by the fluoro- (**4h**; $IC_{50} = 48.1 \mu M$) and lastly the chloro-substituted (**4d**; $IC_{50} = 65.8 \mu M$) derivatives. (c) For the *para*-substituted derivatives, the derivative substituted with the chloro-group (**4a**; $IC_{50} = 46.0 \mu M$) was less potent than the fluoro-substituted derivative (**4g**; $IC_{50} = 27.4 \mu M$) while the thiomethyl derivative (**4e**) was least potent.

The most potent MAO-B derivative was **4f** ($IC_{50} = 7.28 \mu M$) with a trifluoromethyl-substituent on the *meta*-position of the B-ring. The second most active derivative is the 3,4-dichloro-substituted derivative (**4c**; $IC_{50} = 7.81 \mu M$). The following structure-activity relationships were noted for the inhibition of MAO-B: (a) Considering the *meta*-substituted derivatives, the trifluoromethyl-substituted derivative (**4f**) was most active, the second most active derivative was the chloro-containing derivative (**4d**; $IC_{50} = 13.2 \mu M$) and the least active is the fluoro-substituted derivative (**4h**; $IC_{50} = 33.5 \mu M$). (b) For the *para*-substituted derivatives, the derivative substituted with the nitro-group (**4i**; $IC_{50} = 11.3 \mu M$) was most active while the least potent was the fluoro-substituted derivative (**4g**; $IC_{50} = 21.5 \mu M$).

In summary, although the nitrocatechol-chalcone and nitroguaiacol-chalcone derivatives showed a preference for MAO-B inhibition, the most potent derivatives *i.e.*, **1i** ($IC_{50} = 4.2 \mu M$) and **4f** ($IC_{50} = 7.28 \mu M$) were less active than safinamide. For all 4 series, derivatives containing chloro-substituents proved to be potent MAO-B inhibitors with derivative **3c** (3,4-dichloro) being the most active. Furthermore, for the nitrocatechol-, guaiacol- and nitroguaiacol-chalcones, the addition of the trifluoromethyl-substituent (**1i**, **3f**, **4f**) also yielded good potency MAO-B inhibition. Lastly, the pyrazoline derivatives were the least active against both MAO-A and MAO-B.

Mechanism of MAO-A and MAO-B inhibition

It was previously reported that nitrocatechol-chalcone derivatives act as competitive inhibitors of MAO-B (Engelbrecht *et al.*, 2018:123). Lineweaver-Burk graphs were constructed for the inhibition of MAO-A and MAO-B to determine if the highly potent guaiacol-chalcone derivative (**3c**) may also share this property. It was found that both Lineweaver-Burk graphs (Figure 5.3) intersected on the y-axis, indicating **3c** inhibits both MAO-A and MAO-B competitively. Furthermore, replots of the Lineweaver-Burk slopes versus inhibitor concentration yielded linear lines from which the enzyme-inhibitor dissociation constants (K_i) were estimated as 0.29 μM and 0.02 μM for MAO-A and MAO-B, respectively.

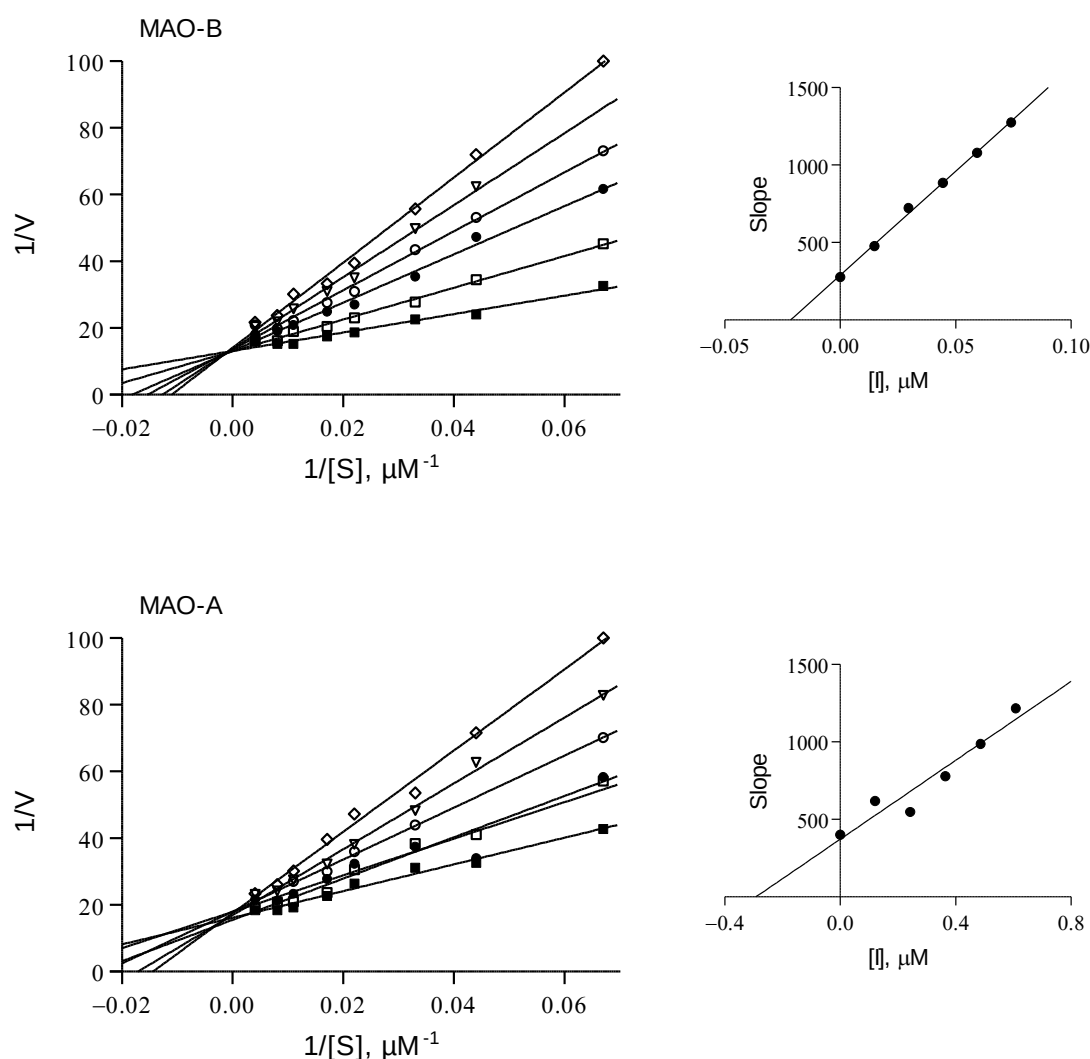


Figure 5.3: Lineweaver-Burk graphs of the inhibition of MAO-A and MAO-B by **3c** and the replots of the slopes of the Lineweaver-Burk versus inhibitor concentration.

Common feature pharmacophore modelling

Training and test sets were created by utilising the *in vitro* MAO inhibition data as a metric to determine which derivatives to include in the respective sets. Using the training set, ten pharmacophore hypotheses (hypos.) were created and subsequently ranked based on their rank score (Table 5.2) and AUC-ROC (Table 5.3). The generated hypotheses presented with rank scores ranging from 60.72-64.84 indicating they were not generated by chance. The hypos. identified numerous common features, *i.e.*, hypo. 1 and 3 identified a hydrophobic (H) feature, a hydrogen bond donor (D) and two hydrogen bond acceptors (A). Hypo. 2 and 4 identified a hydrophobic feature and three hydrogen bond acceptors. Hypo. 5, 6 and 8 identified two hydrophobic features, a hydrogen bond donor and a hydrogen bond acceptor. Hypo. 7 and 10 identified a ring aromatic (R), two hydrophobic features and a hydrogen bond acceptor. Lastly, hypo. 9 identified two hydrophobic features and two hydrogen bond acceptors.

Comparing the 10 generated hypotheses, hypo. 5 had the best EF^{10%} and AUC-ROC values (Table 5.3). As mentioned, hypo. 5 reported that two hydrophobic features (H), a hydrogen bond donor (D) and a hydrogen bond acceptor (A) are important for MAO inhibition (Figure 5.4). By mapping **3c** with hypo. 5 (Figure 5.4) it is evident that the methoxy-group on ring A and the *para*-chloro substituent on ring B act as hydrophobic features. Furthermore, the hydroxy-group on ring A and the carbonyl-group of the bridge act as a hydrogen bond donor and acceptor, respectively.

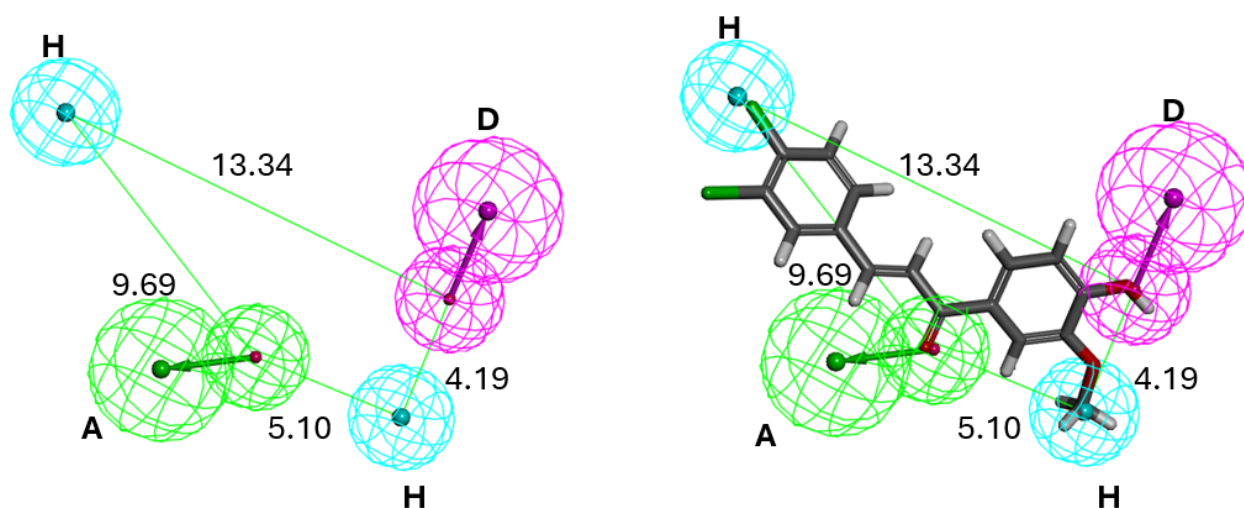


Figure 5.4: Hypo. 5 (left) with inter-feature constraints and (right) with **3c** mapped. Hydrogen bond acceptor (A), hydrogen bond donor (D), ring aromatic (R), hydrophobic (H).

Table 5.2: Summary of the pharmacophore hypotheses.

Hypo.	Features ^a	Rank	Direct Hit	Partial Hit	Max Fit
01	HDAA	64.84	111111	000000	4
02	HAAA	63.64	111111	000000	4
03	HDAA	63.47	111111	000000	4
04	HAAA	62.27	111111	000000	4
05	HHDA	62.24	111111	000000	4
06	HHDA	61.61	111111	000000	4
07	RHHA	61.31	111111	000000	4
08	HHDA	61.30	111111	000000	4
09	HHAA	61.04	111111	000000	4
10	RHHA	60.72	111111	000000	4

^a R = Ring aromatic, H = Hydrophobic, A = Hydrogen bond acceptor, D = Hydrogen bond donor.

Table 5.3: Enrichment factor and AUC-ROC of the pharmacophore hypotheses.

Hypo.	Enrichment factor at 10%	ROC
Hypo. 1	7.5	0.82 ± 0.16
Hypo. 2	7.5	0.81 ± 0.17
Hypo. 3	7.5	0.85 ± 0.11
Hypo. 4	7.5	0.81 ± 0.12
Hypo. 5	7.5	1.00 ± 0.002
Hypo. 6	7.5	0.85 ± 0.12
Hypo. 7	5.0	0.67 ± 0.19
Hypo. 8	7.5	0.85 ± 0.12
Hypo. 9	7.5	0.75 ± 0.21
Hypo. 10	7.5	0.78 ± 0.18

Conclusion

This study investigated nitrocatechol-, guaiacol-, nitroguaiacol-chalcone and pyrazoline chalcone derivatives as potential inhibitors of MAO. Overall, the chalcone derivatives were more potent inhibitors of MAO-B, whereas the pyrazoline derivatives had comparable inhibitory activities against MAO-A and MAO-B. The most potent inhibitors were the

guaiacol-chalcone derivatives. Derivative **3c** was the most active MAO-A inhibitor, however, it was less active than the reference inhibitor, harmine. This derivative was also the most potent MAO-B inhibitor and more active than the reference inhibitor, safinamide. Derivatives **3b** and **3f** exhibited activity comparable to that of safinamide. These *in vitro* data suggested that the guaiacol moiety is preferred over the nitroguaiacol and nitrocatechol moiety for MAO activity. This can be attributed to the absence of nitro-group on the *meta*-position in guaiacol-containing derivatives. Lineweaver-Burk graphs showed that derivative **3c** inhibited MAO-A and MAO-B competitively with enzyme-inhibitor dissociation constants (K_i) of 0.29 μM and 0.02 μM for MAO-A and MAO-B, respectively. Lastly, the *in vitro* inhibition data was utilised to generate common feature pharmacophore models for activity against MAO, which were evaluated by calculating the rank scores, $\text{EF}^{10\%}$ and AUC-ROC values. The best performing model was hypo. 5. This hypothesis indicated that four features are important for activity *i.e.*, a hydrogen bond donor, a hydrogen bond acceptor and two hydrophobic features.

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CHAPTER 6: CONCLUSION

Global healthcare has significantly improved since the discovery of penicillin and subsequent antibiotics. However, bacteria have increasingly developed resistance against these antibiotics. Antibiotic resistance is a natural phenomenon that occurs in bacteria; however, it has been accelerated by numerous factors including the misuse of antibiotics (Poudel *et al.*, 2023:2). Antimicrobial resistance (AMR) is the cause of more than 700 000 deaths annually, highlighting the need for the design and discovery of novel antibiotic compounds (Akram *et al.*, 2023:1; Church & McKillip, 2021:1535).

The concept of drug repurposing, *i.e.*, finding novel indications for existing drugs, is a practical approach to identifying novel leads for medical conditions. This study aimed to determine if compounds with known activity against monoamine oxidase (MAO) will also have antibacterial activity. The *in vitro* antibacterial data were used to construct a common feature pharmacophore model which were used to design novel compounds that were synthesised. The antibacterial activity of the novel derivatives was determined against some of the ESKAPE pathogens, whereafter a checkerboard assay was used to indicate a possible antibacterial mode of action. The novel derivatives were also screened for MAO-A and MAO-B inhibition activity and their mechanism of inhibition was determined by Lineweaver-Burke plots.

As part of the first article for this study, previously synthesised nitrocatechol-chalcone and nitrocatechol-pyrazoline derivatives, with proven *in vitro* activity against MAO-A, MAO-B and/or COMT, were screened for activity against *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. The initial derivatives only had activity against *S. aureus*, with compounds **2a**, **2b** and **1b** (1 - 2 µg/ml) (article 1) having comparable activity to tetracycline (2 µg/ml). Ligand-based drug design (LBDD) was utilised to create a common feature pharmacophore model based on the *in vitro* data. This pharmacophore model was validated by determining the rank score, enrichment factor (EF), and area under the receiver operating characteristic (ROC-AUC). The pharmacophore model performed well and had a maximum fit value of 4, and a rank score of 84.02, indicating the model was not created by coincidence. The EF^{10%} (6.7) and ROC-AUC (0.85) values proved the pharmacophore model can accurately identify active compounds. The model indicated that four hydrogen bond acceptors are important for antibacterial activity. By mapping *R-2b* with the model, it was found that the oxygen atoms of the nitro-group, one of the hydroxy-groups on ring A, the carbonyl-moiety in the pyrazoline ring as well as the nitrile

substituent on ring B act as important hydrogen bond acceptors that influence the antibacterial activity. Using this data, novel nitrocatechol-chalcone and pyrazoline derivatives were synthesised after which the minimum inhibitory concentration (MIC) of each derivative was determined against both the drug-susceptible and resistant *S. aureus* (MRSA) strains. The most active compounds were **3i** (0.5 µg/ml) and **3c** (0.5 µg/ml) (article 1) against the susceptible and resistant strains respectively, which were more active than tetracycline. The most active pyrazoline derivatives were **4a** and **4c** (article 1), exhibiting activities of 16 µg/ml and 32 µg/ml against the susceptible and resistant strains, respectively. Our findings suggested the importance of an electron-withdrawing substituent on ring B for antibacterial activity in nitrocatechol-chalcones.

As part of the second article, the importance of the catechol moiety with regards to antibacterial activity was investigated by synthesising two series of compounds, one where the nitro-moiety was omitted, and the *meta*-hydroxy moiety was replaced by a *meta*-methoxy (guaiacol-chalcone derivatives) and another series where the nitro-moiety was still present, and the *meta*-hydroxy moiety was replaced by a *meta*-methoxy (nitroguaiacol-chalcone derivatives). The antibacterial activity of these derivatives was determined against *S. aureus*, MRSA, *K. pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *K. aerogenes*. The most active derivative was nitroguaiacol-chalcone derivative **2c** (8 µg/ml) (article 2), with activity against the susceptible *S. aureus* and MRSA strains. It is noteworthy that none of the guaiacol-chalcone derivatives demonstrated antibacterial activity, with the sole exception of compound **1j** (article 2). To investigate a potential mode of action, the checkerboard assay was employed to assess the possibility of synergistic interactions between existing antibiotics and the derivatives synthesised in articles 1 and 2. One nitroguaiacol-chalcone derivative (**2a**) (article 2) had a synergistic effect when combined with chloramphenicol with a fractional inhibitory concentration index (FICI) of 0.45 ± 0.09 , implying its antibacterial mechanism of action might also target bacterial protein synthesis. A pharmacophore model was developed and validated (max. fit: 4, rank score: 52.34, EF^{10%}: 8, ROC-AUC: 0.95 ± 0.02), and indicated four features are important for antibacterial activity *i.e.*, ring aromatic, a hydrophobic moiety and two hydrogen bond acceptors. By mapping **2c** (article 2) with the model it was concluded that, similar to article 1, the two oxygen atoms *i.e.*, the methoxy-group on ring A and the carbonyl-group, act as hydrogen bond acceptors that are important for antibacterial activity. Additionally with the derivatives of article 2, ring B and the chlorine on the *para*-position of ring B act as the ring aromatic and the hydrophobic moiety, respectively. Compared to the pharmacophore model of article 1, this model did not

classify the nitro-group as essential for antibacterial activity; however, the *in vitro* data suggest it is however important for antibacterial activity. When substituting the hydroxy-group with a methoxy-group, the oxygens in both groups were identified as hydrogen bond acceptors. However, a comparison between nitrocatechol (**3c**, article 1) and nitroguaiacol (**2c**, article 2) revealed that nitrocatechol was more active. This indicates the hydroxy-moiety is more crucial for activity than the methoxy-moiety.

As part of the third article, the novel chalcone and pyrazoline derivatives synthesised for both articles 1 and 2 were further evaluated for MAO inhibition by determining their IC₅₀ values. It was determined that the chalcone derivatives were more potent inhibitors of MAO-B than MAO-A, whereas the pyrazoline derivatives had comparable MAO-A and MAO-B activity. The guaiacol-chalcone derivative, **3c** (IC₅₀ = 0.49) (article 2), was the most active against MAO-A, however, it was less potent than harmine (IC₅₀ = 0.01 µM). This derivative also had the most potent inhibition against MAO-B with an IC₅₀ of 0.06 µM, which is more potent than the reference inhibitor safinamide (IC₅₀ = 0.12 µM). The guaiacol-chalcone derivatives, **3b** and **3f** (article 2) exhibited inhibitory action comparable to safinamide with an IC₅₀ value of 0.12 µM against MAO-B. The chalcone derivatives exhibited a marked preference for the inhibition of MAO-B, with guaiacol-chalcone derivatives demonstrating superior potency compared to other derivatives. The main difference between the guaiacol-chalcone and the nitroguaiacol-chalcone derivatives was the absence of the nitro-group. Lineweaver-Burk graphs indicated that **3c** (article 2) inhibits both MAO-A and MAO-B competitively. A pharmacophore model was developed and subsequently validated (max. fit: 4, rank score: 62.24, EF^{10%}: 7.5, ROC-AUC: 1.00 ± 0.002). This pharmacophore model indicated that two hydrophobic features, a hydrogen bond donor and a hydrogen bond acceptor were important for MAO inhibitory activity. After mapping **3c** (article 2) with the model it indicated that the methoxy-group on ring A and the *para*-chlorine substituent on ring B act as hydrophobic features, whereas the hydroxy-group on ring A and the carbonyl-group act as a hydrogen bond donor and acceptor respectively.

In summary, *in vitro* data reported in this study showed that nitrocatechol-chalcone, nitroguaiacol-chalcone and nitrocatechol-pyrazoline derivatives have good activity against *S. aureus* and MRSA with some derivatives having activity comparable to that of tetracycline. The guaiacol-chalcone derivatives however, had very little antibacterial activity. The generated pharmacophore models highlighted several groups as important for antibacterial activity in chalcone derivatives (Figure 6.1). A checkerboard assay suggested

that the mechanism of antibacterial action of nitroguaiacol-chalcone derivatives was similar to that of chloramphenicol. Furthermore, nitrocatechol-chalcone, guaiacol-chalcone and nitroguaiacol-chalcone derivatives showed a preference for MAO-B inhibition with guaiacol-chalcone derivatives being the most active. A selected derivative also inhibited MAO-A and MAO-B competitively. A generated pharmacophore model highlighted several groups as important for MAO inhibitory activity in chalcone derivatives (Figure 6.2).

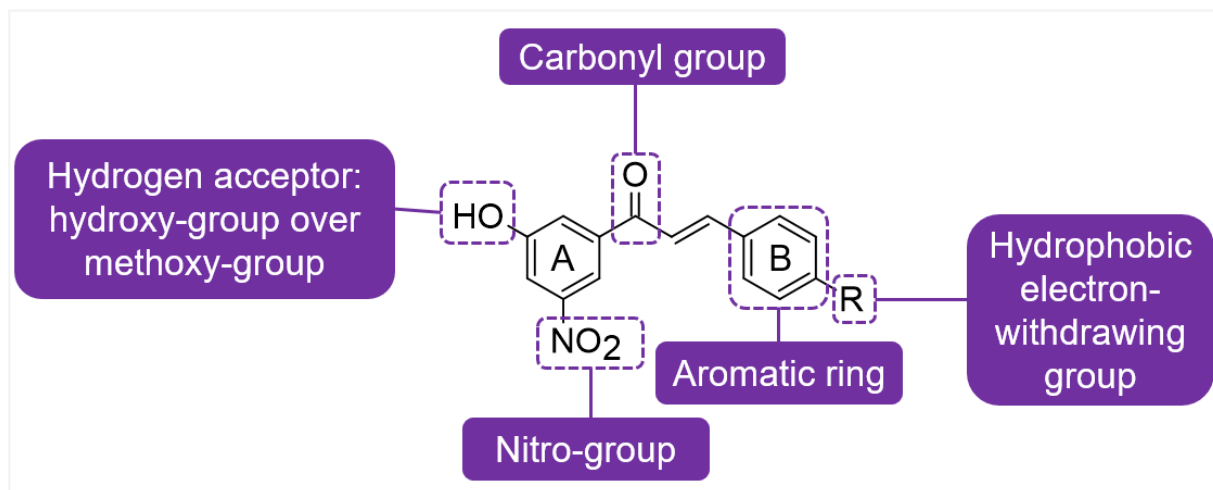


Figure 6.1: Summary of suggested alterations to chalcone derivatives for antibacterial activity.

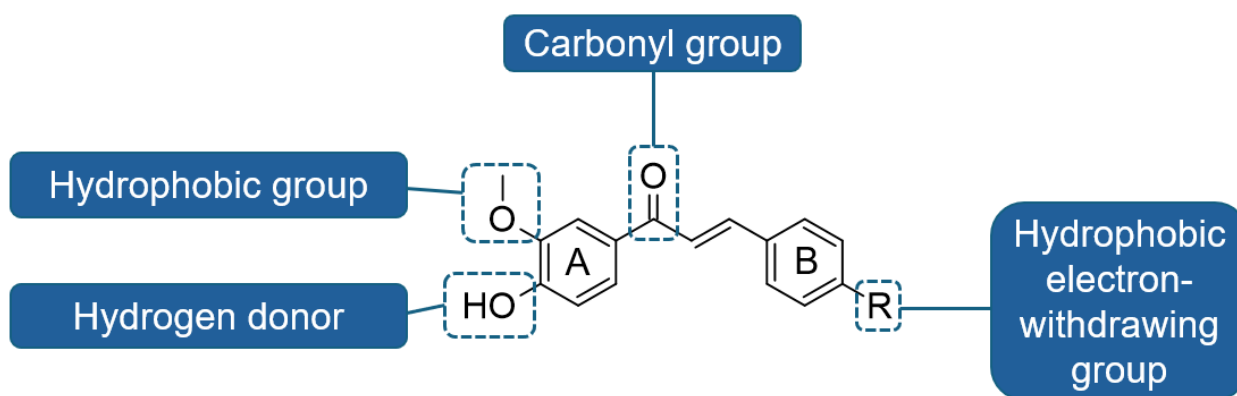


Figure 6.2: Summary of suggested alterations to chalcone derivatives for MAO inhibitory activity.

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Church, N.A. & McKillip, J.L. 2021. Antibiotic resistance crisis: challenges and imperatives. *Biologia*. 76: 1535–1550. <https://doi.org/10.1007/s11756-021-00697-x>

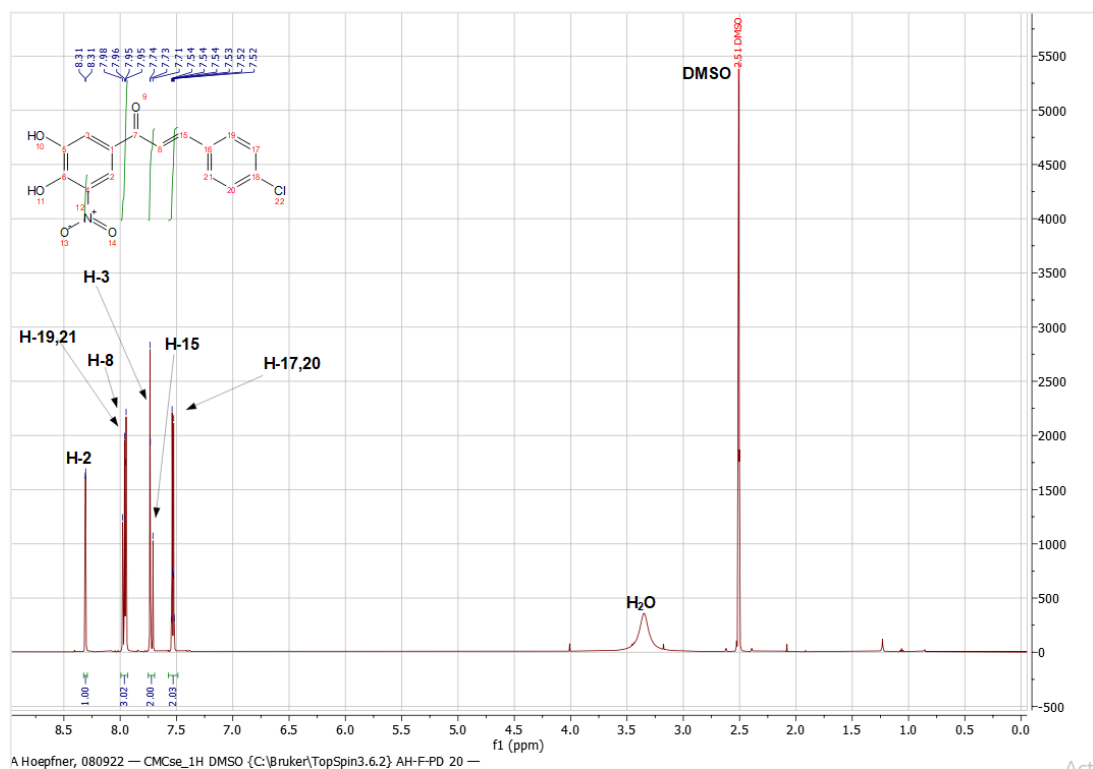
Poudel, A.N., Zhu, S., Cooper, N., Little, P., Tarrant, C., Hickman, M. & Yao, G. 2023. The economic burden of antibiotic resistance: a systematic review and meta-analysis. *PLoS One*. 18(5), e0285170. <https://doi.org/10.1371/journal.pone.0285170>

ANNEXURE A

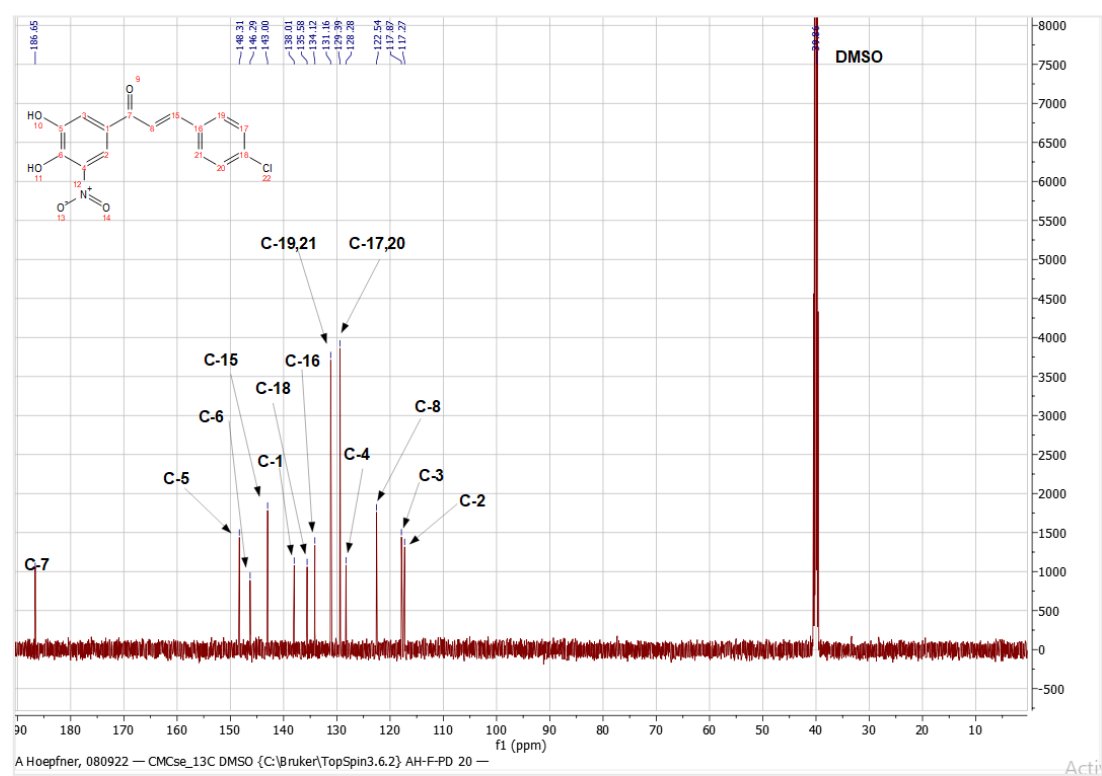
Article 1: Nuclear Magnetic Resonance (^1H , C^{13} and HSQC) and Mass spectrometry (MS) spectra of compounds

(2E)-3-(4-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3a)

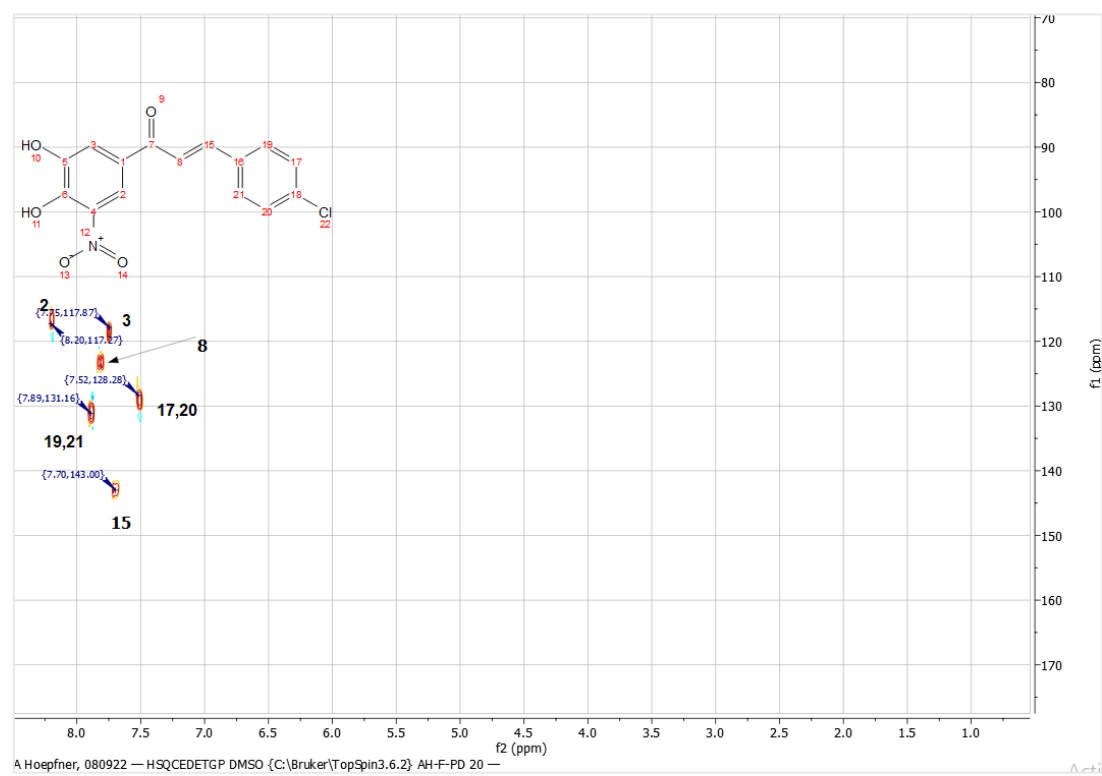
Proton (^1H) NMR spectrum



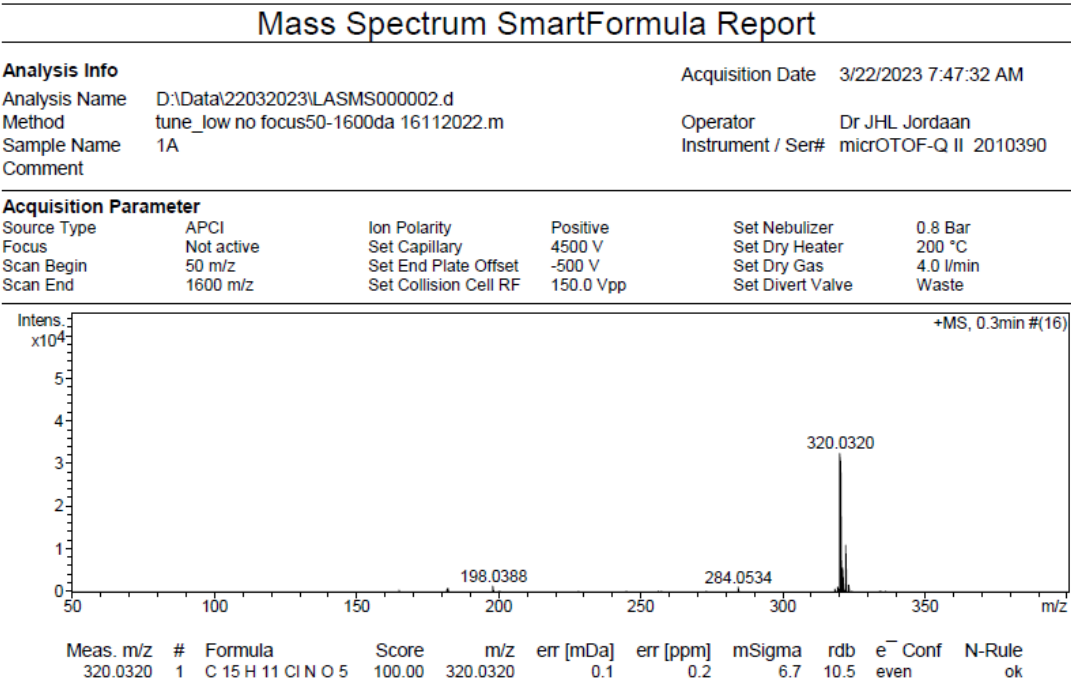
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

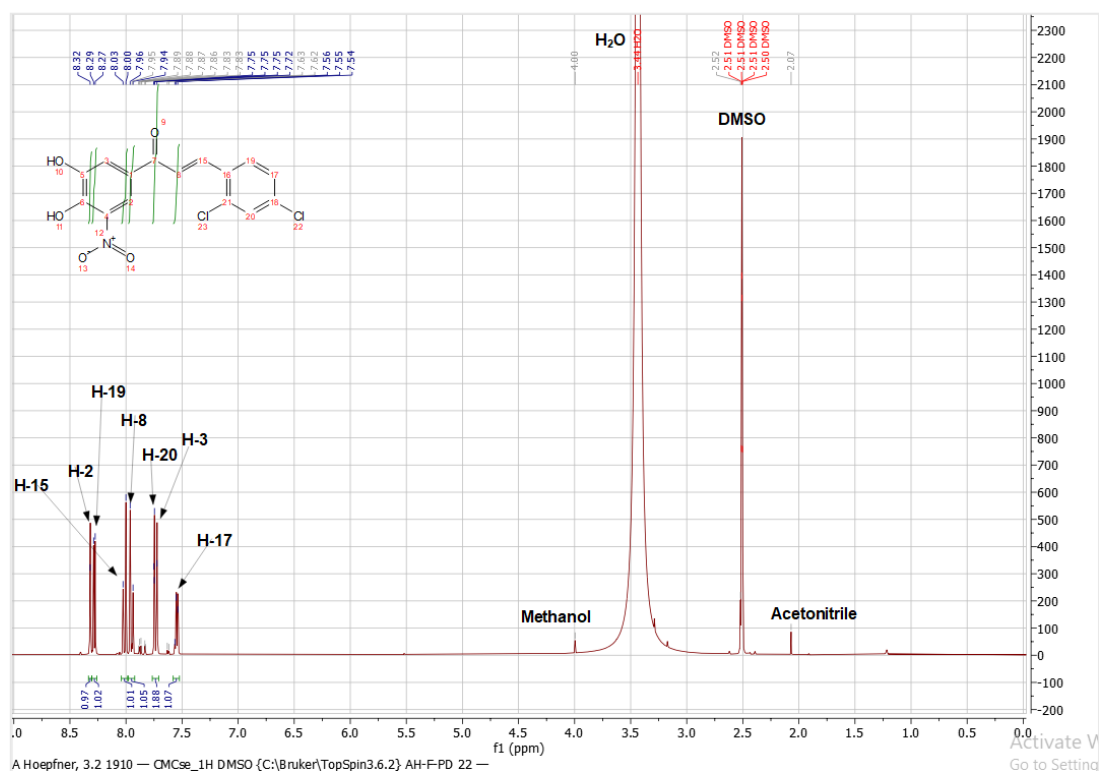


Mass spectrometry (MS) spectrum

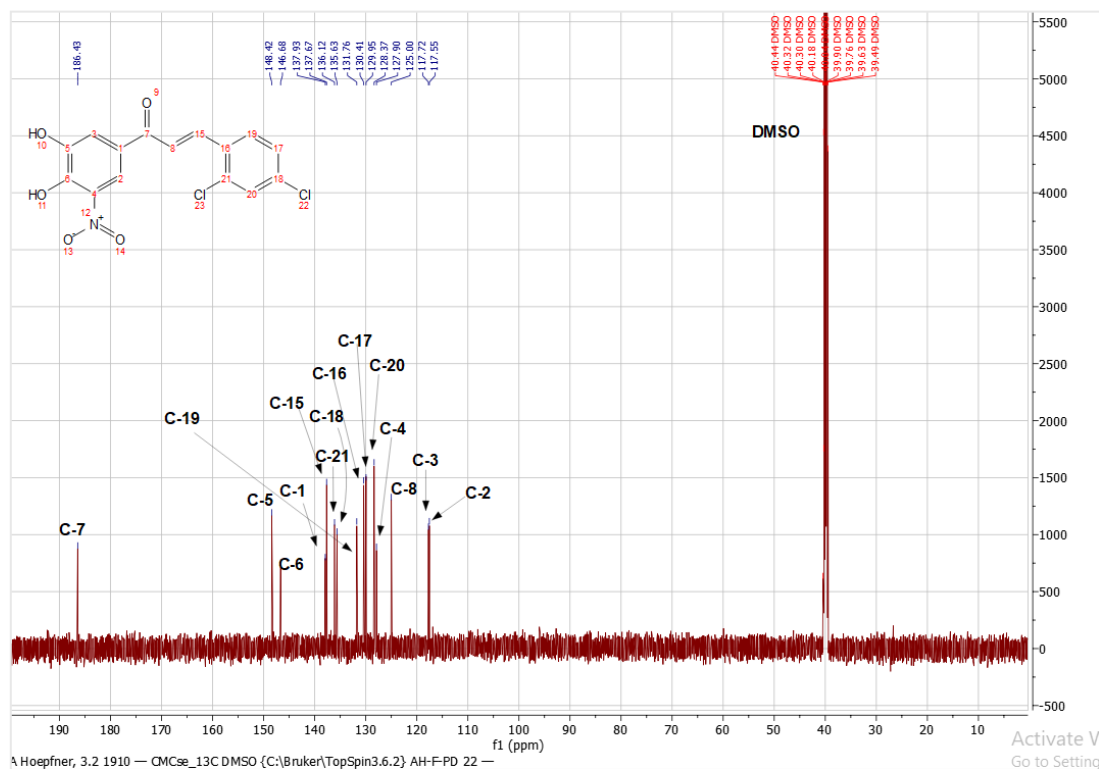


(2E)-3-(2,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3b)

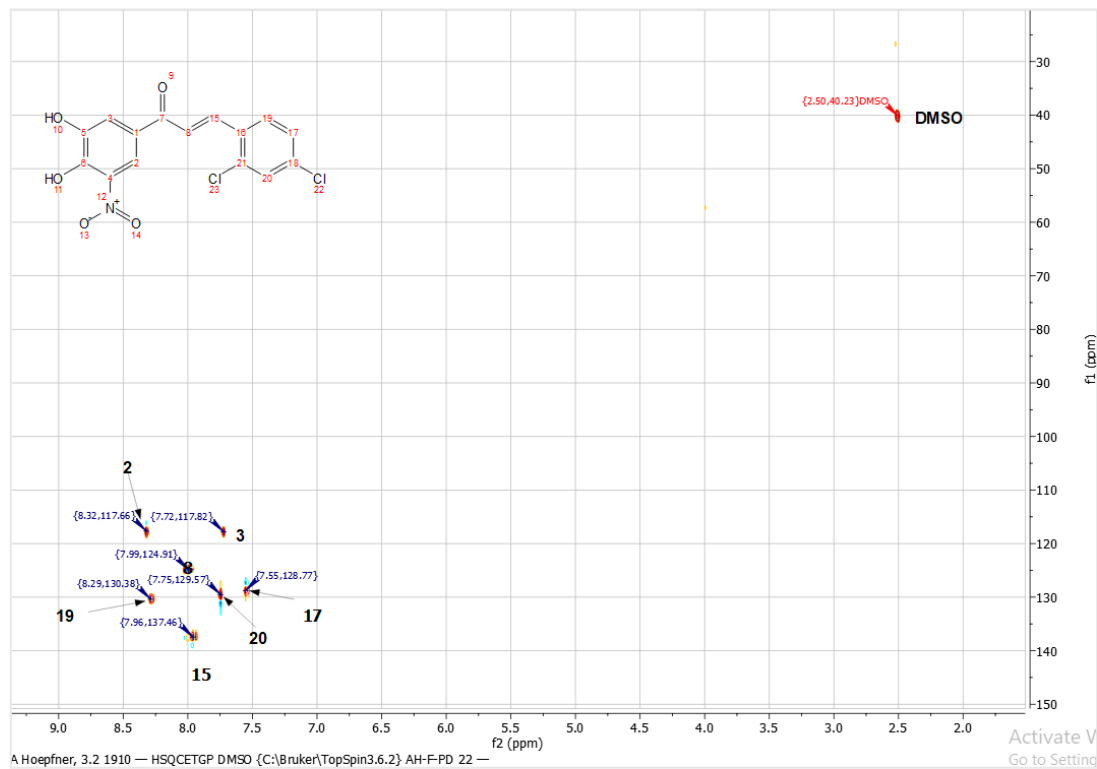
Proton (^1H) NMR spectrum



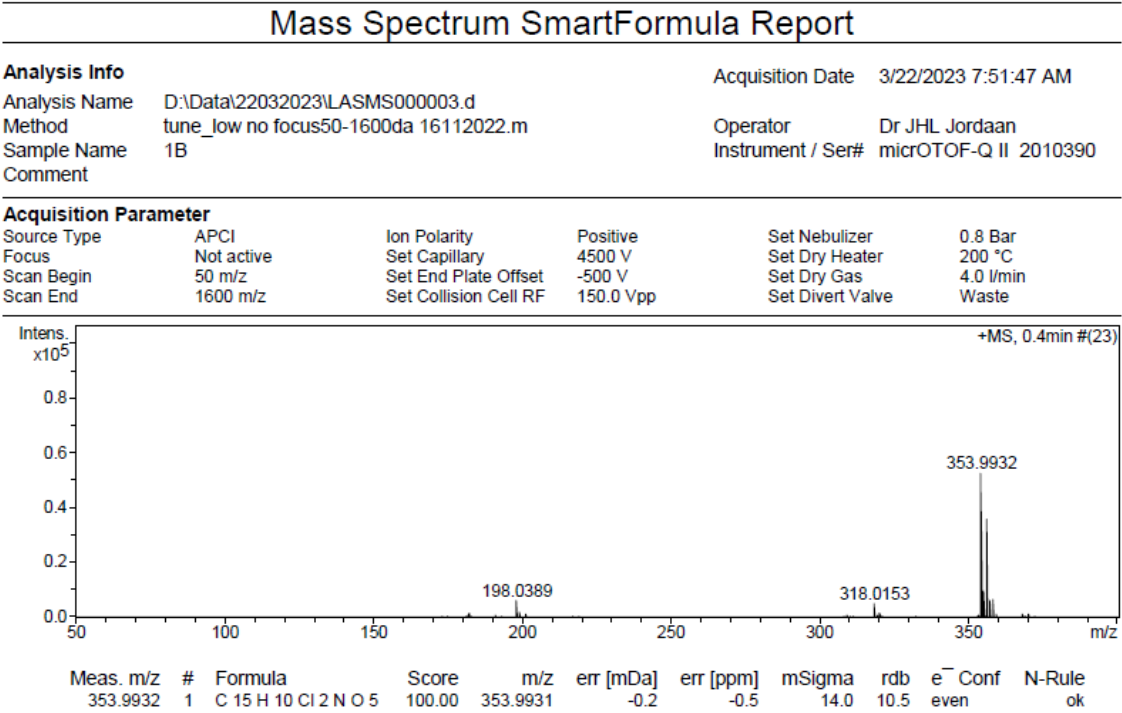
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

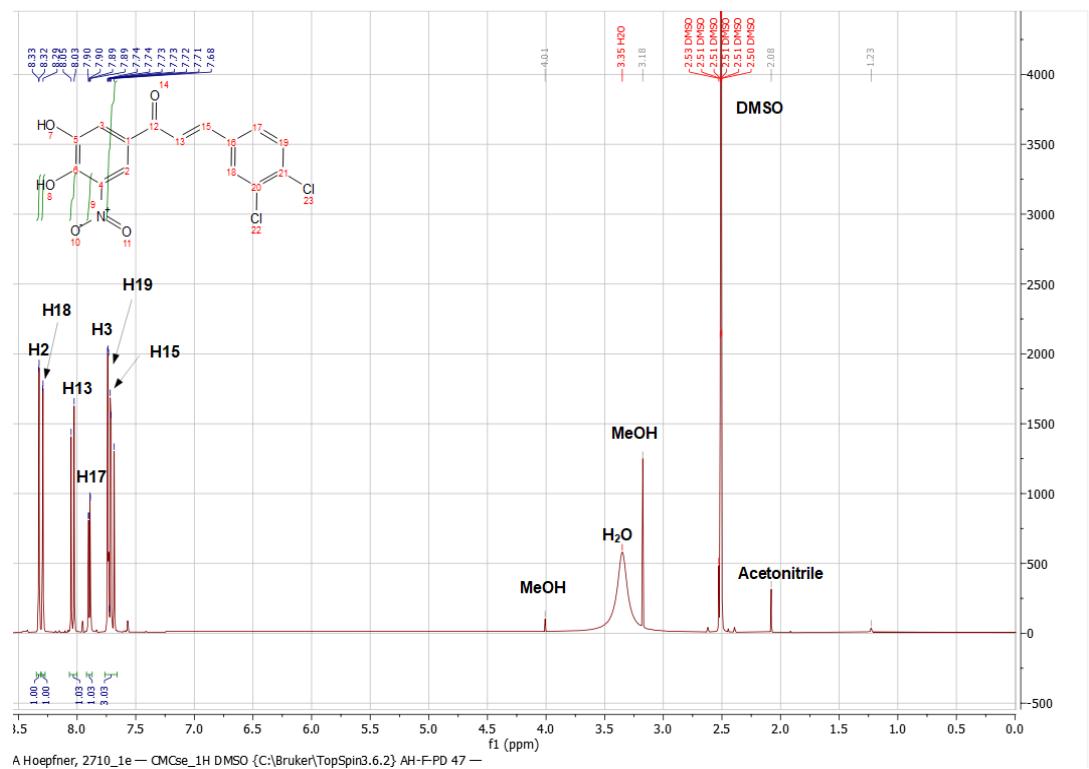


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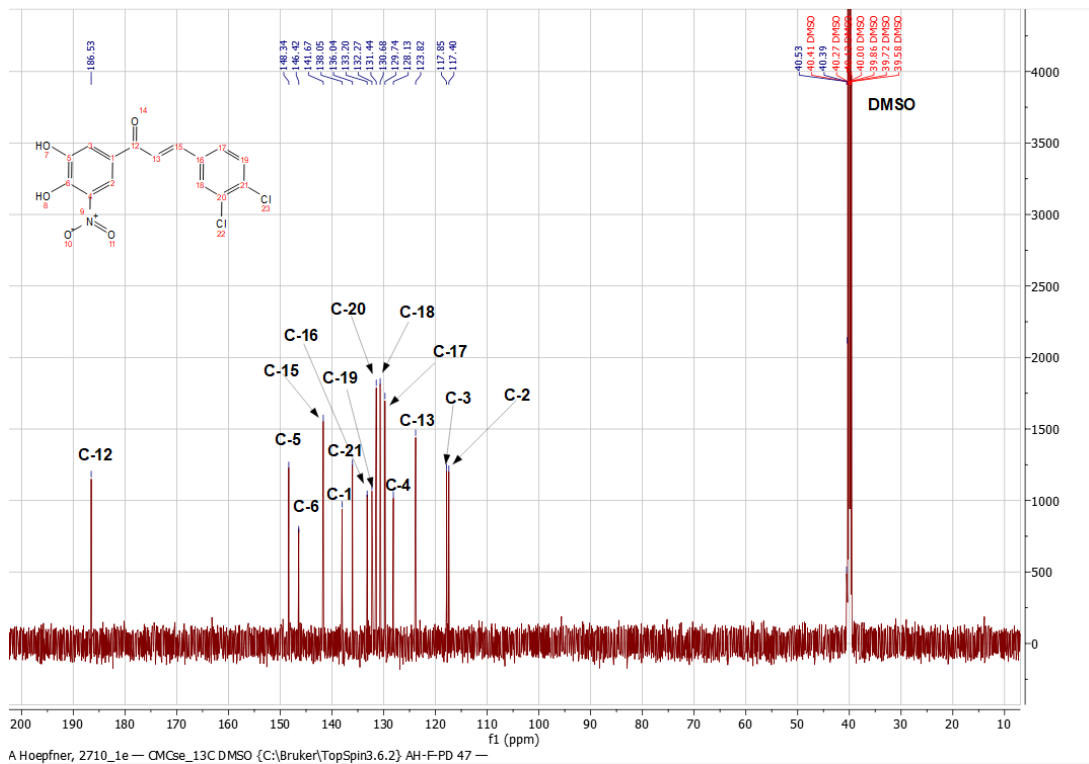


(2E)-3-(3,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3c)

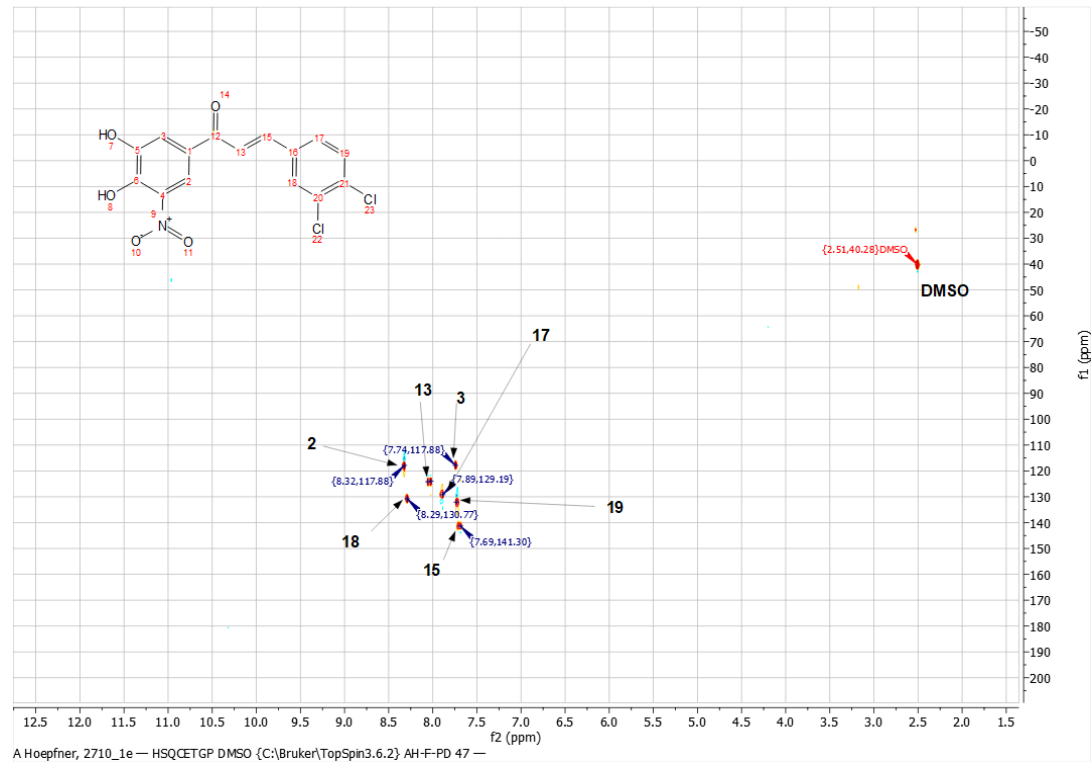
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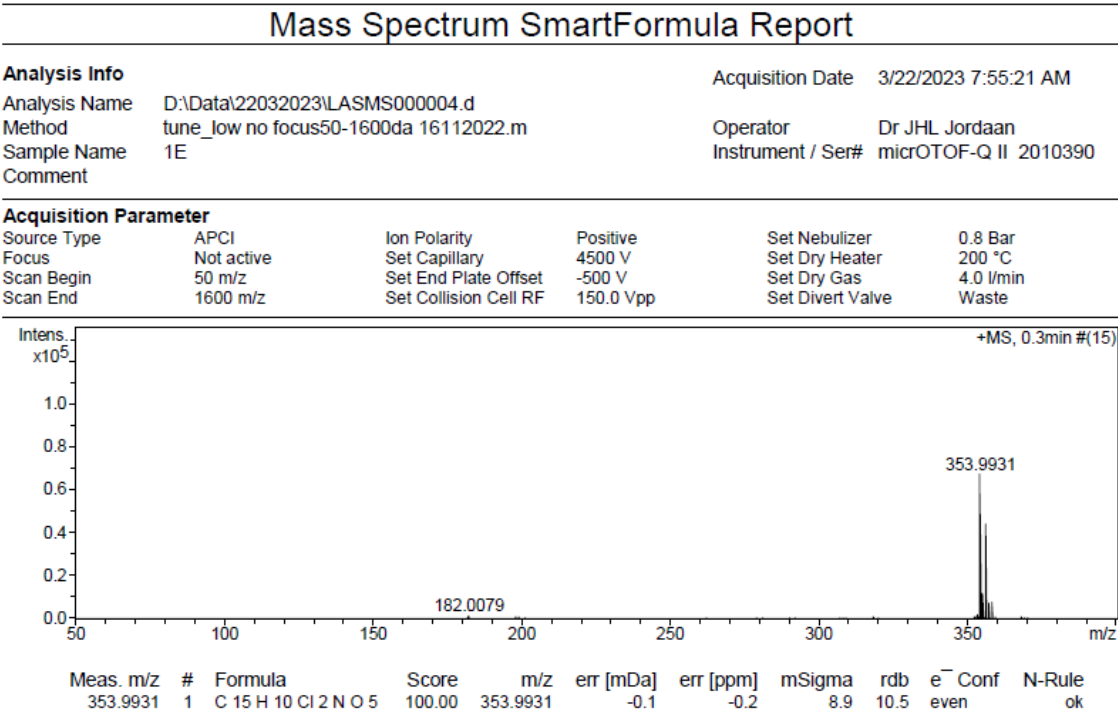
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

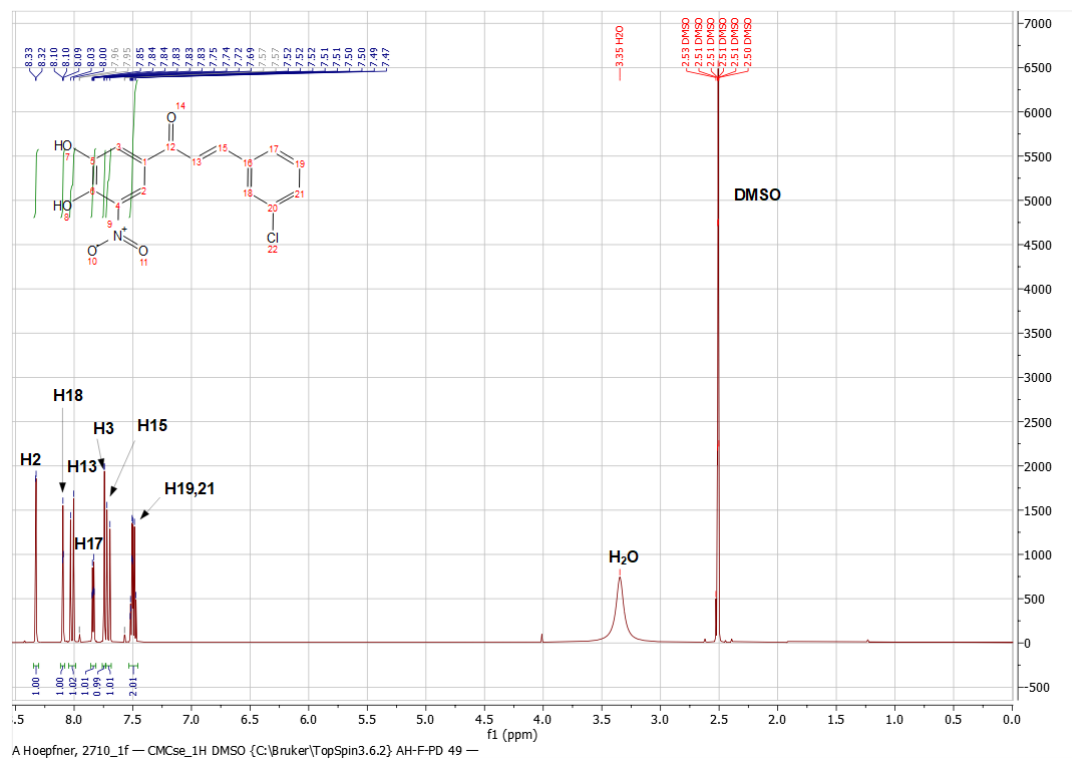


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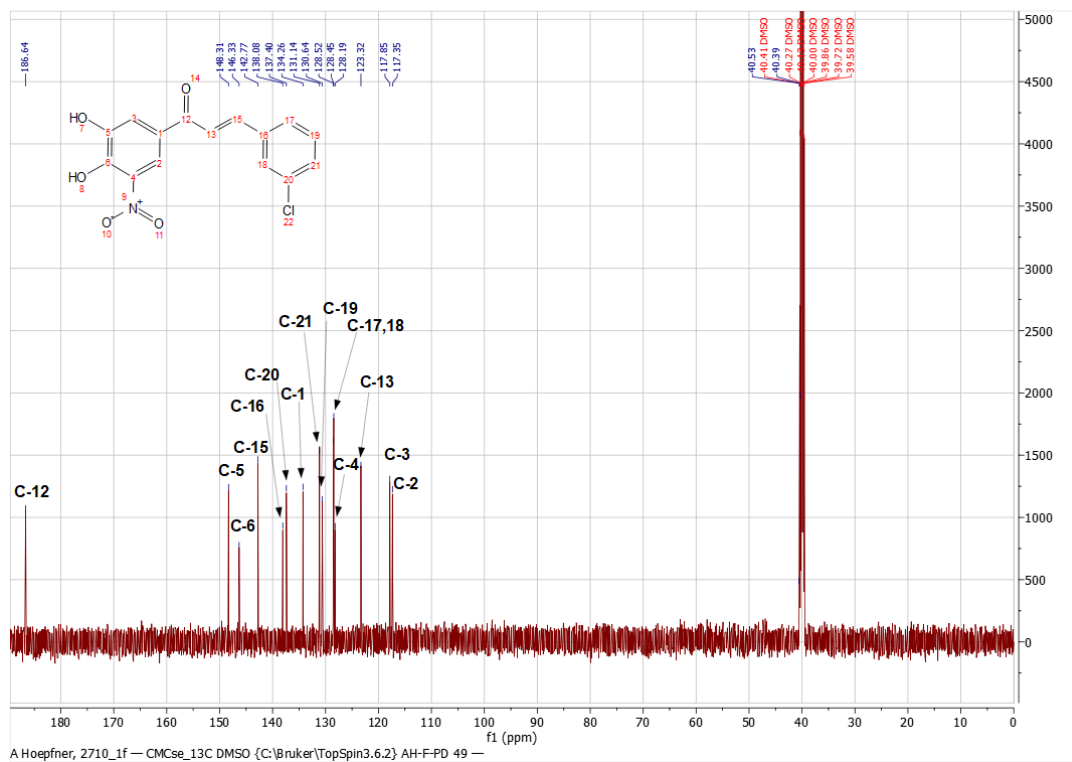


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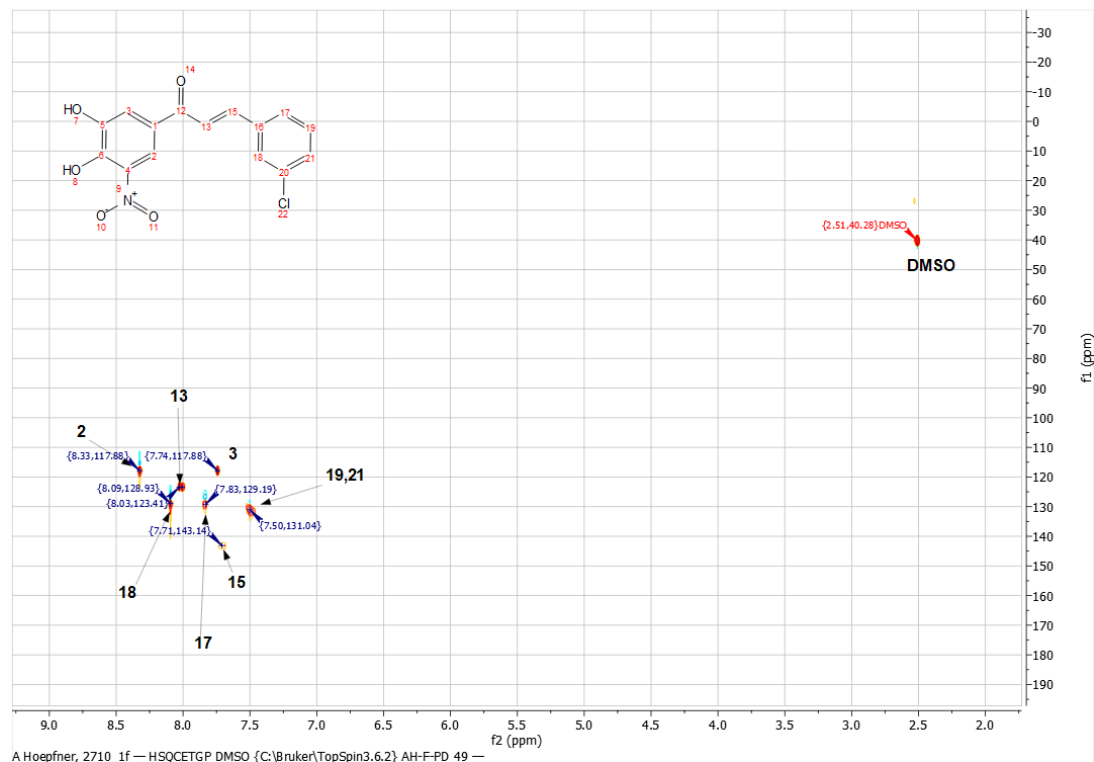
Proton (^1H) NMR spectrum



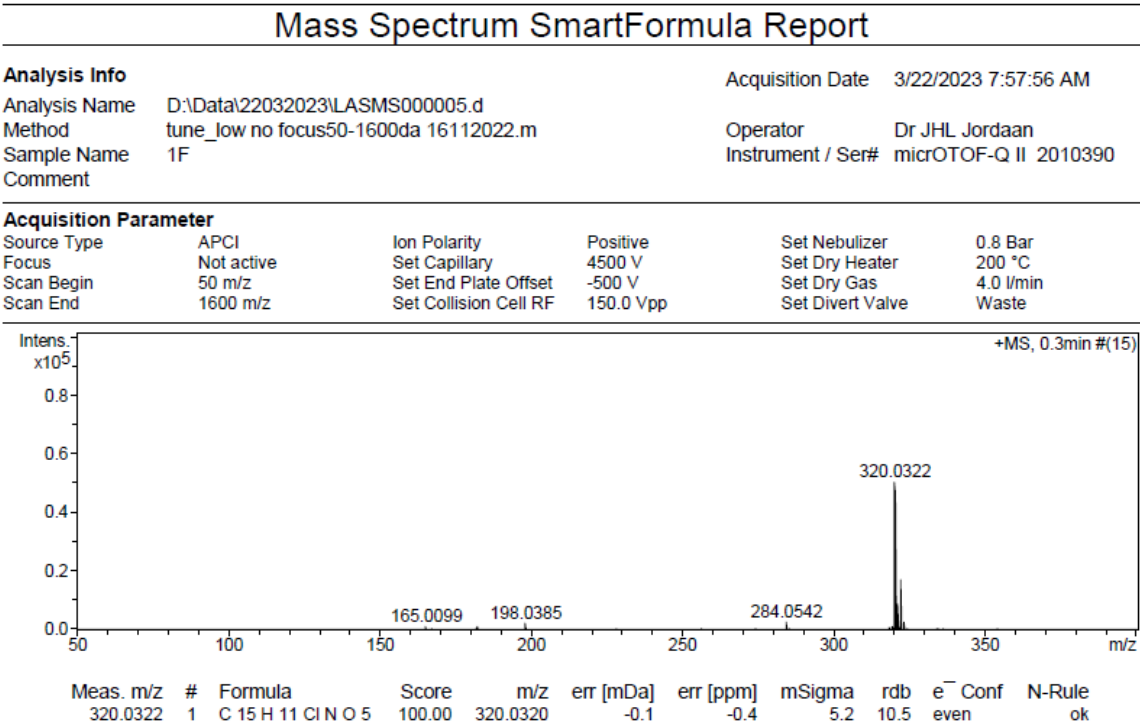
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

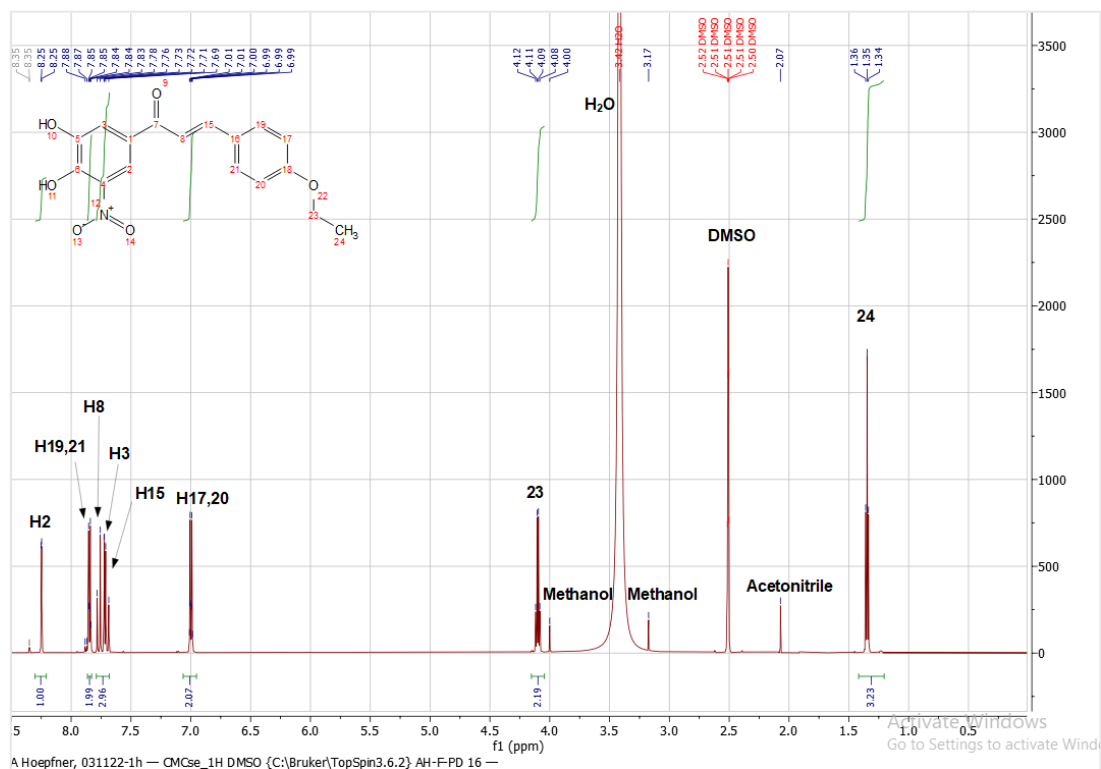


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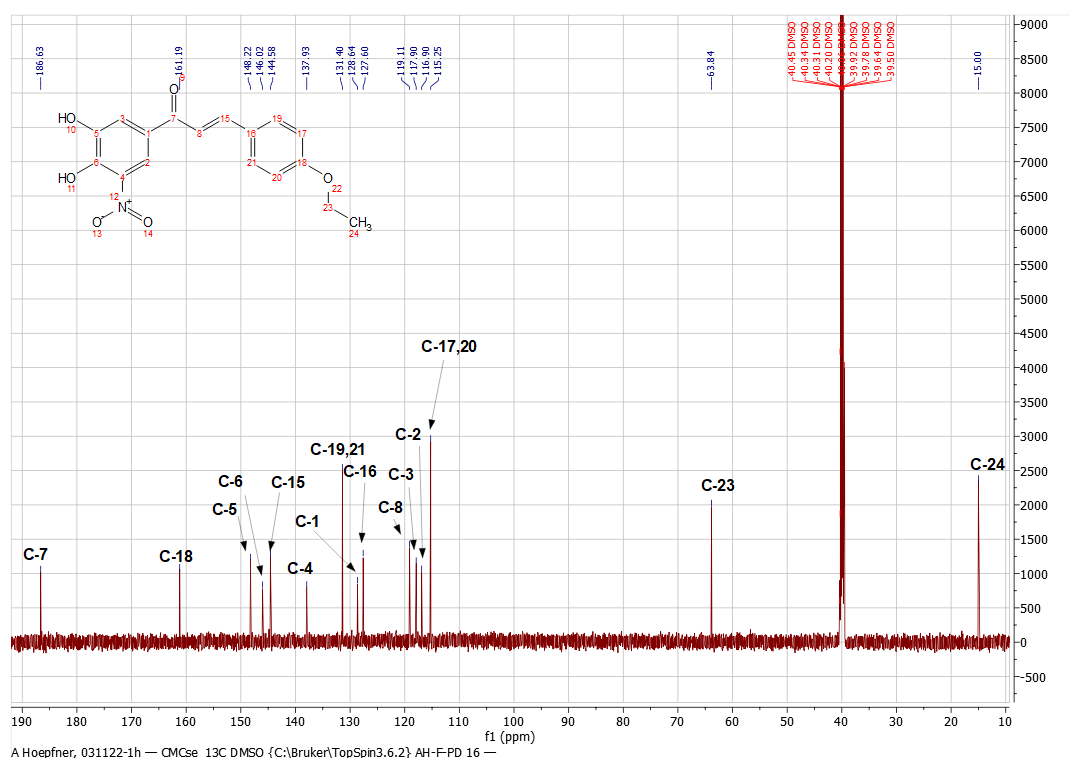


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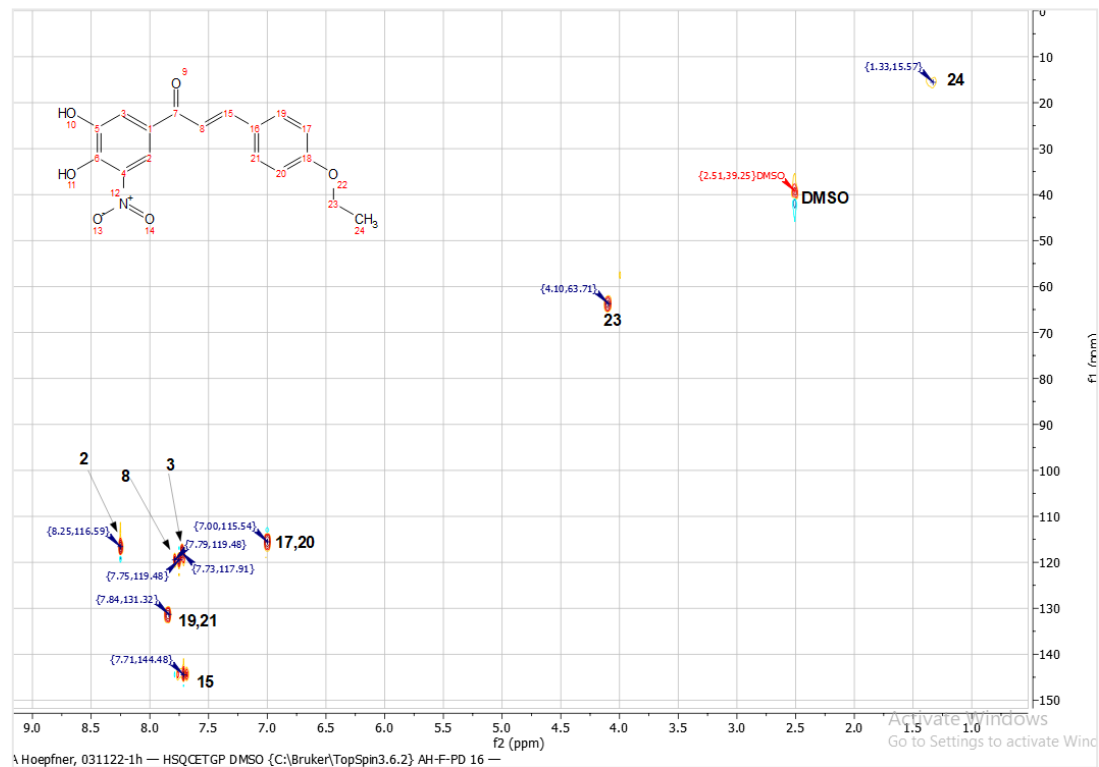
Proton (^1H) NMR spectrum



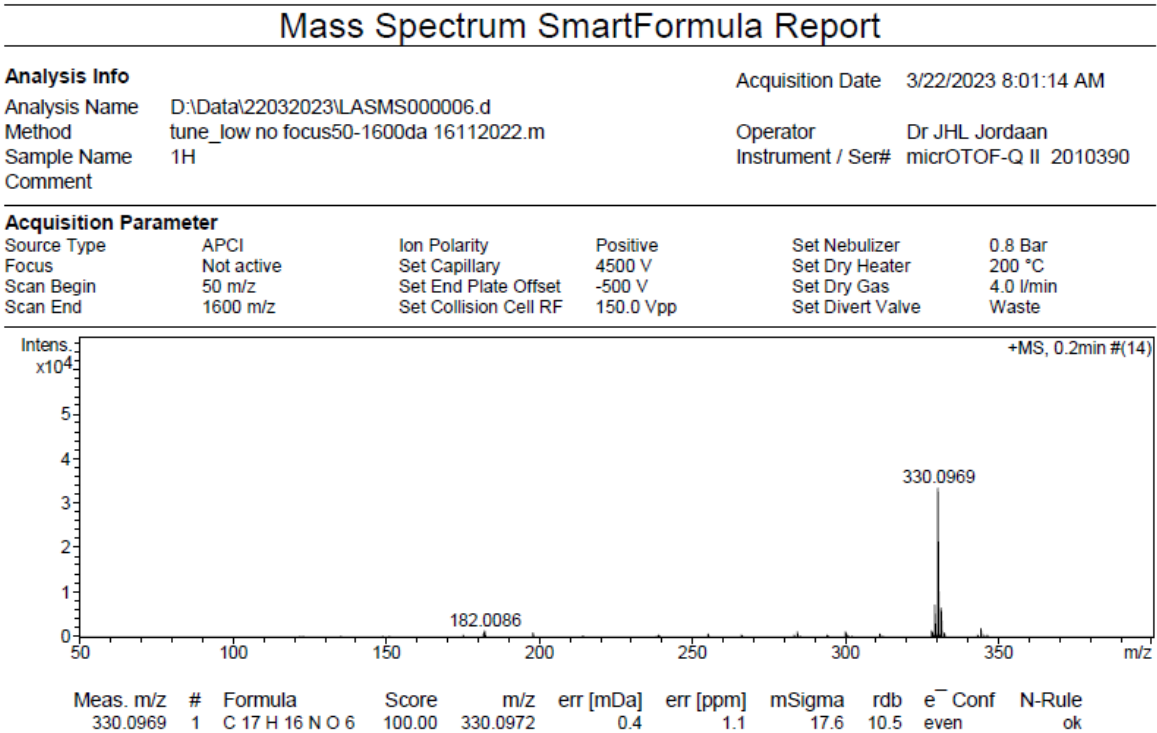
Carbon (^{13}C) NMR spectrum



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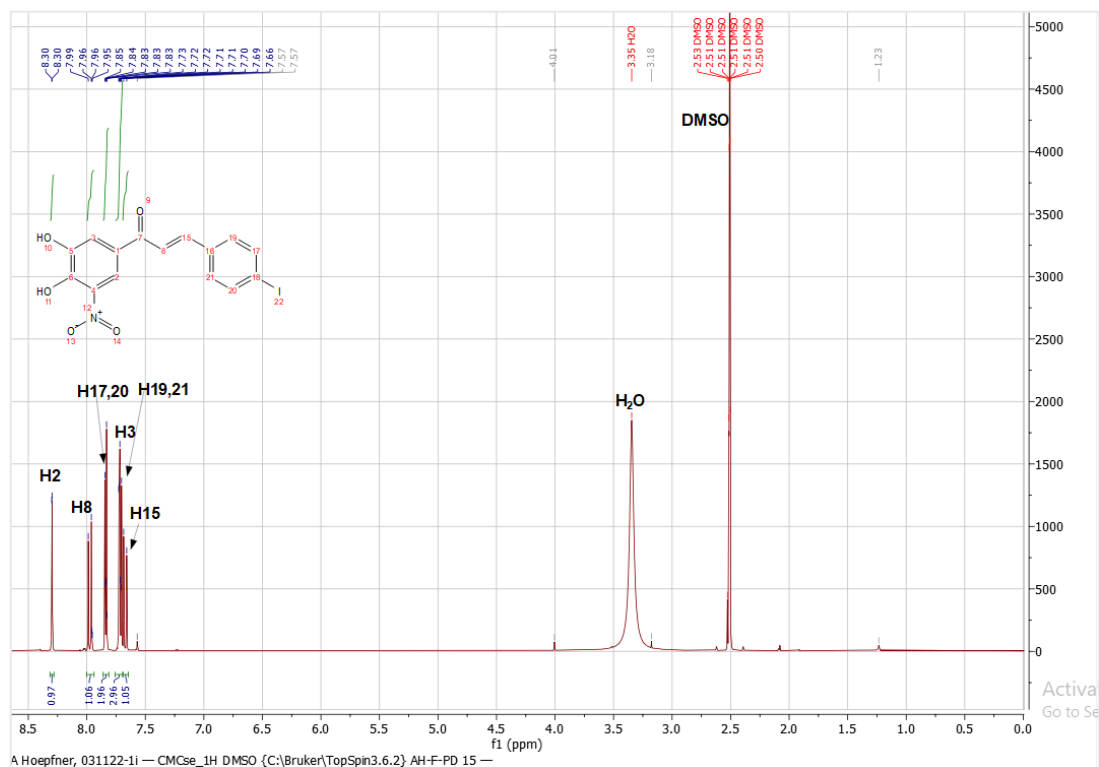


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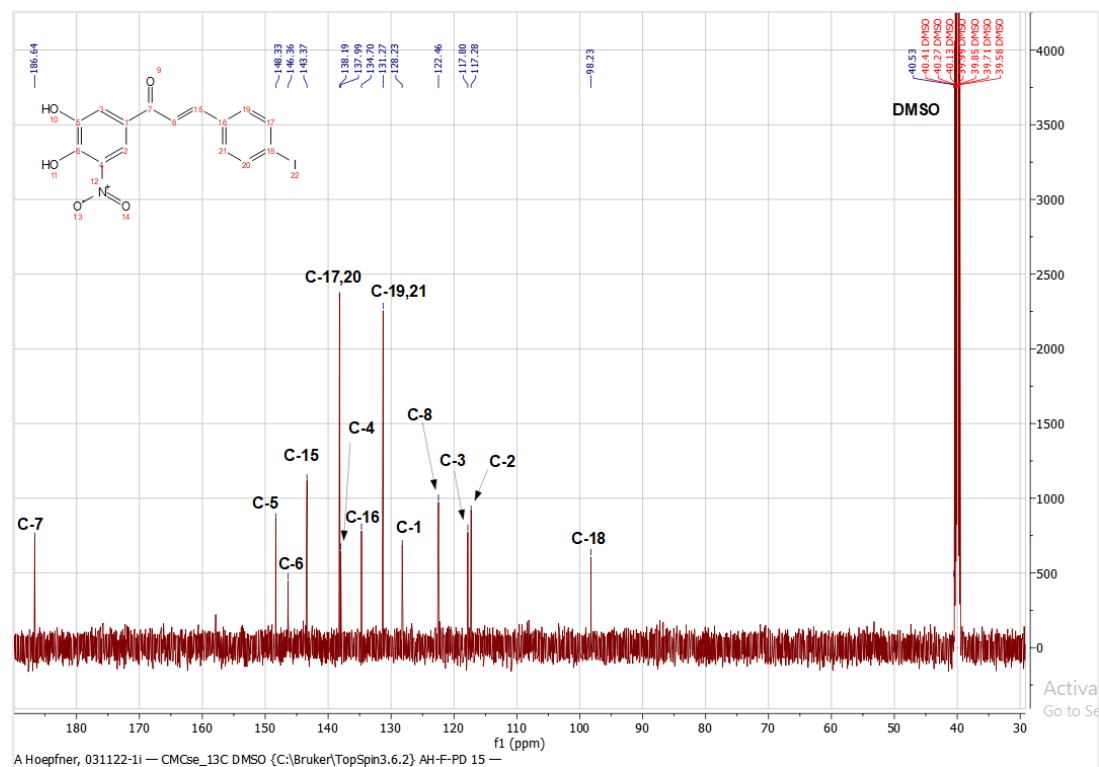


(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-iodophenyl)prop-2-en-1-one (3f)

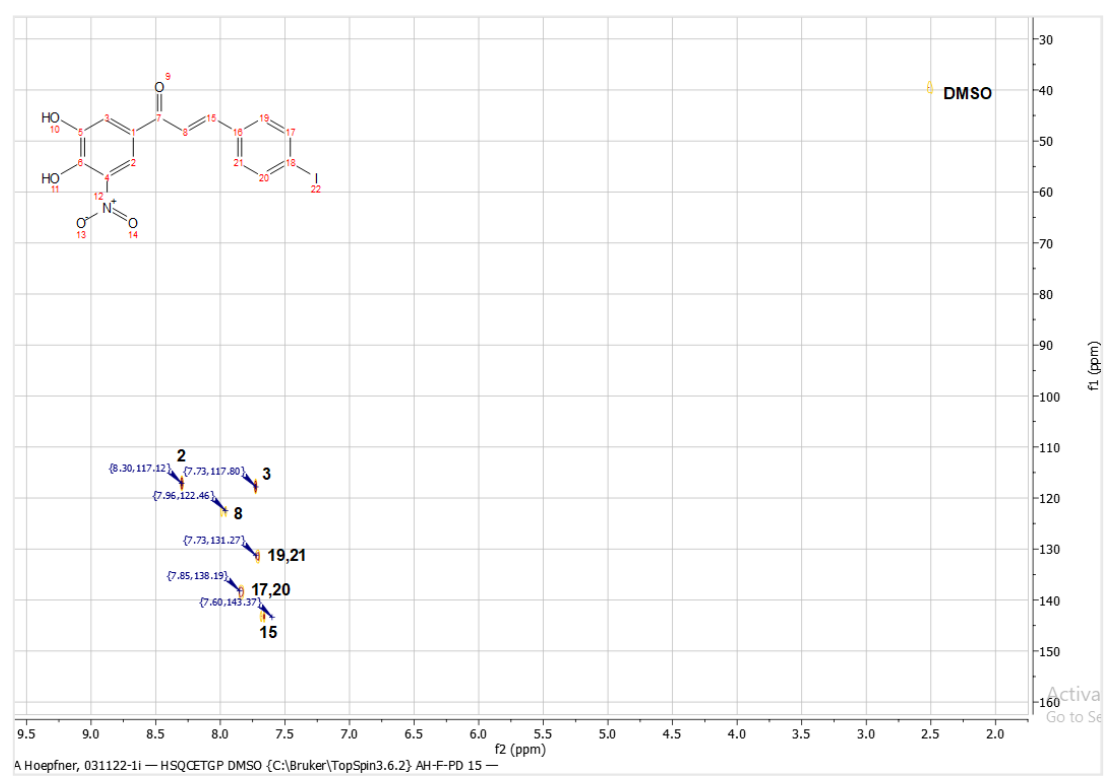
Proton (^1H) NMR spectrum



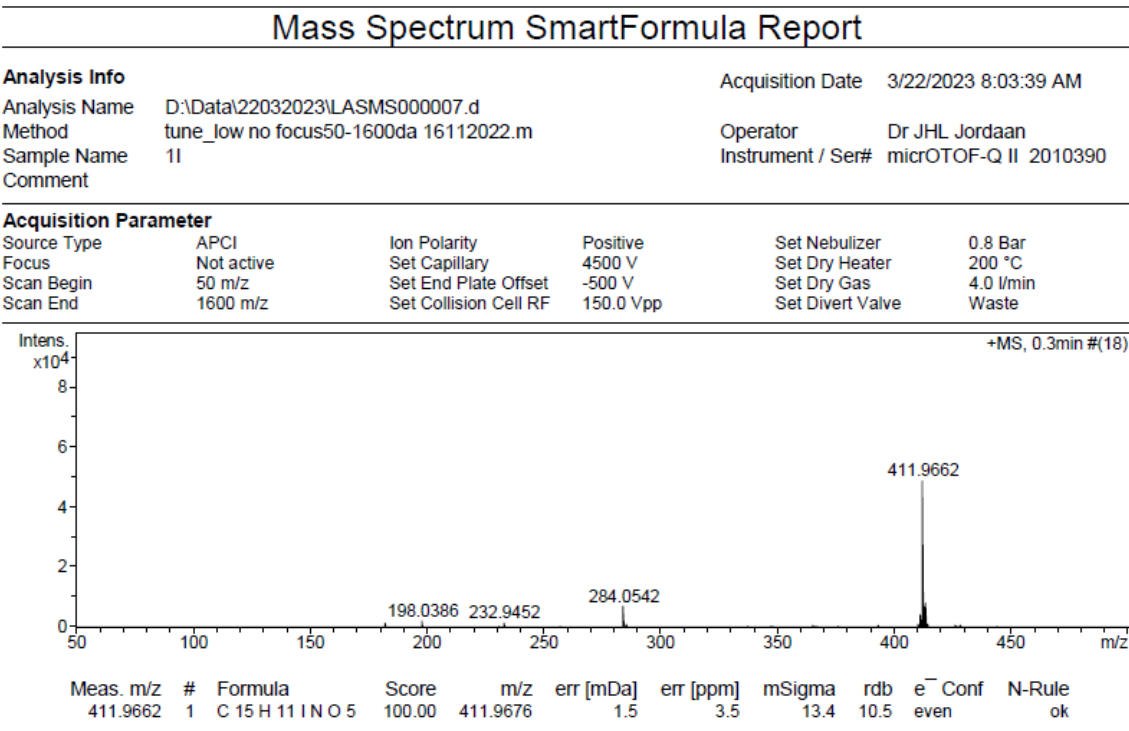
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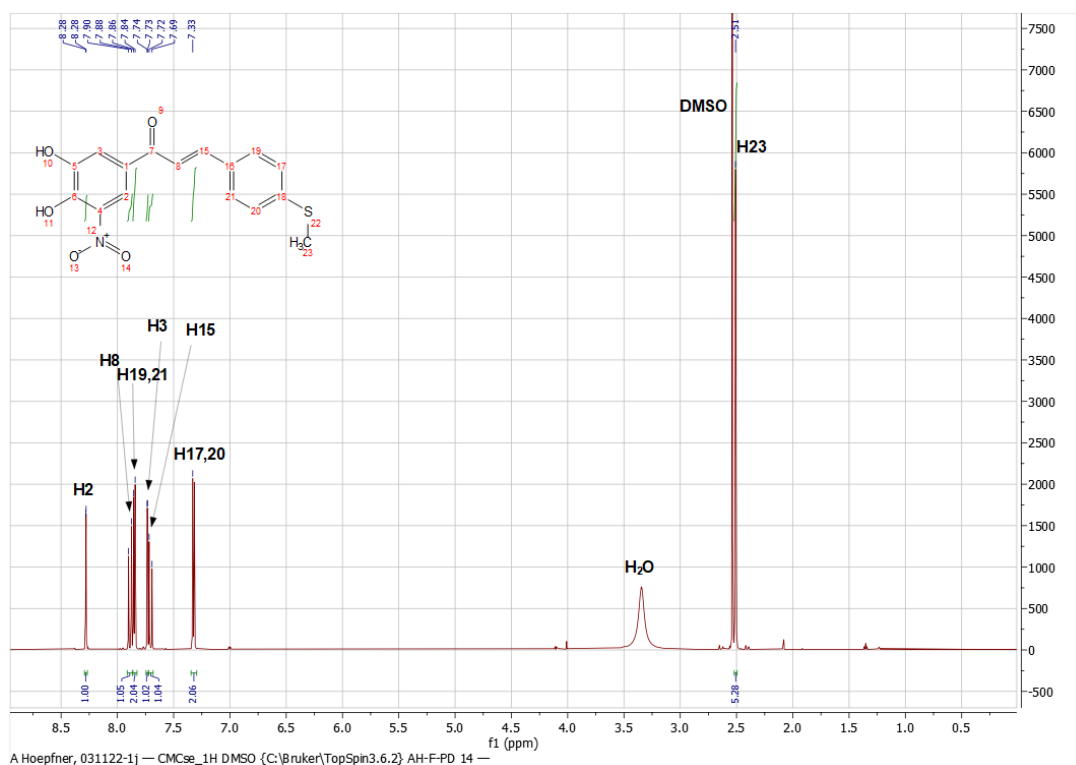


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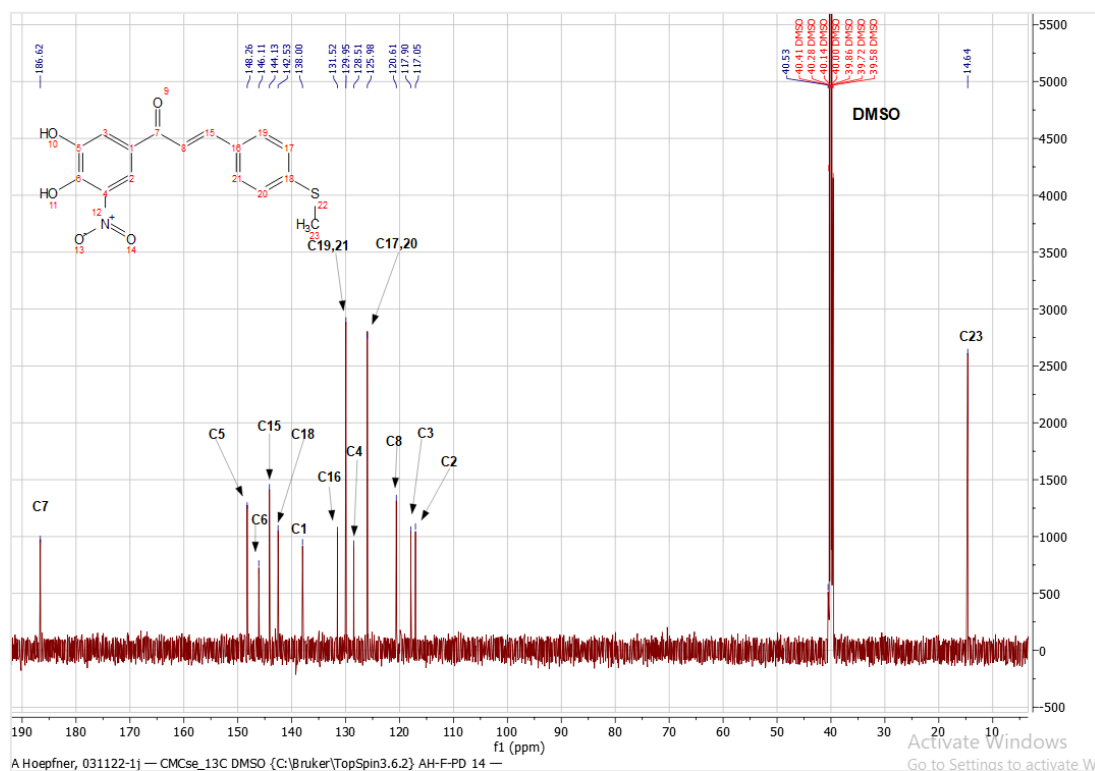


(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-[4-(methylsulfonyl)phenyl]prop-2-en-1-one (3g)

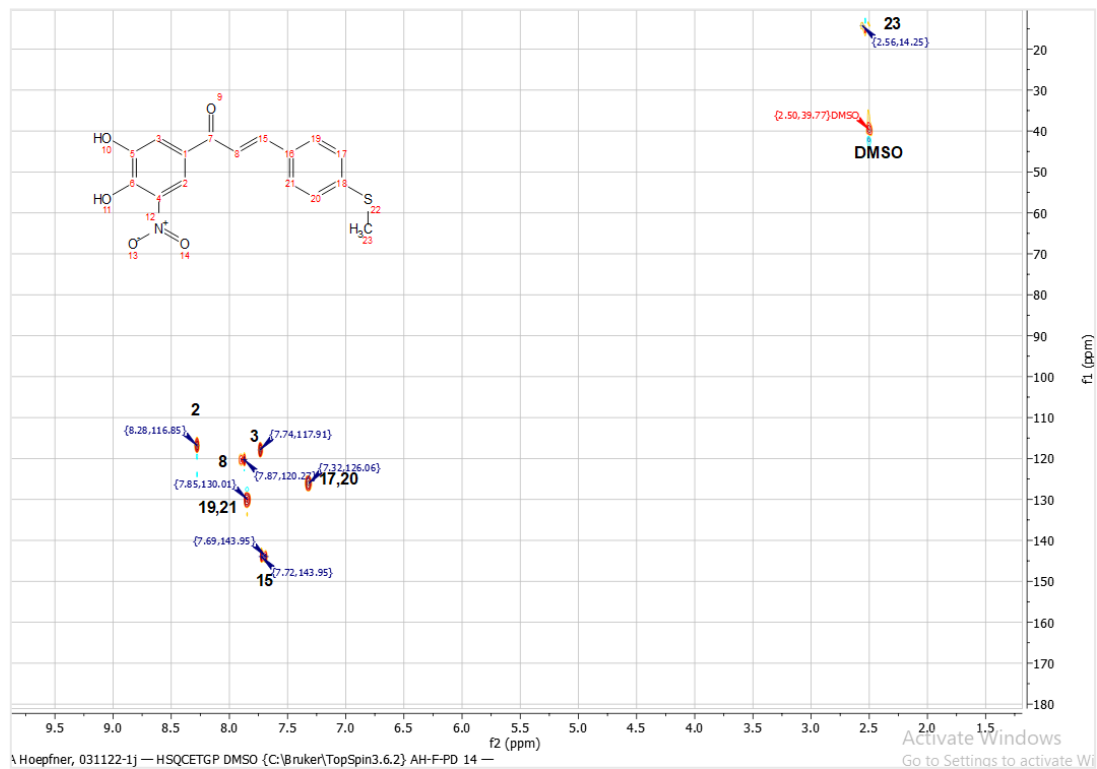
Proton (^1H) NMR spectrum



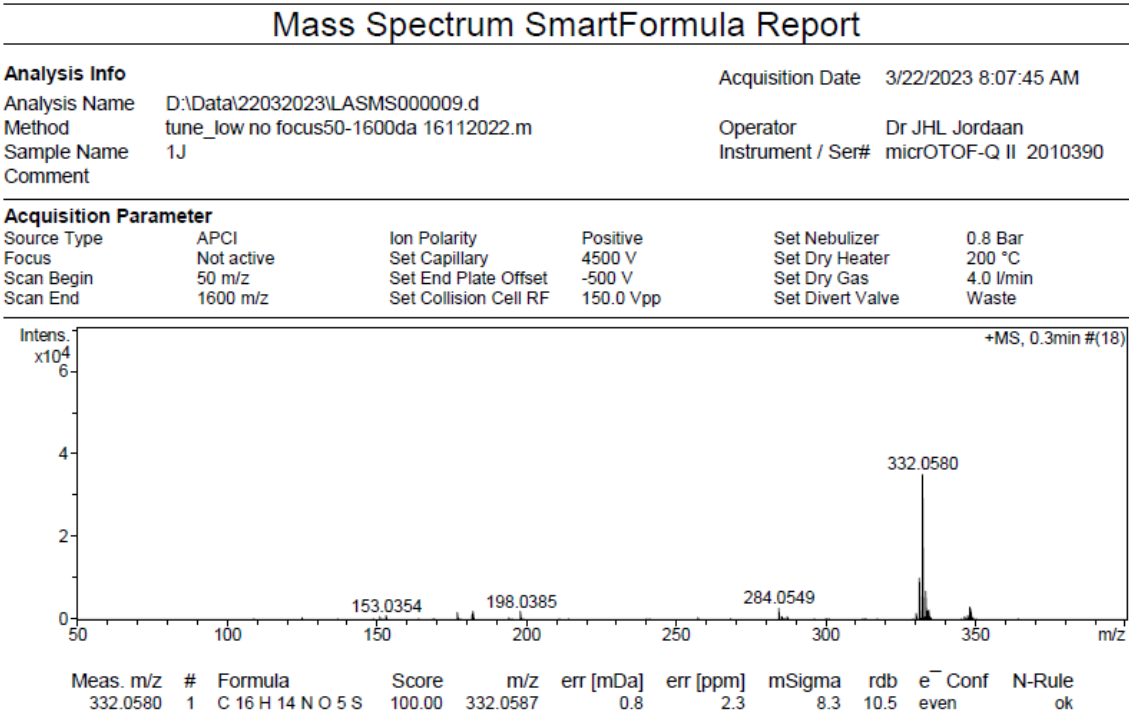
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

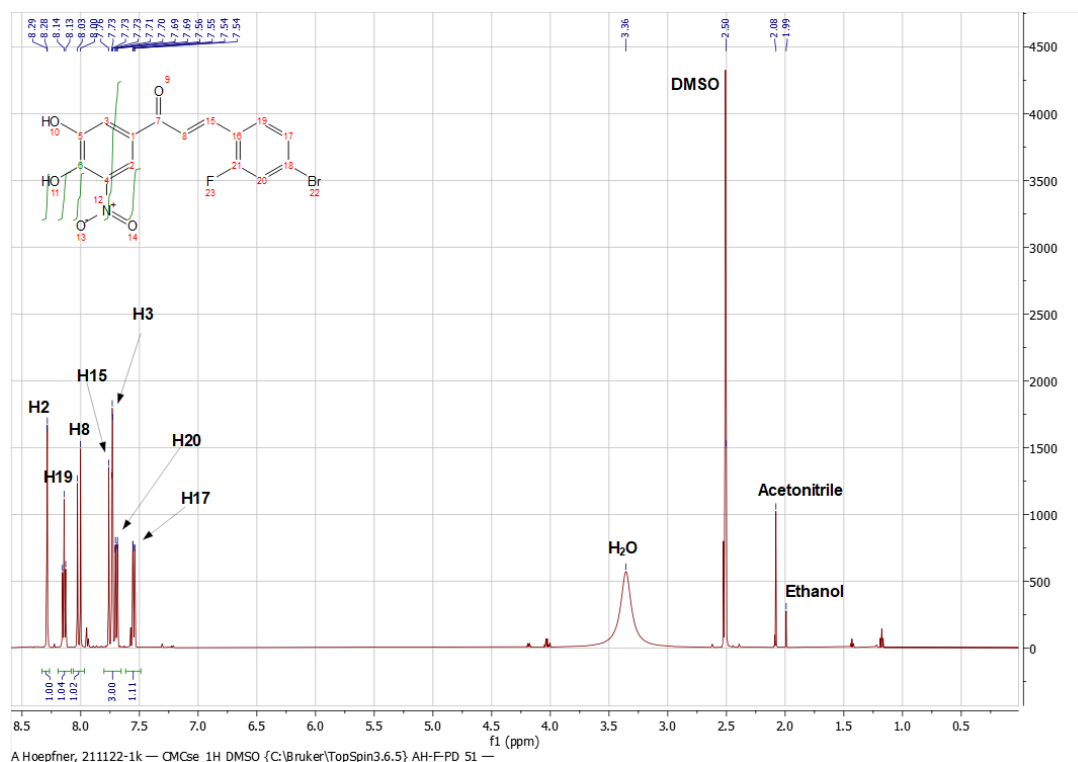


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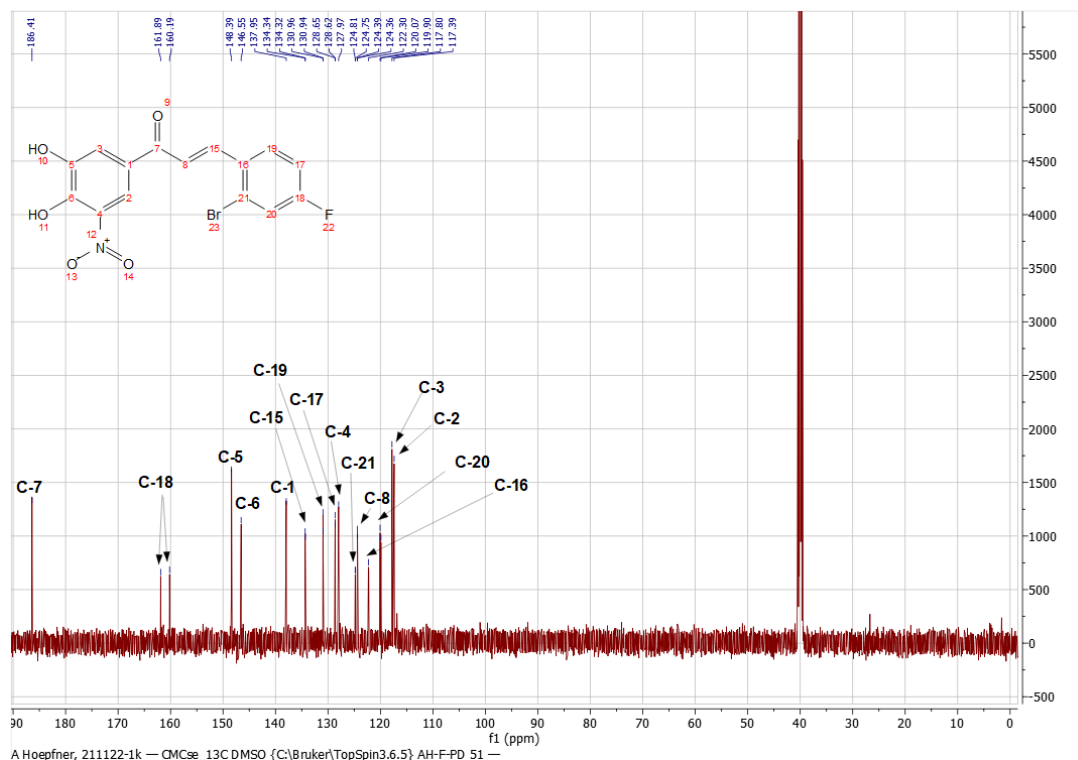


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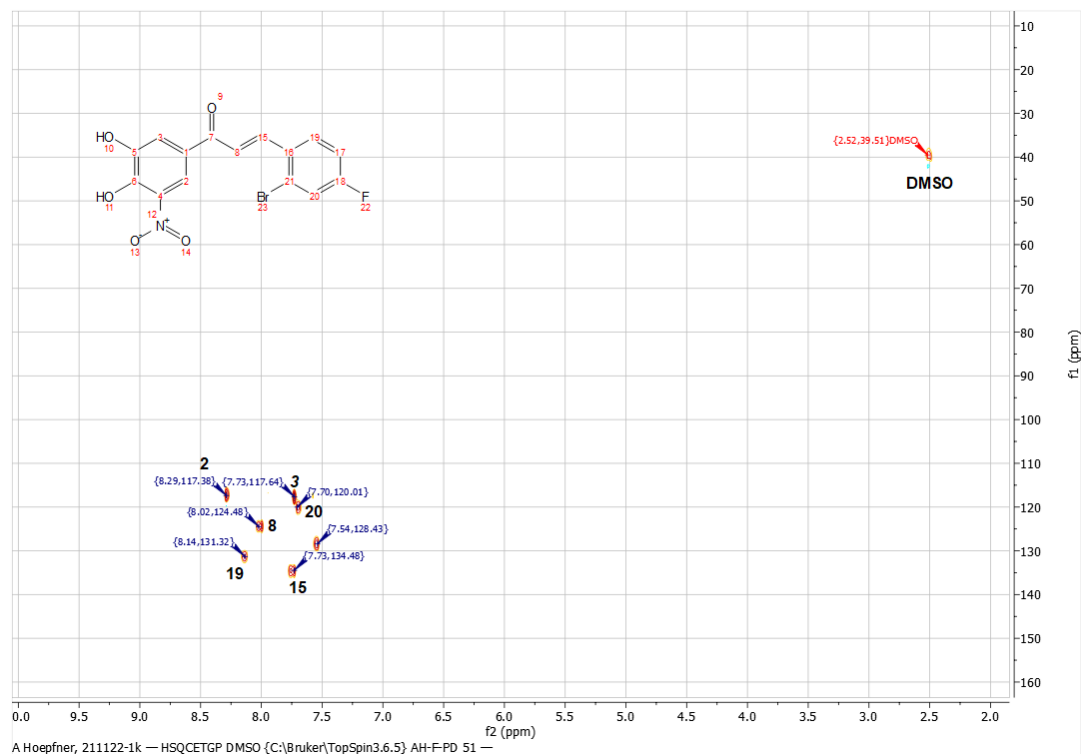
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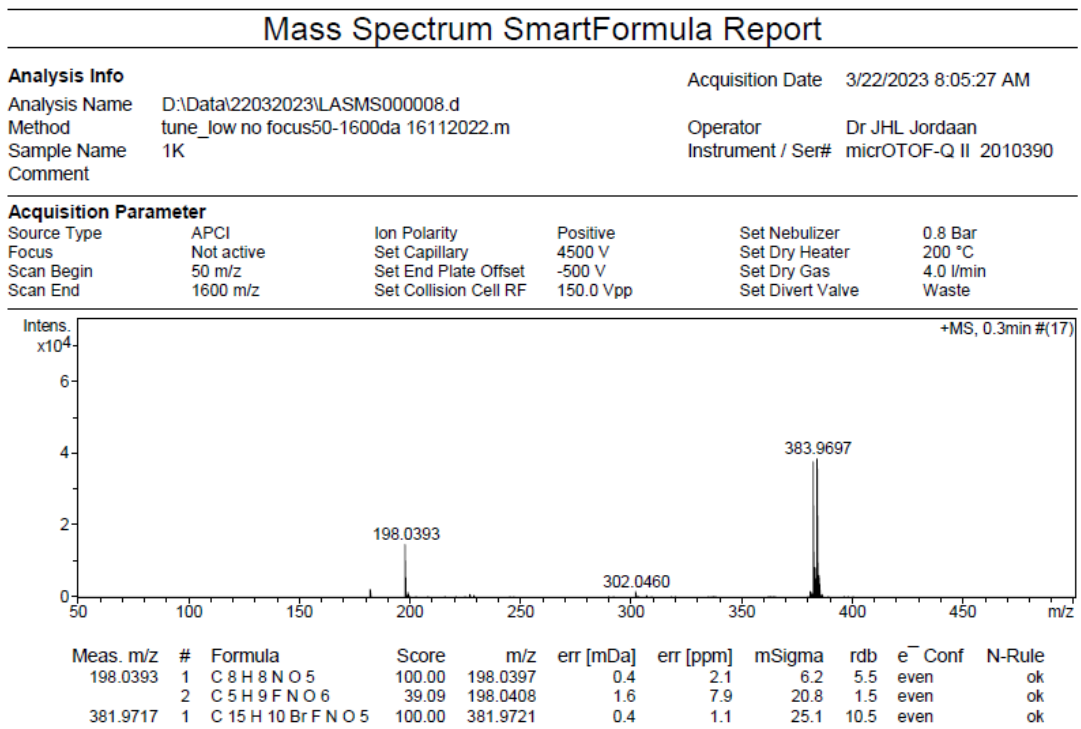
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Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

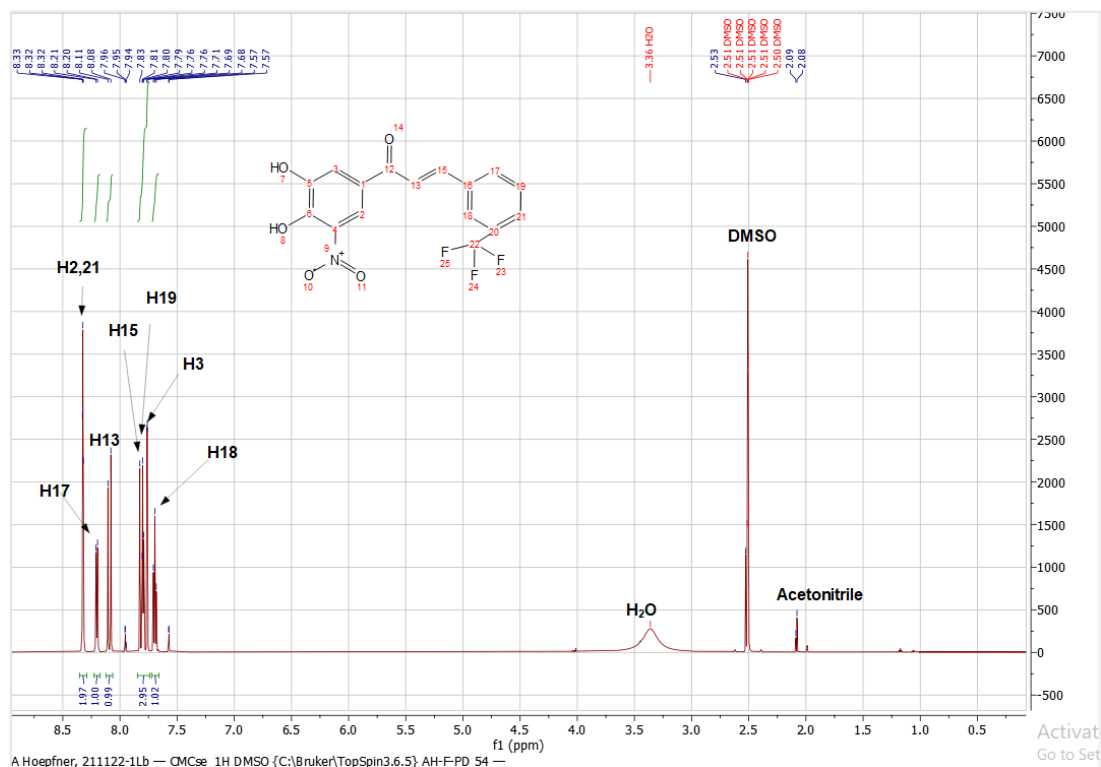


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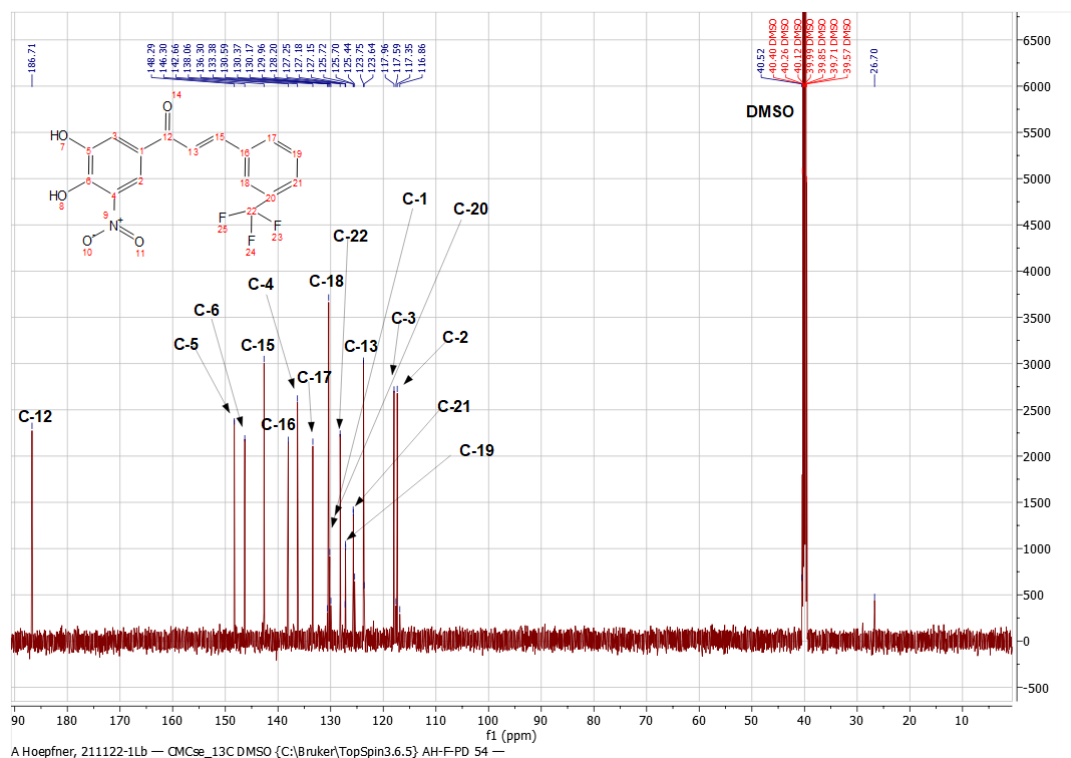


(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (3i)

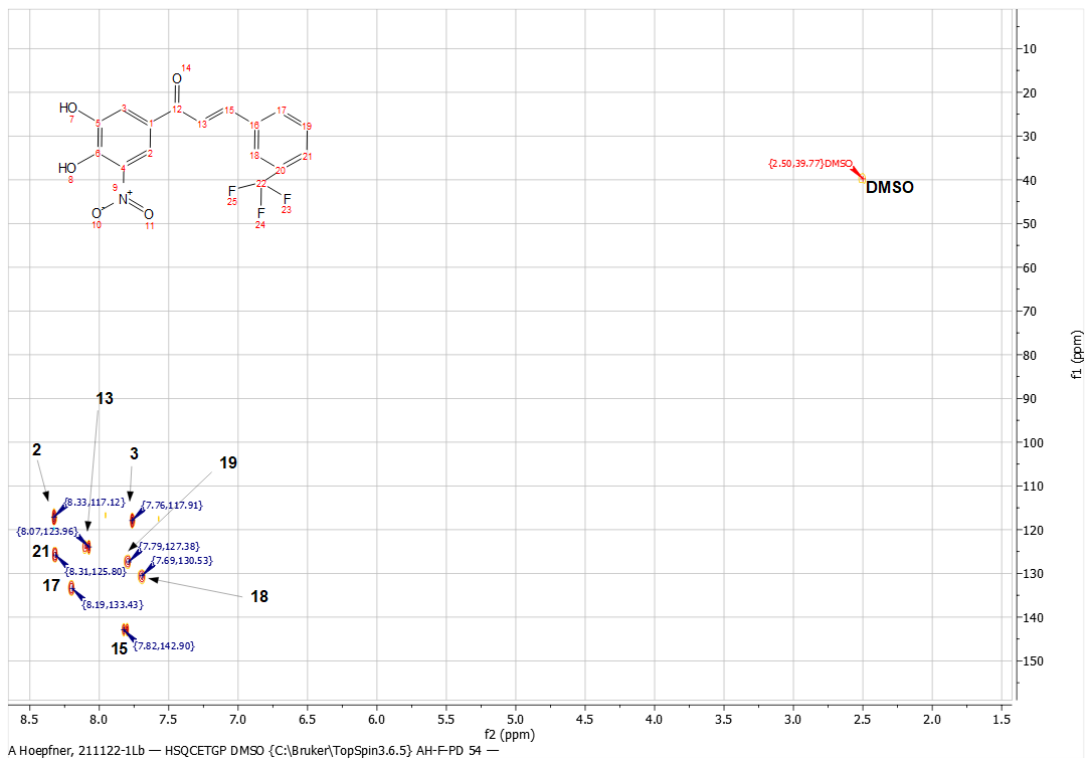
Proton (^1H) NMR spectrum



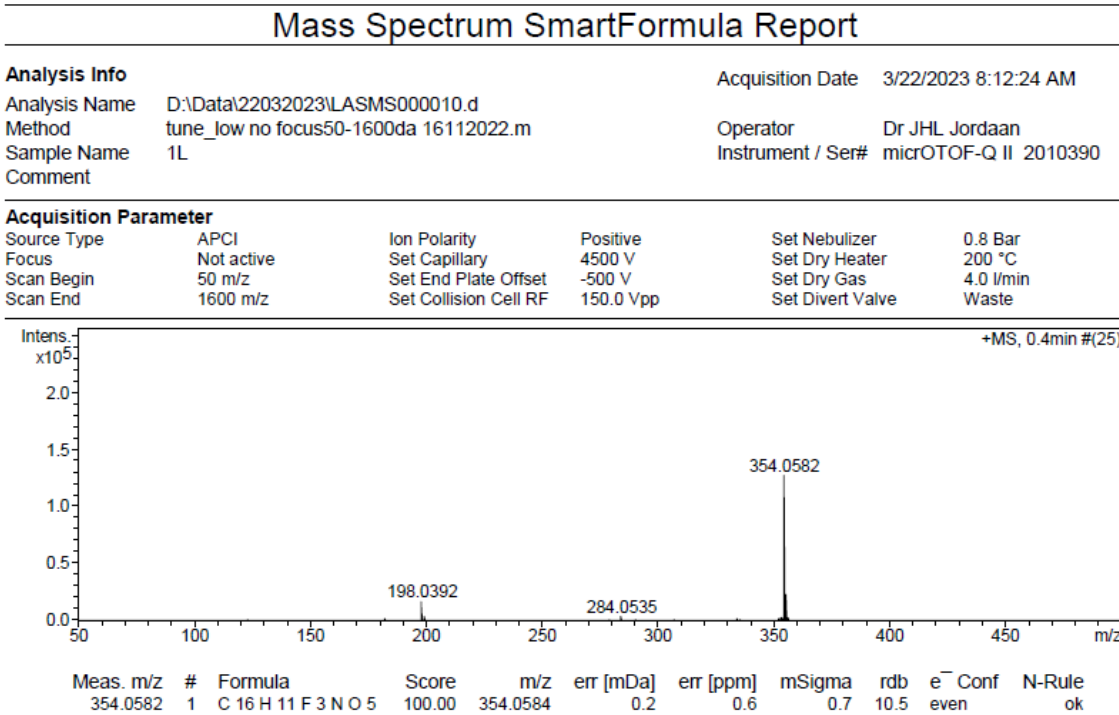
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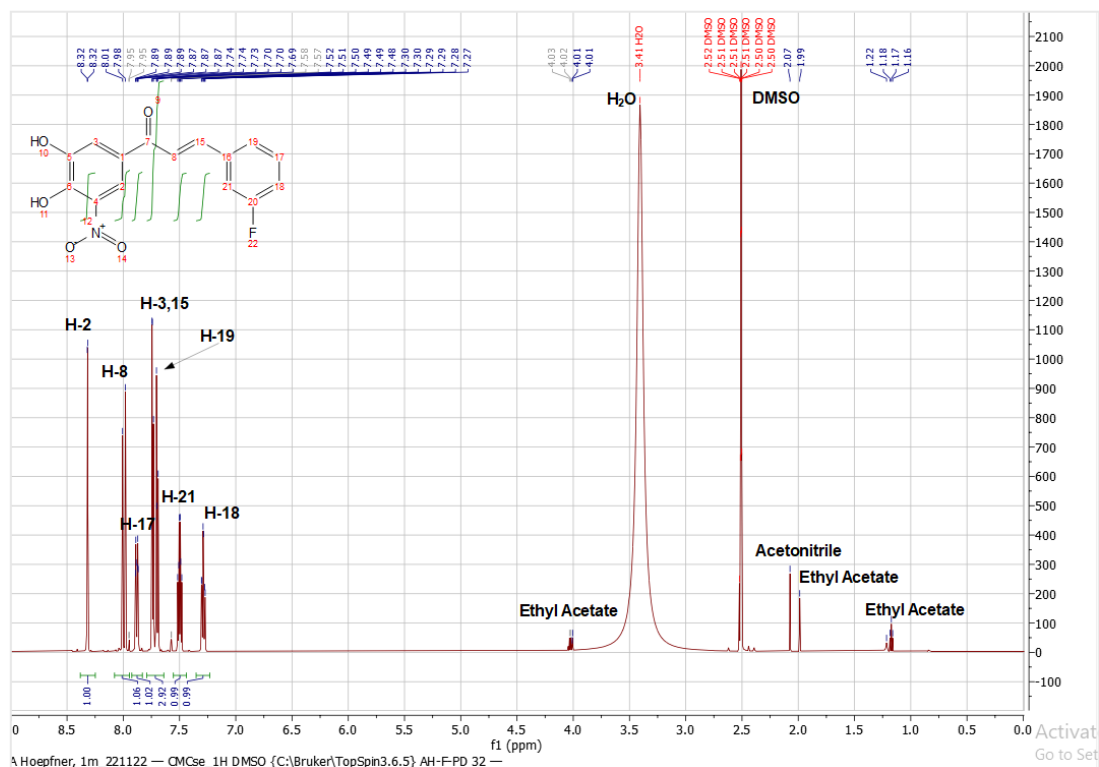


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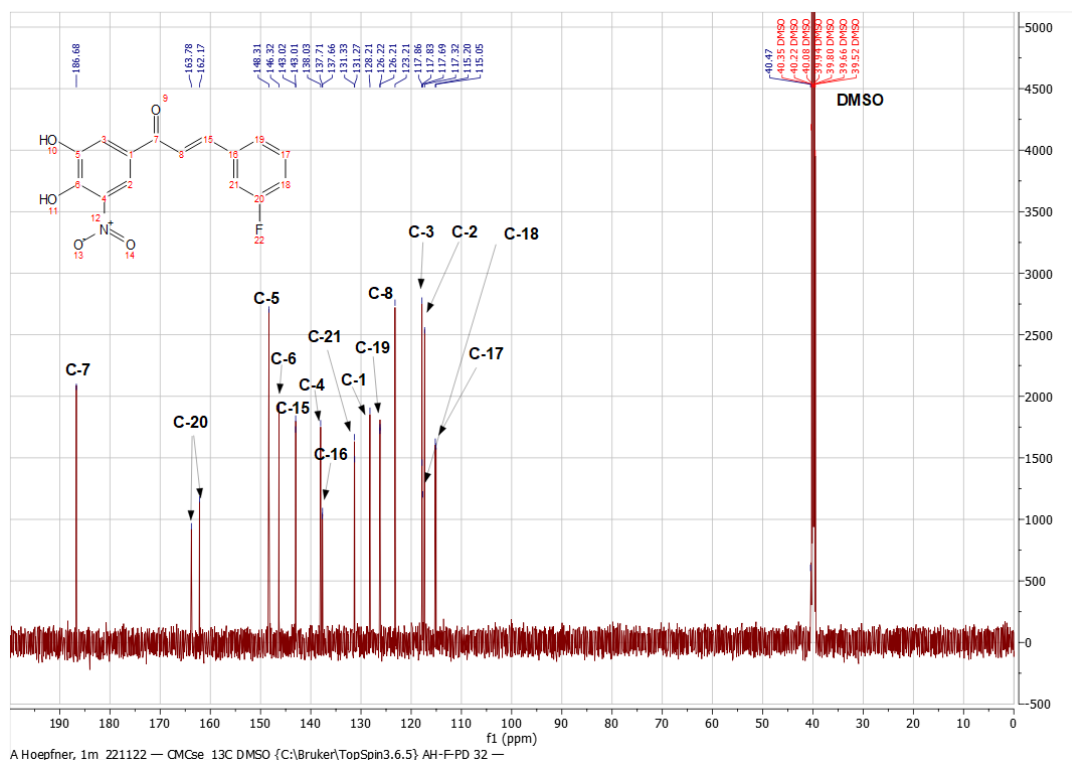


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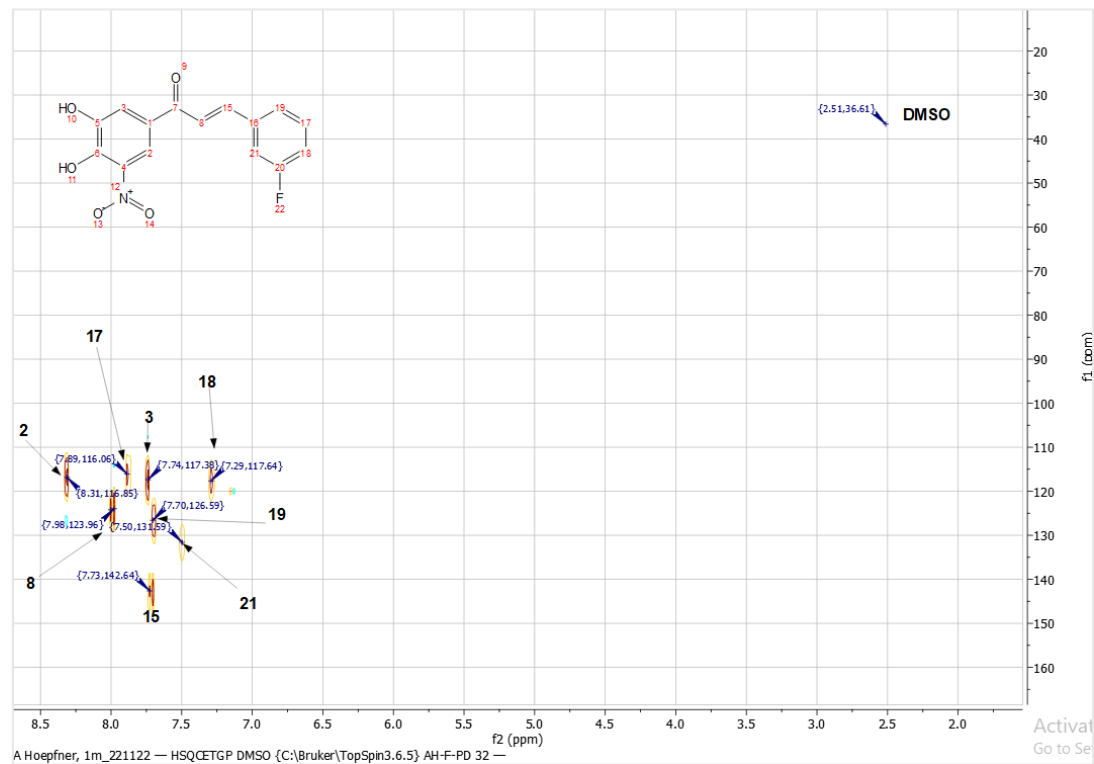
Proton (^1H) NMR spectrum



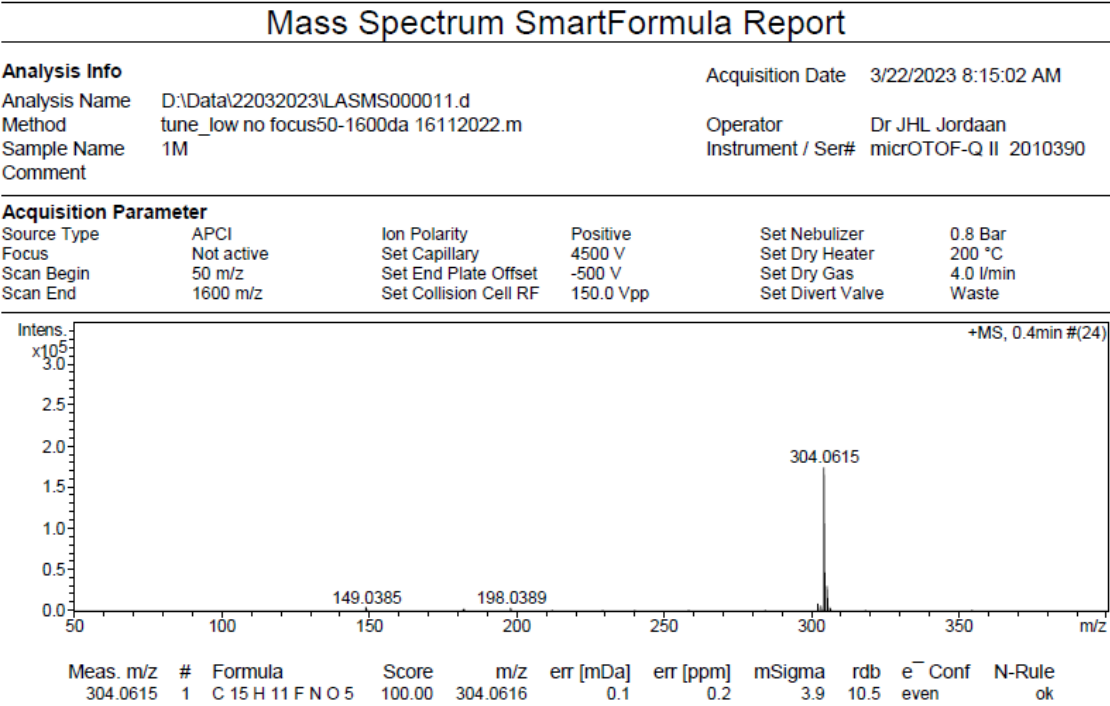
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Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

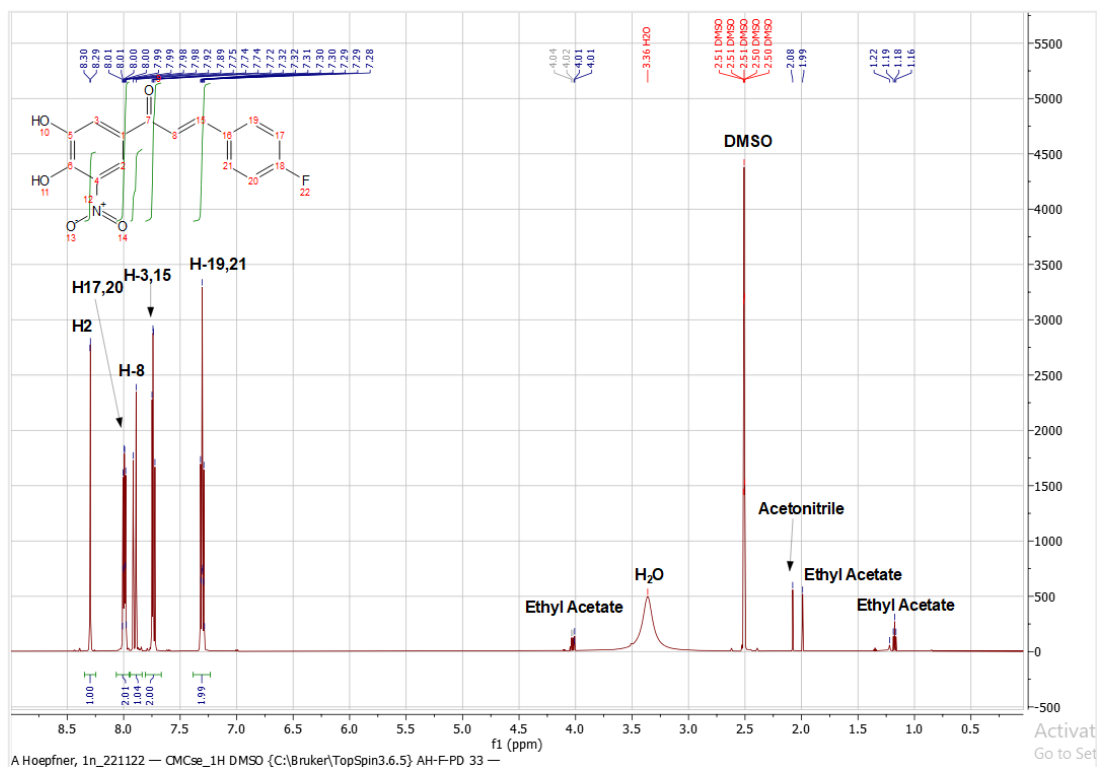


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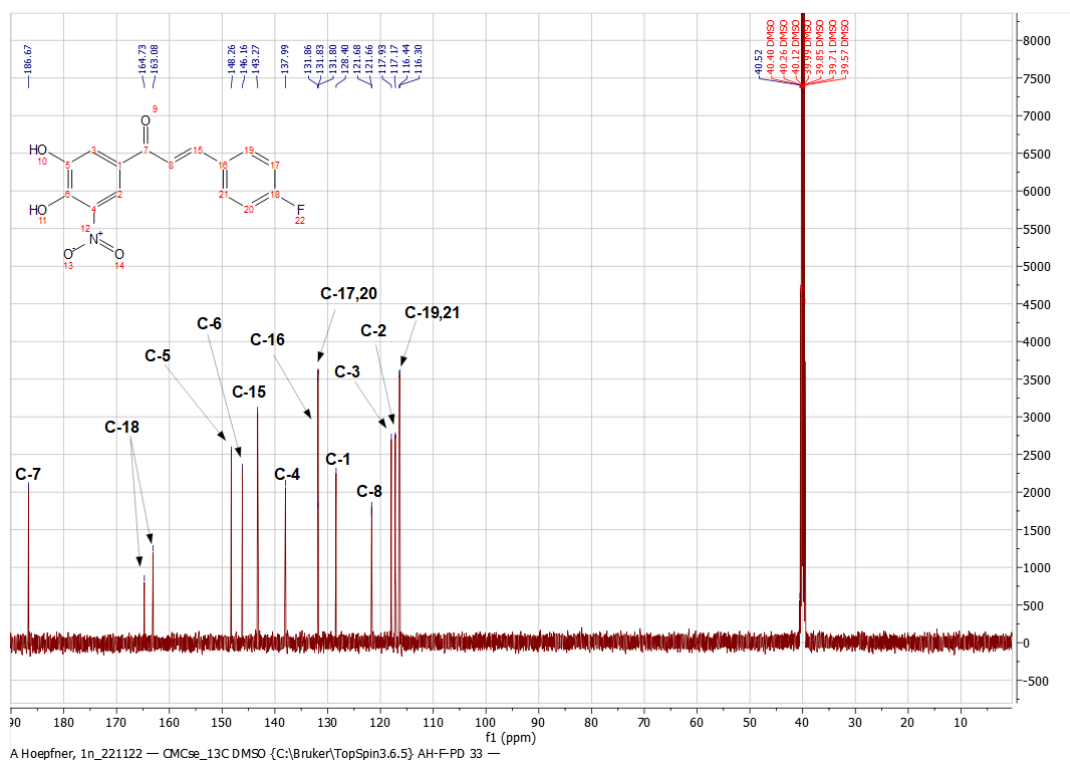


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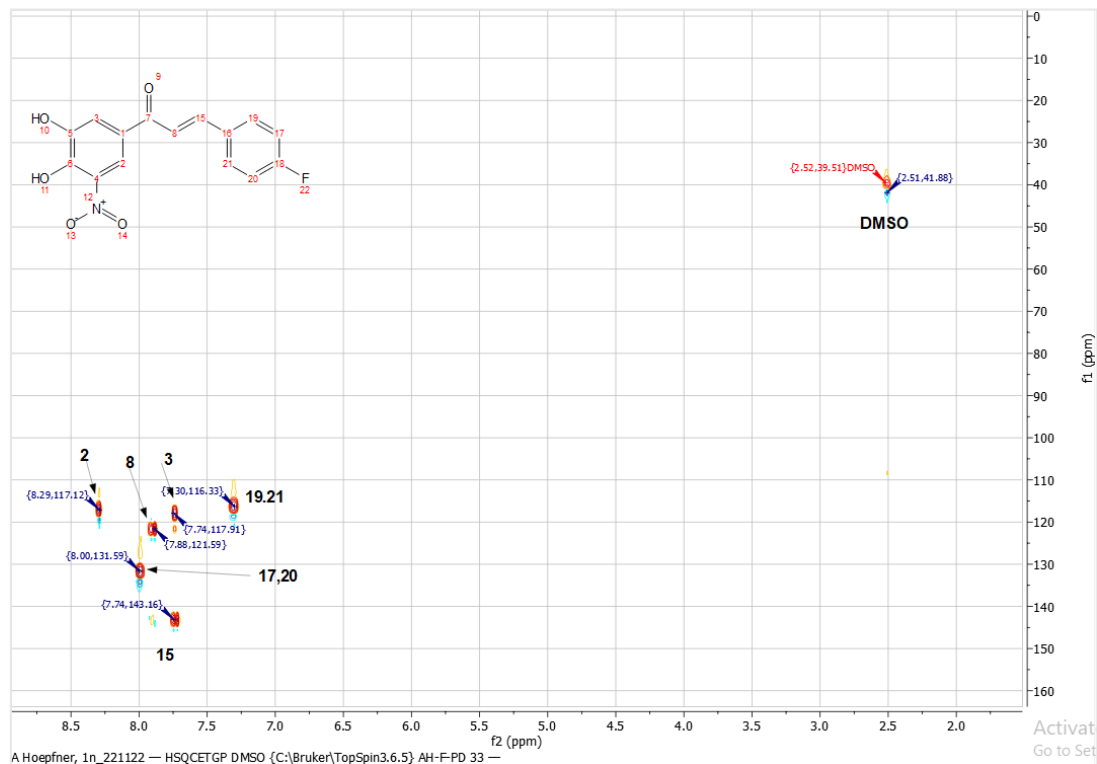
Proton (^1H) NMR spectrum



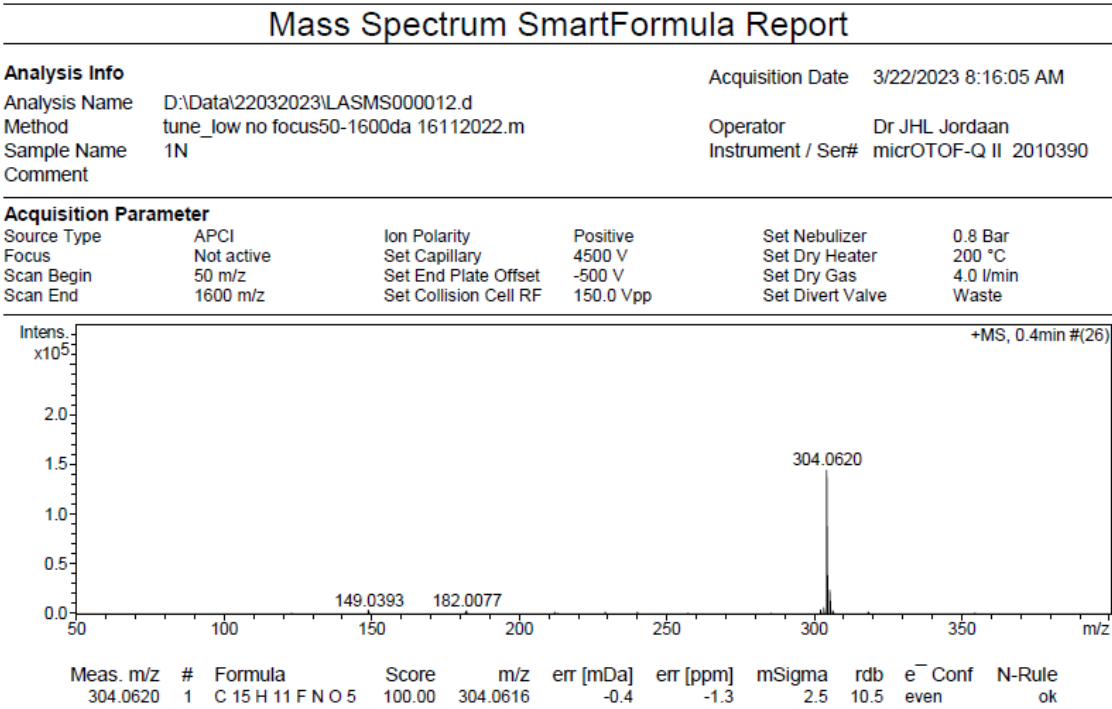
Carbon (^{13}C) NMR spectrum



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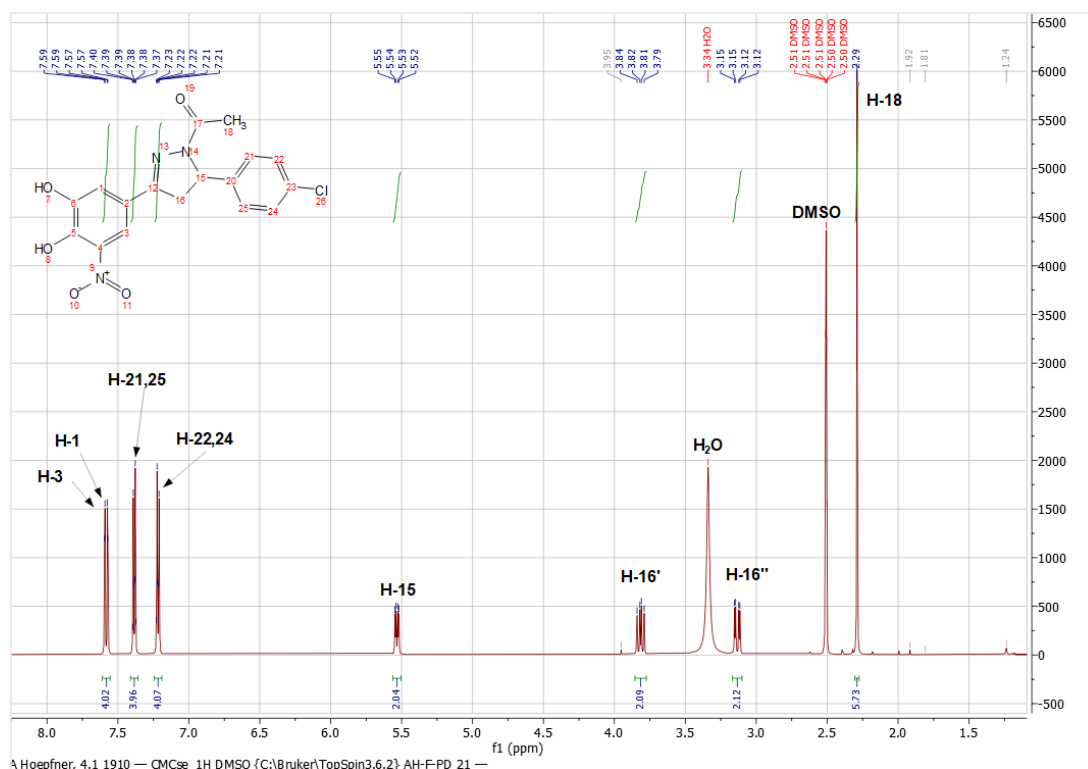


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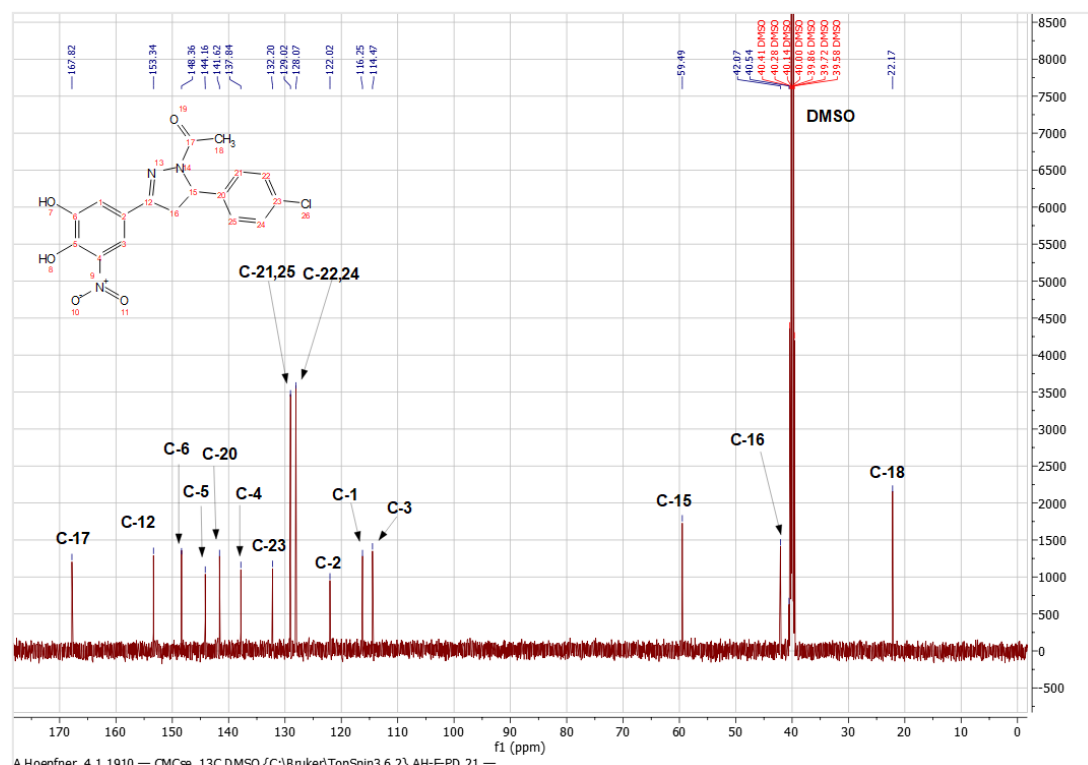


1-[5-(4-chlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4a)

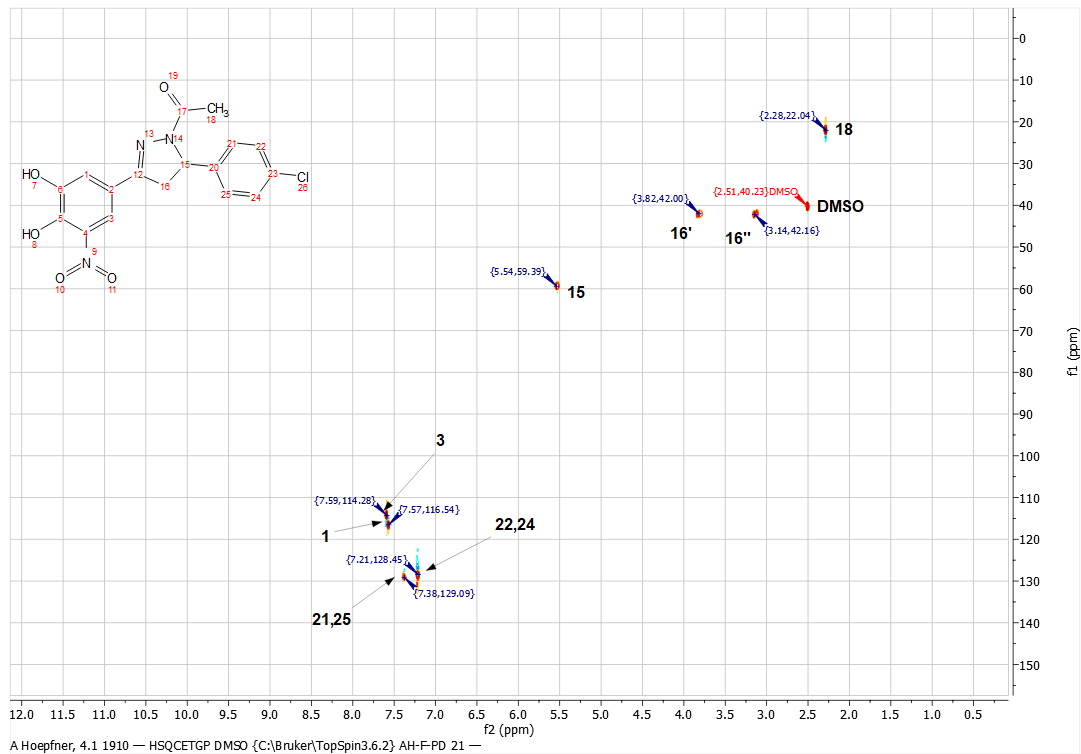
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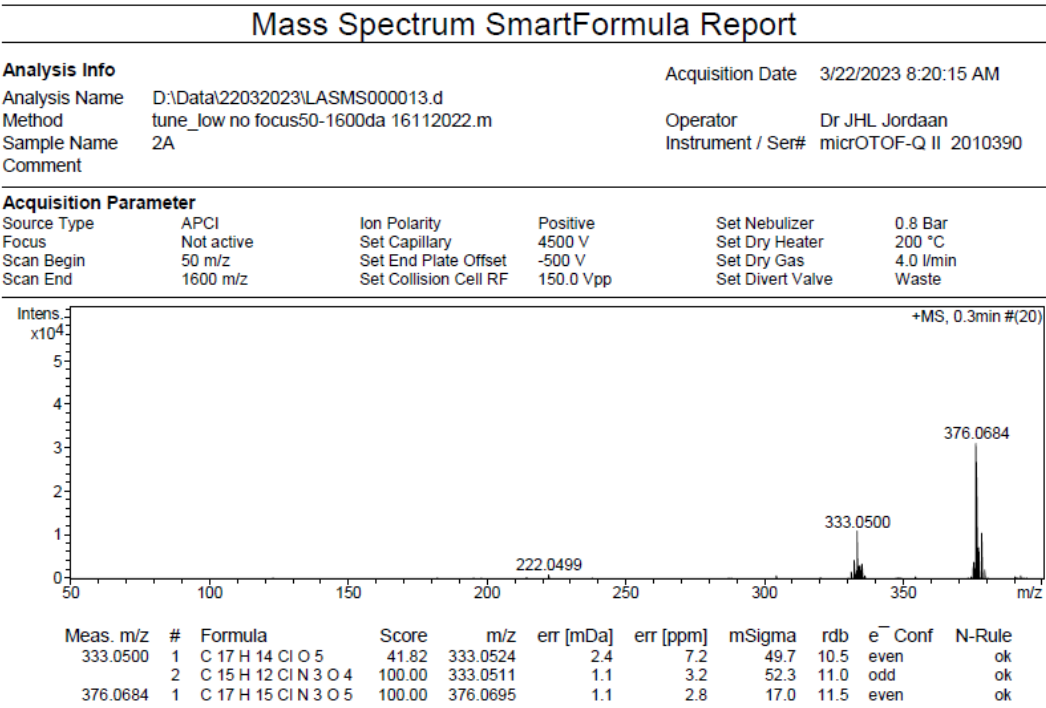
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

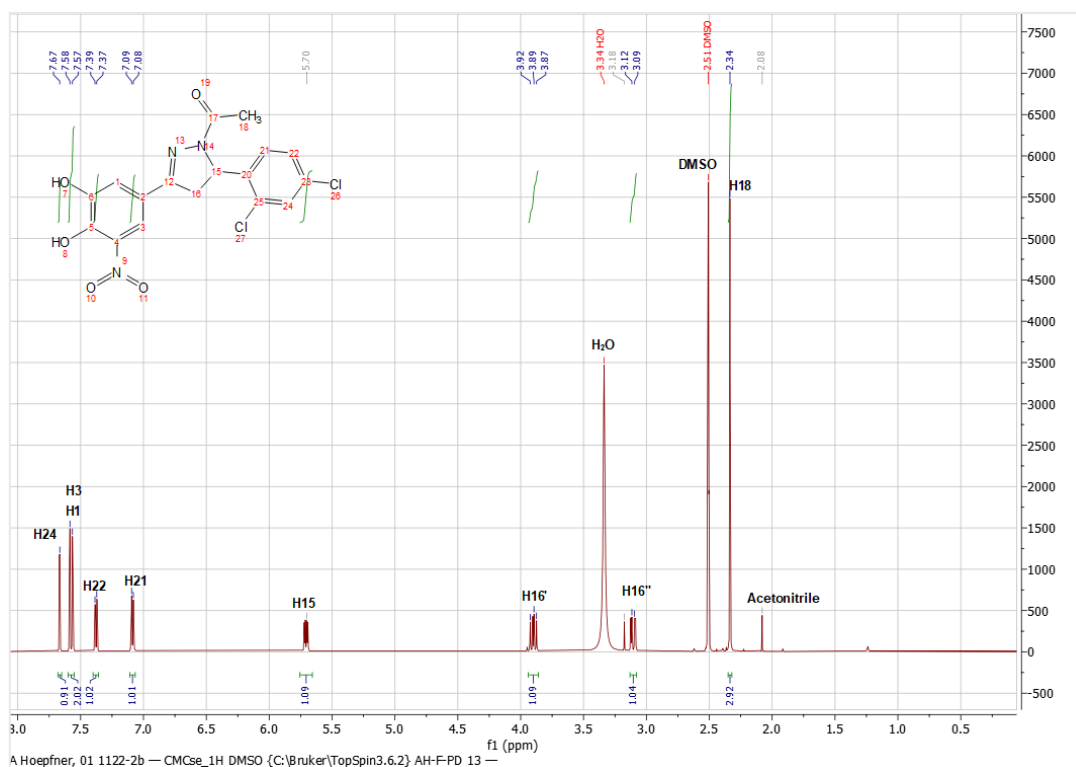


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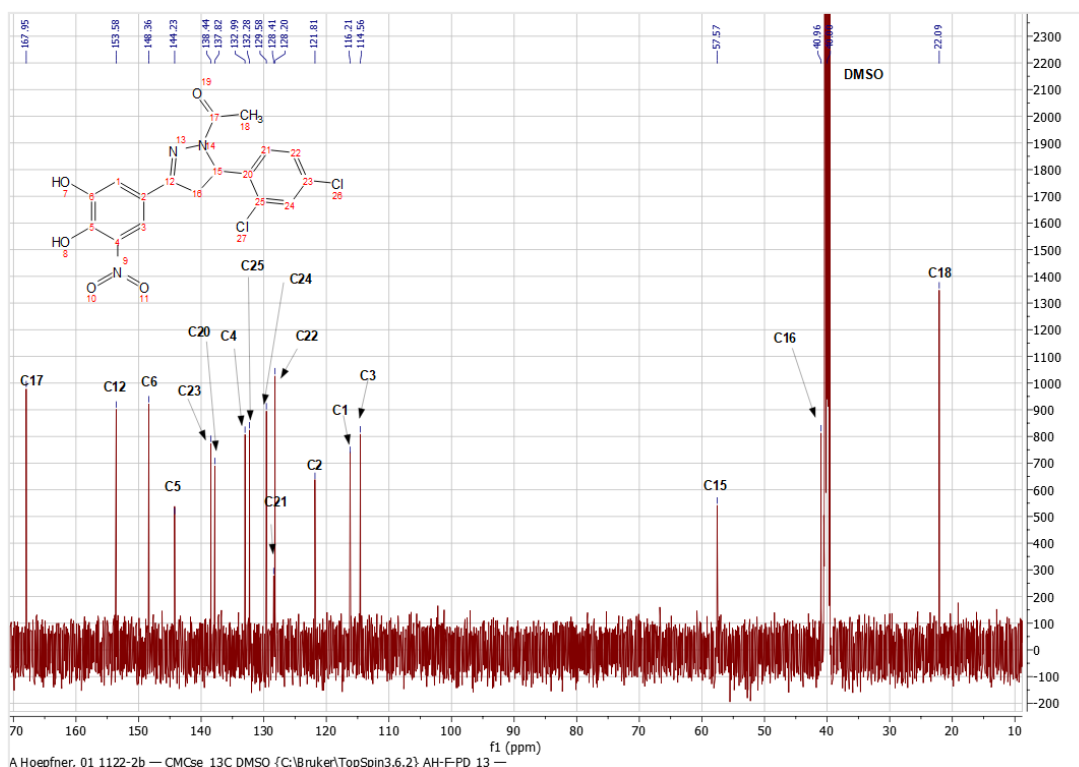


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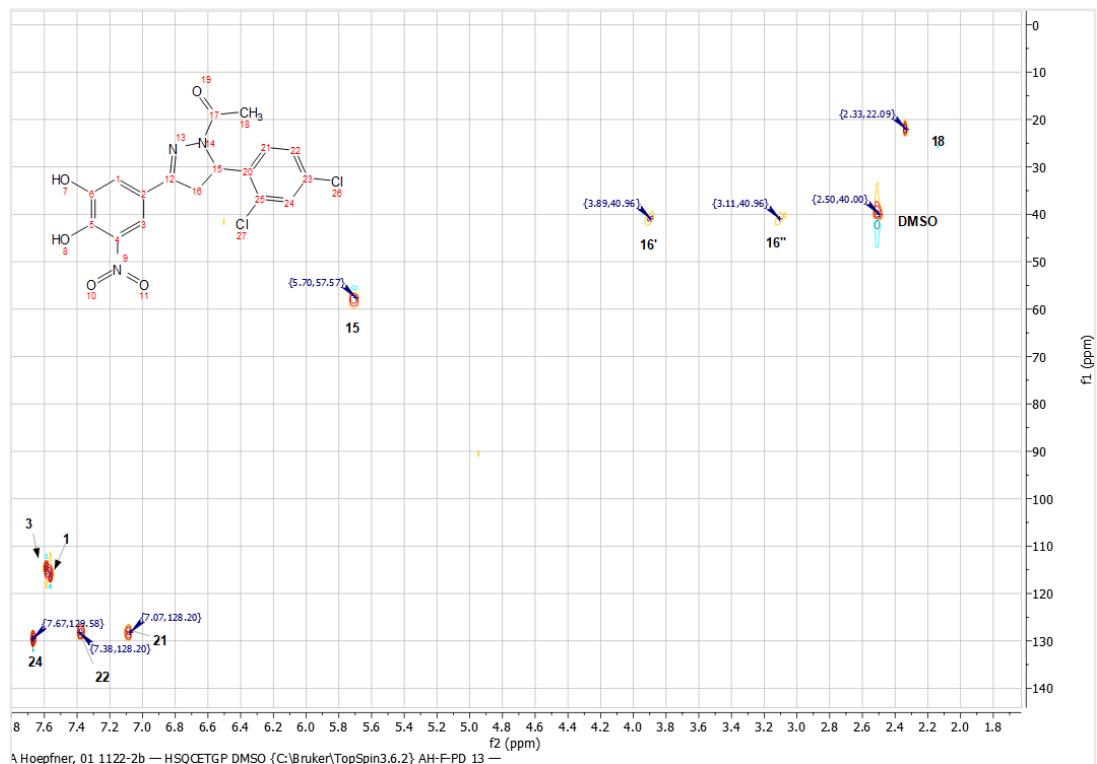
Proton (^1H) NMR spectrum



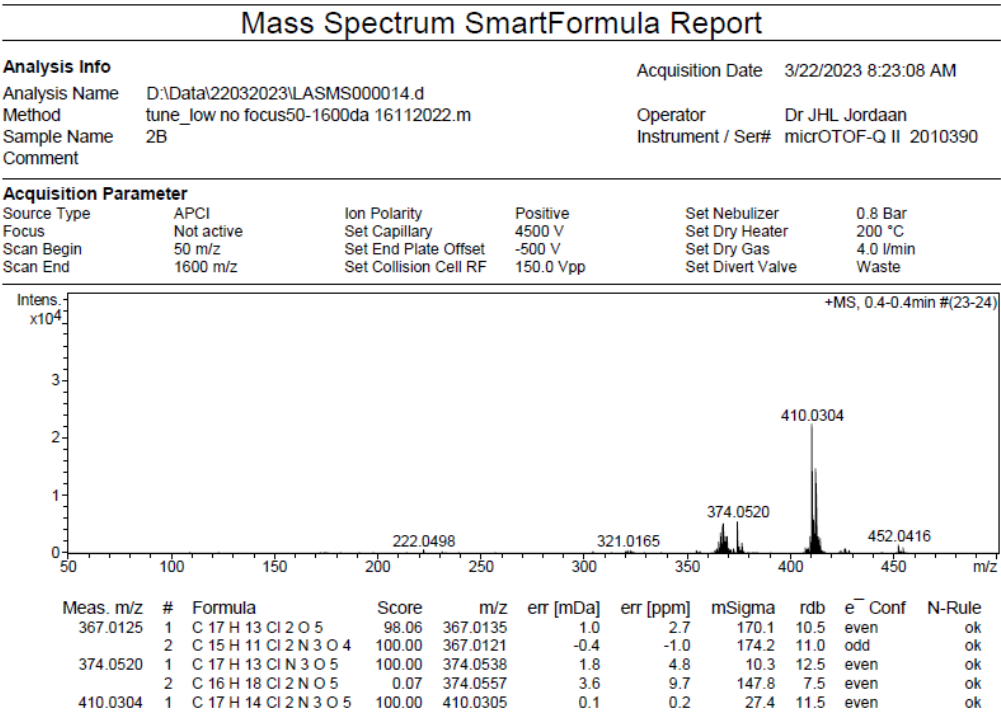
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

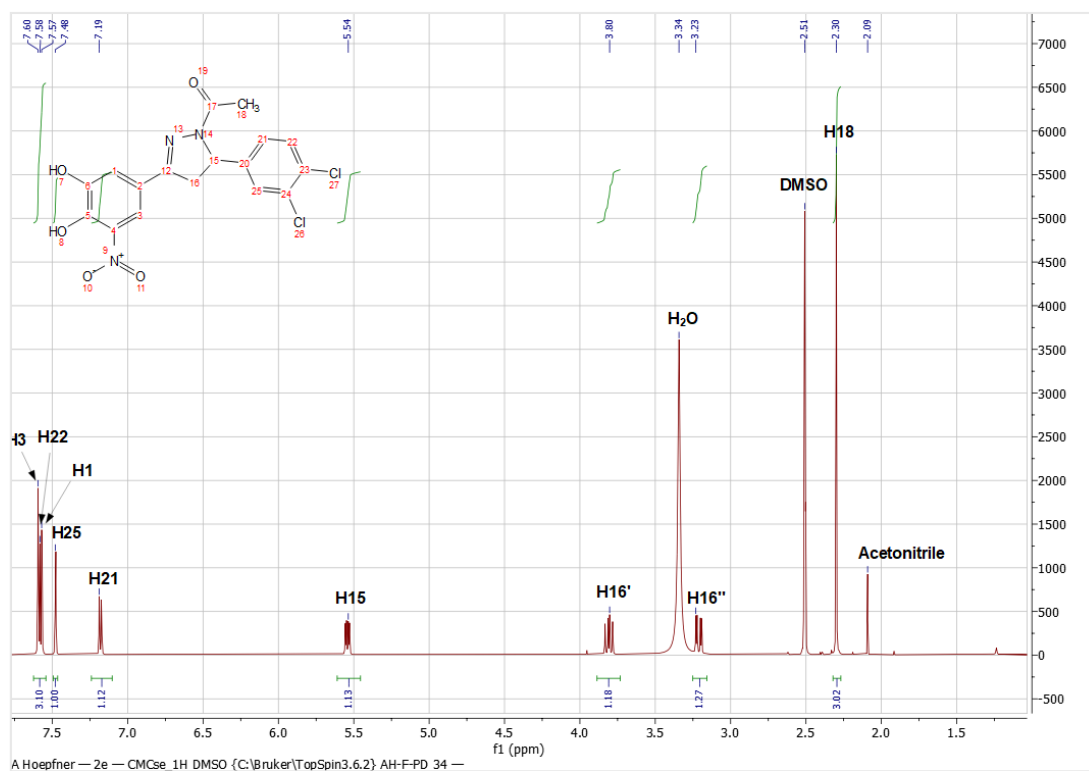


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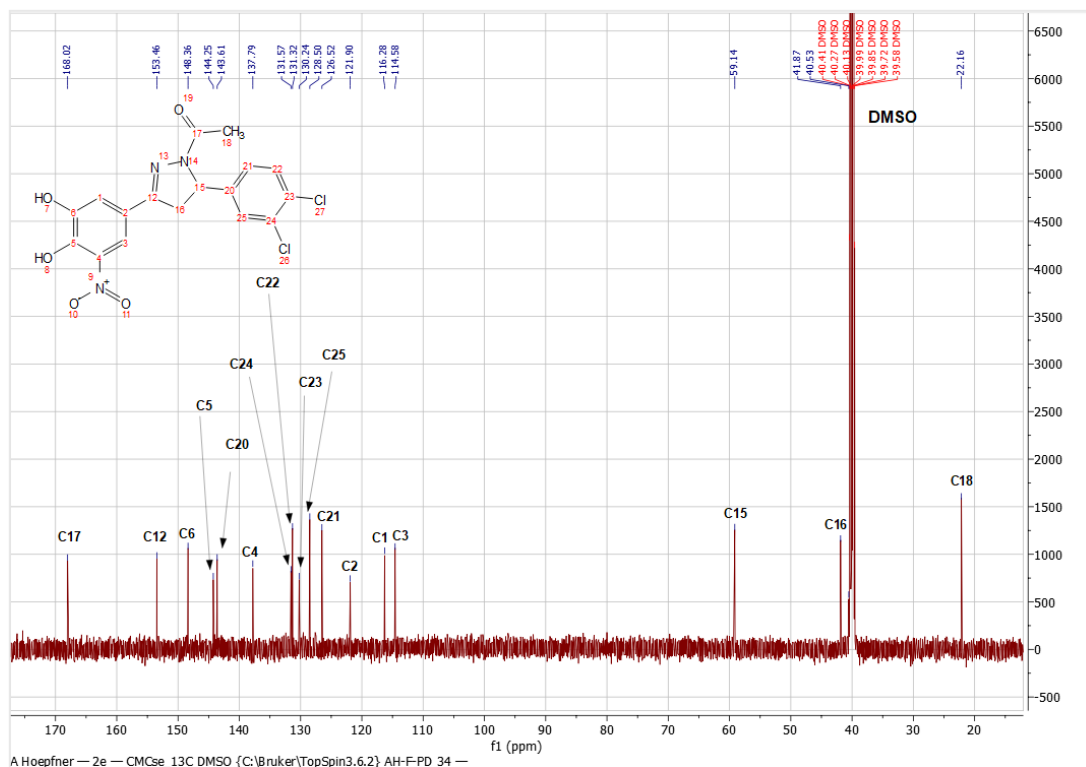


1-[5-(3,4-dichlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4c)

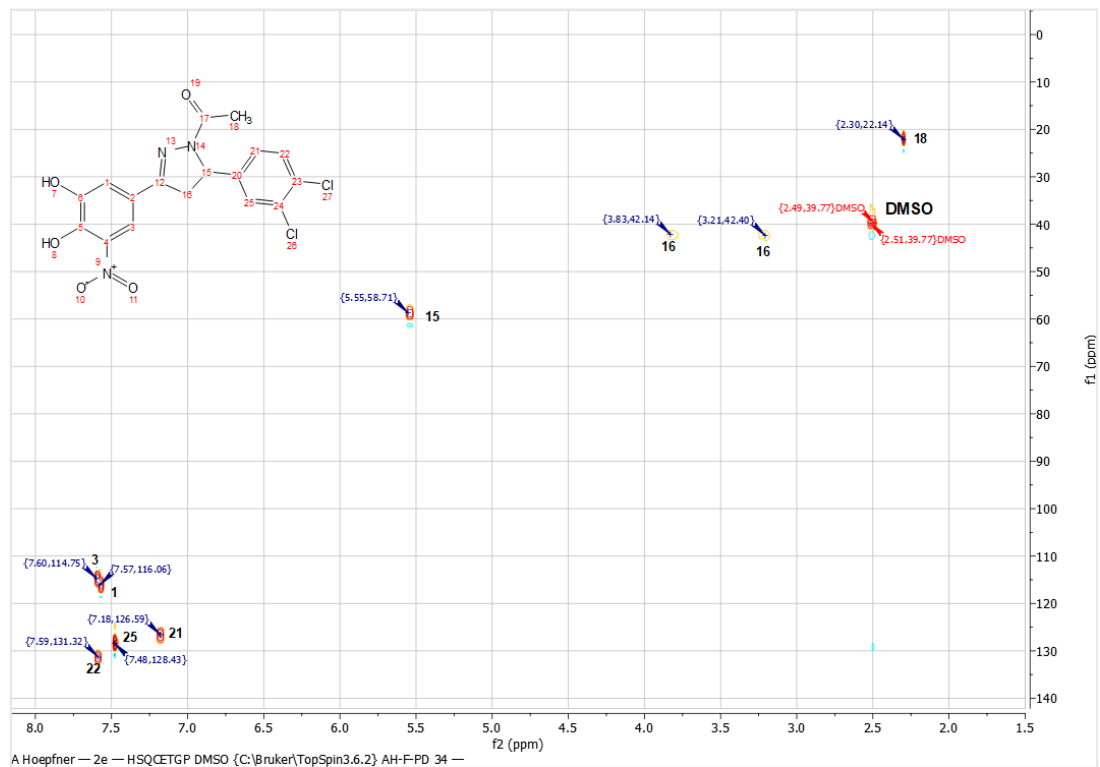
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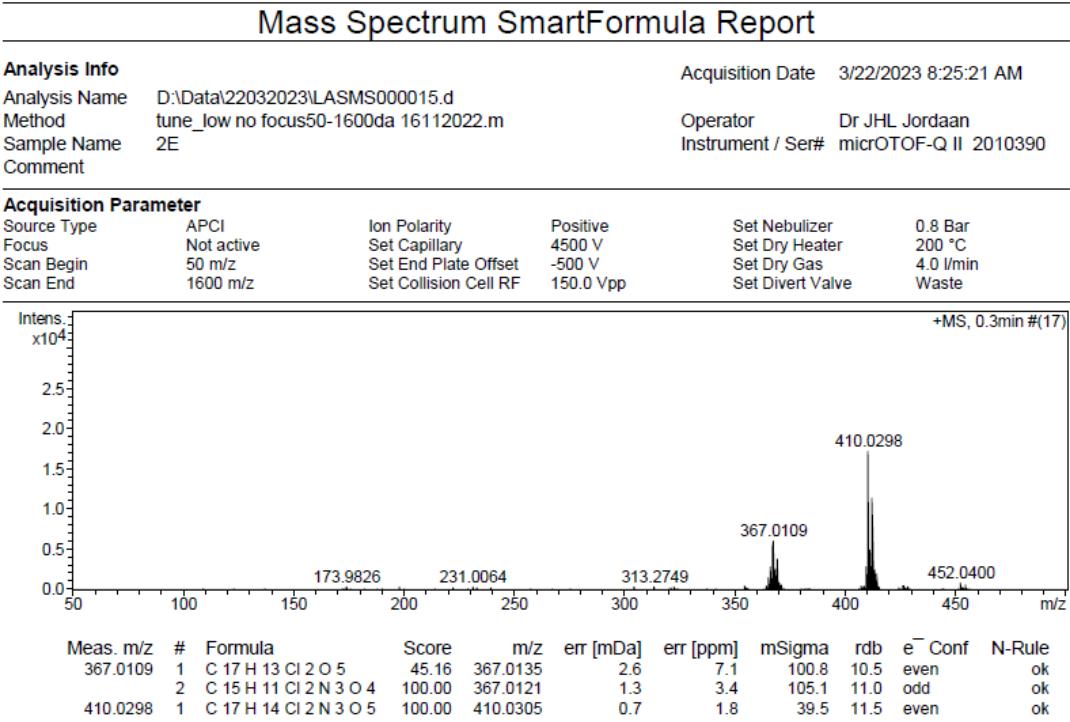
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

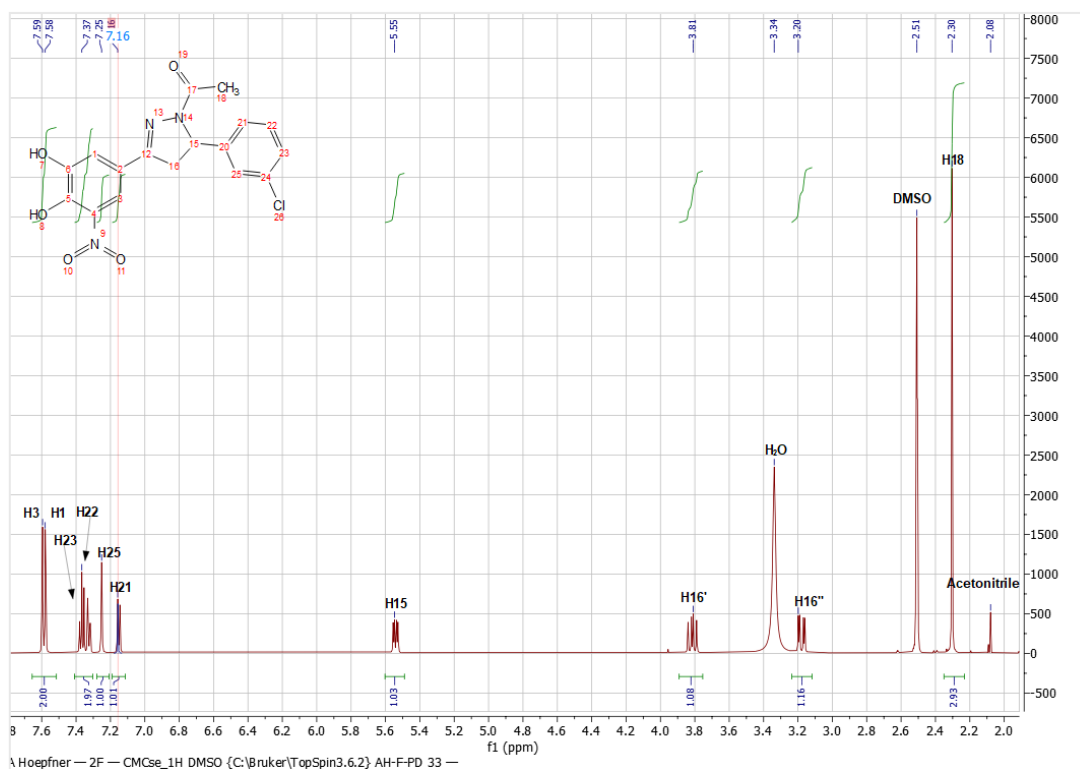


Mass spectrometry (MS) spectrum

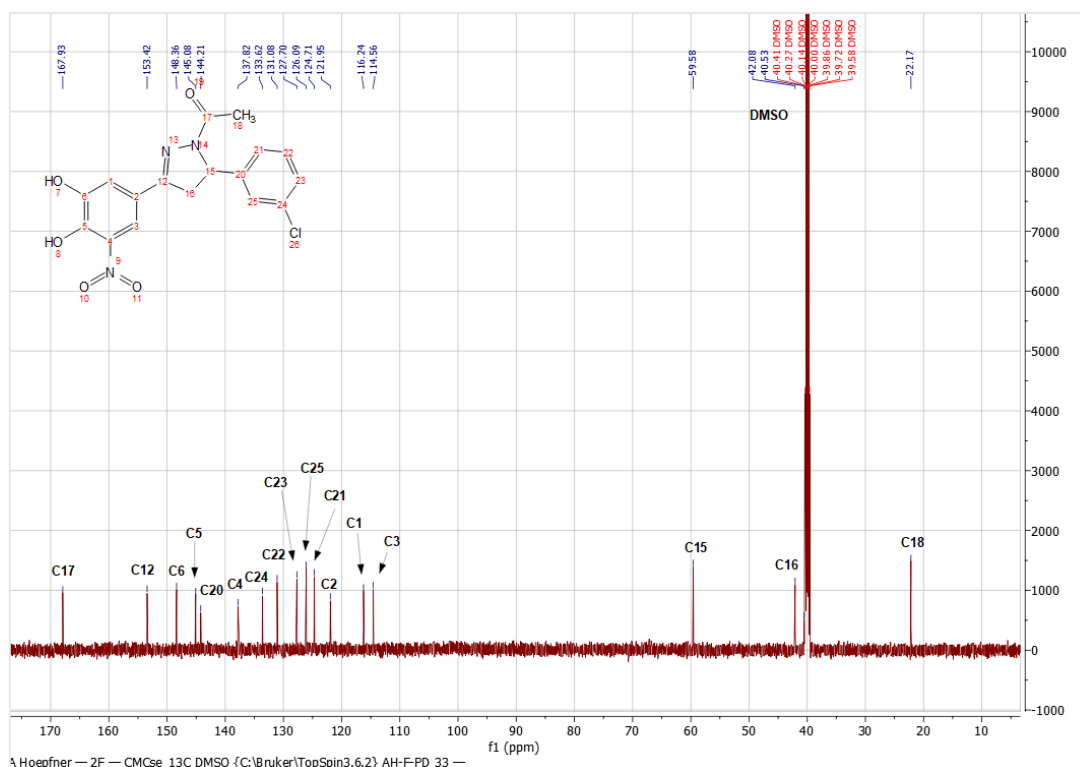


1-[5-(3-chlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4d)

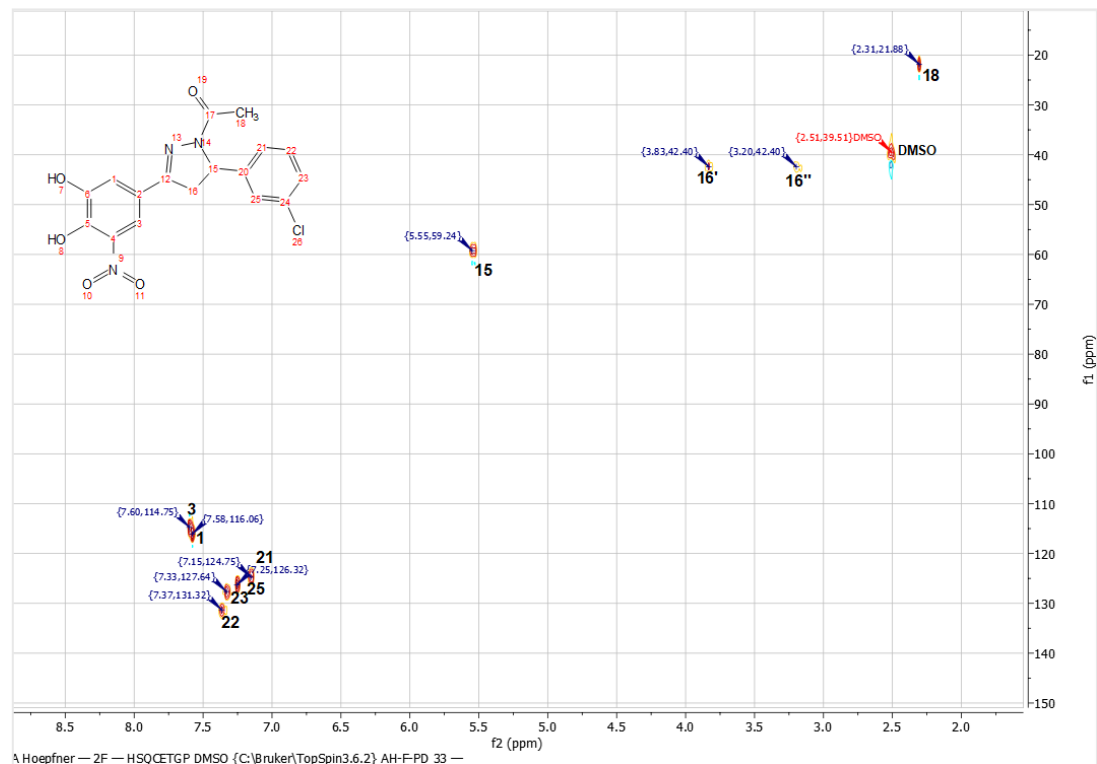
Proton (^1H) NMR spectrum



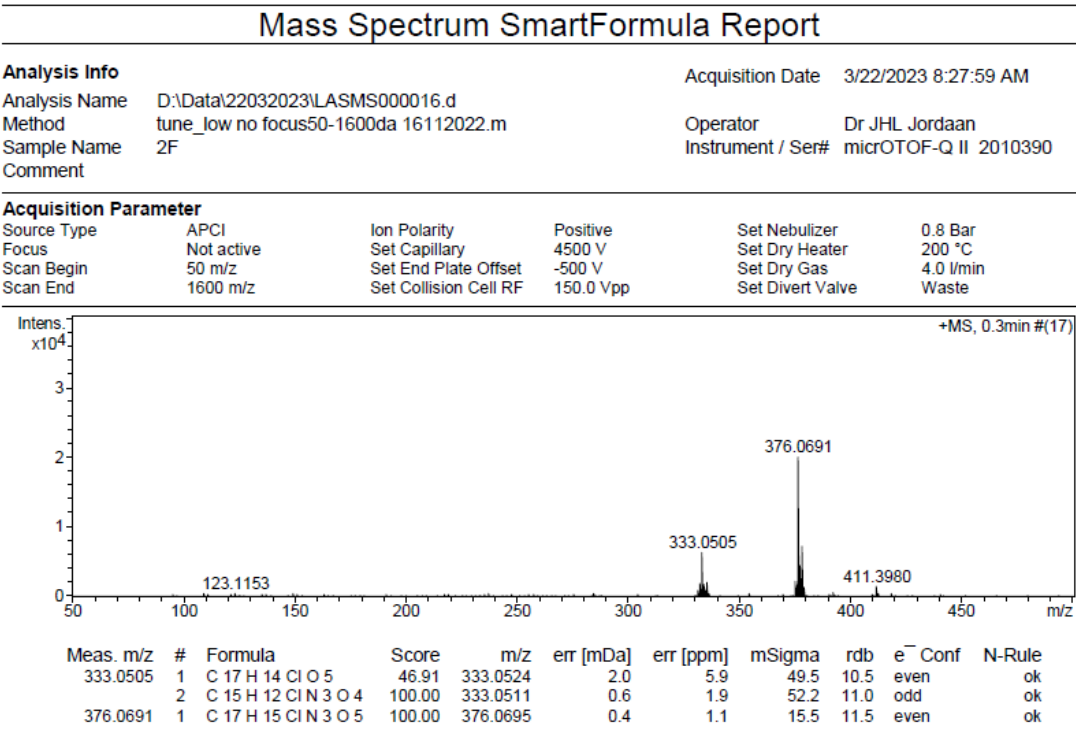
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

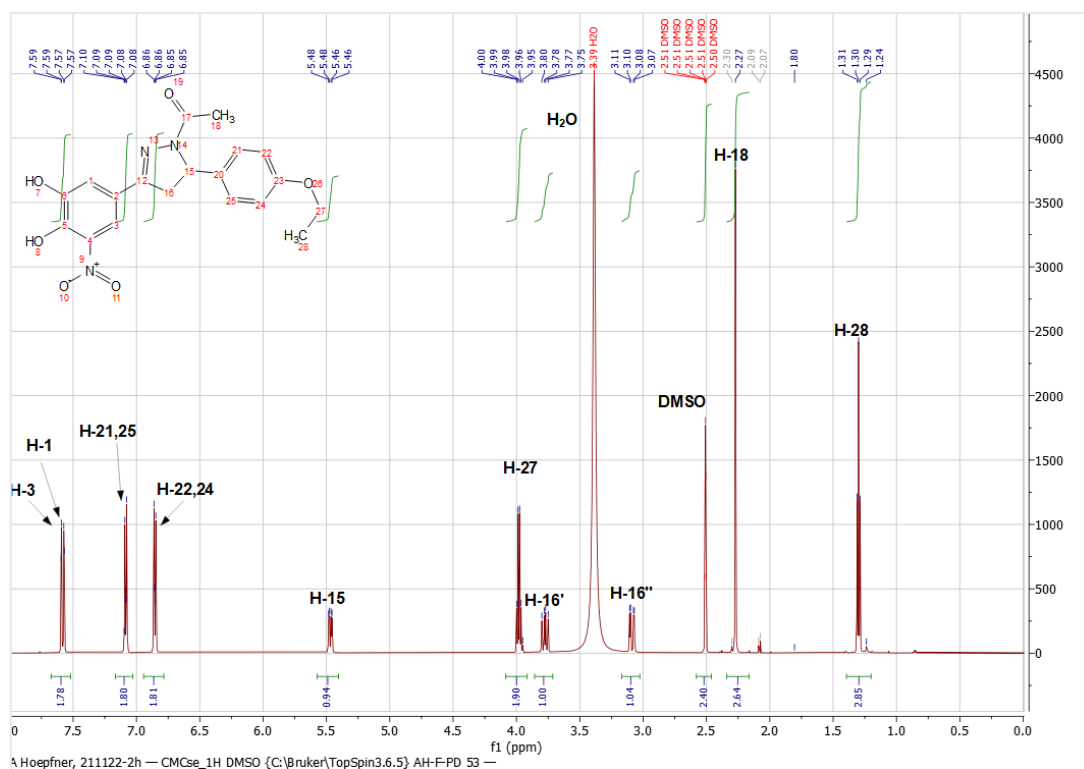


Mass spectrometry (MS) spectrum

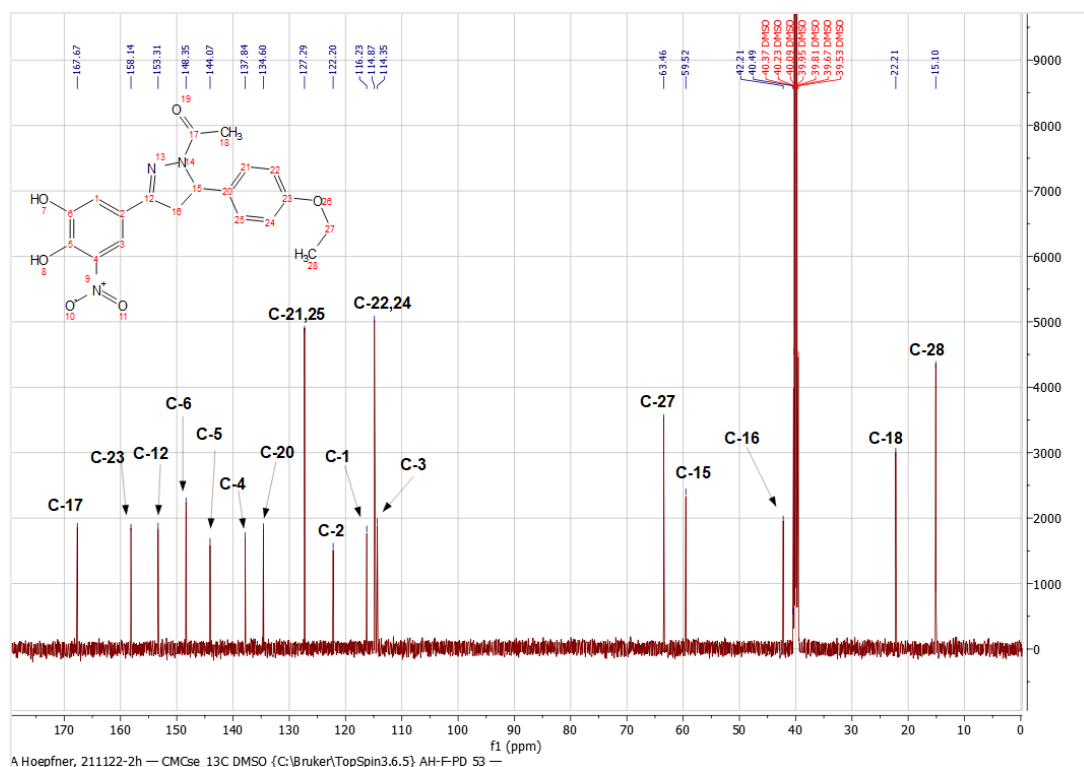


1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-ethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4e)

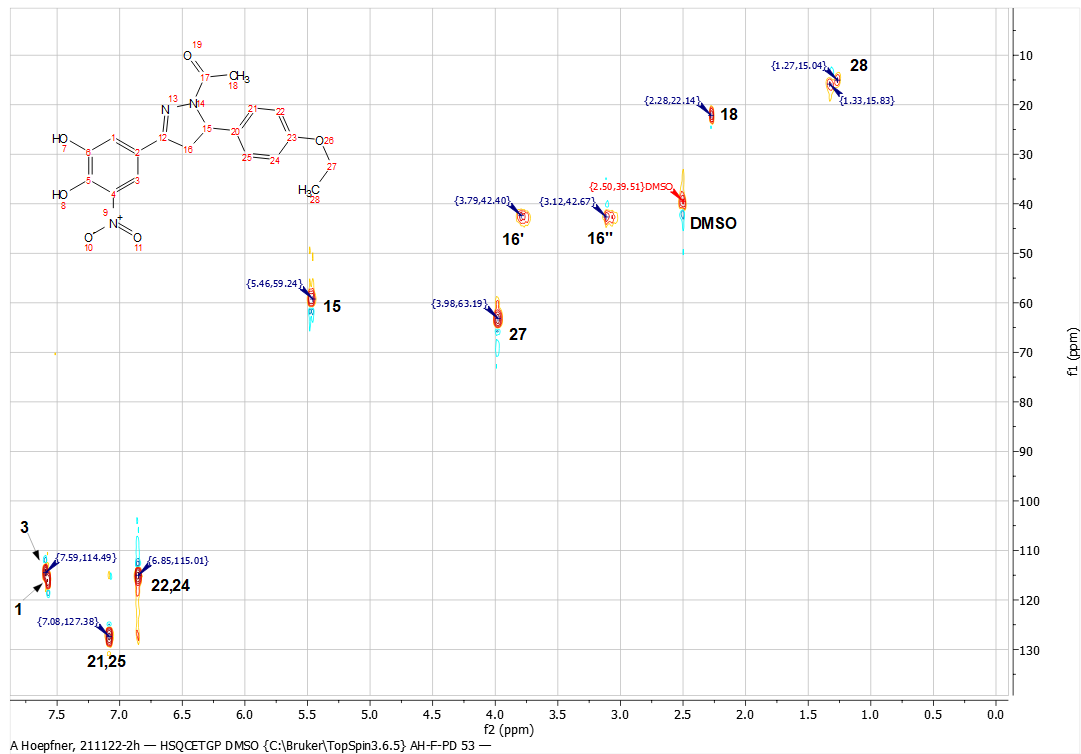
Proton (¹H) NMR spectrum



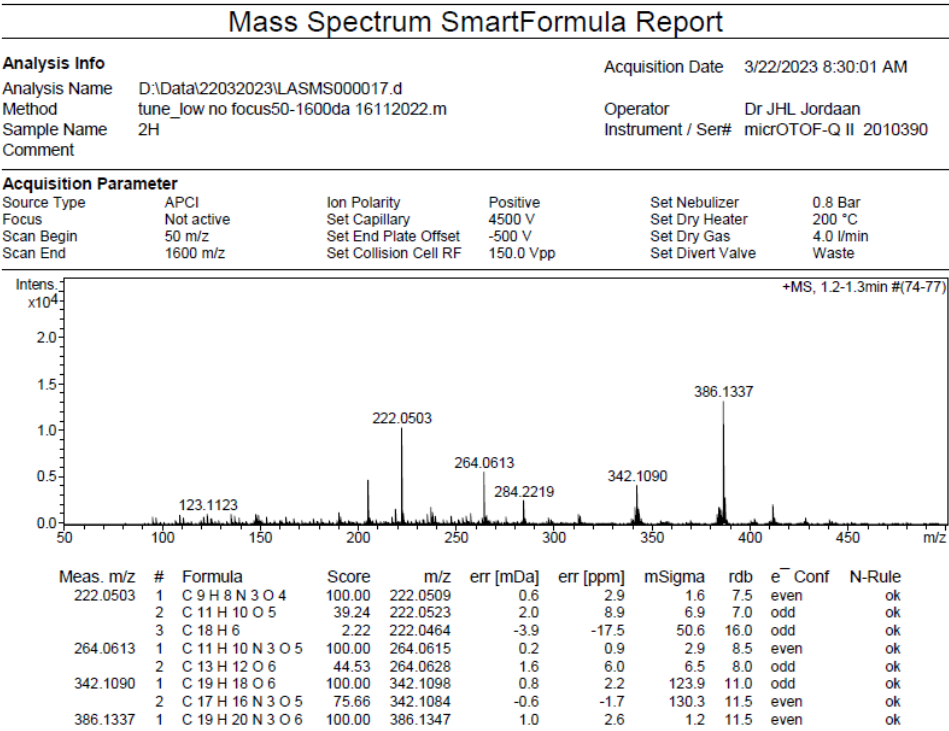
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

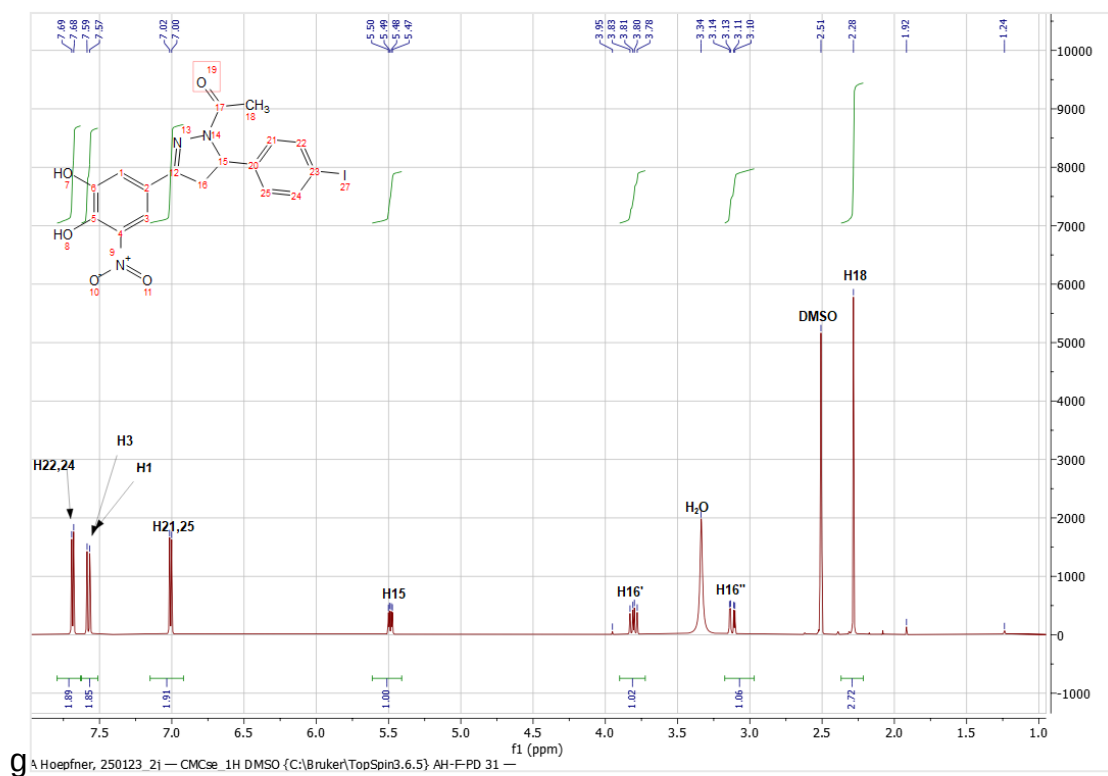


Mass spectrometry (MS) spectrum

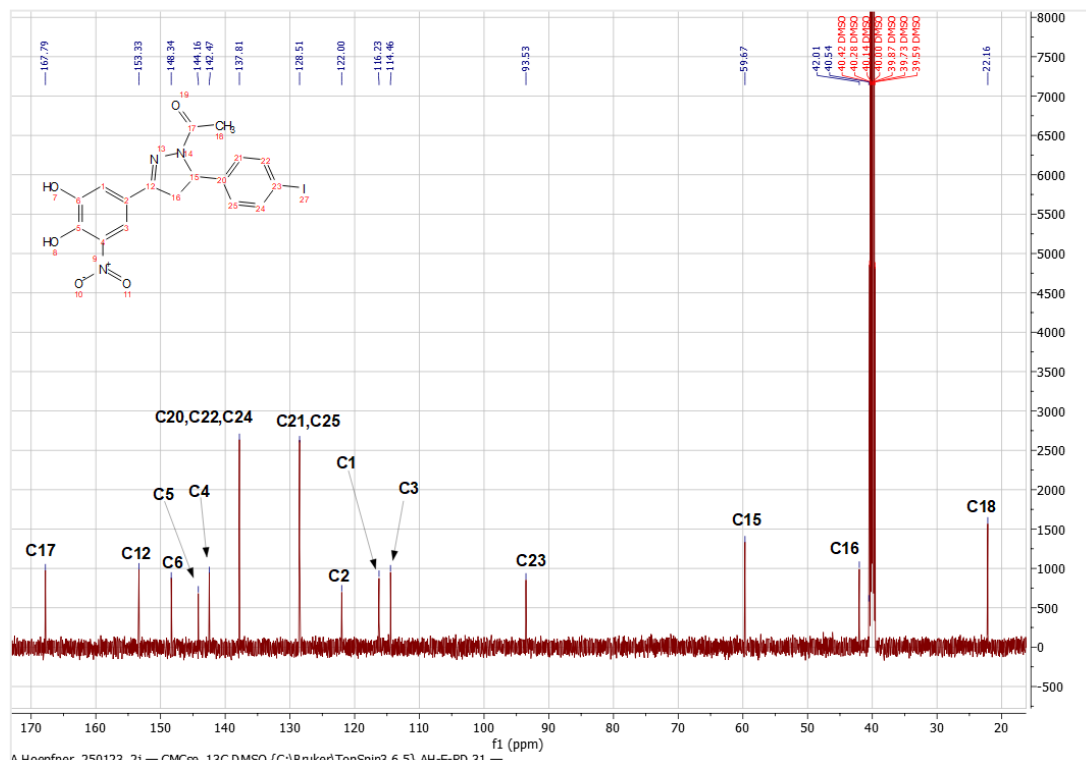


1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-iodophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4f)

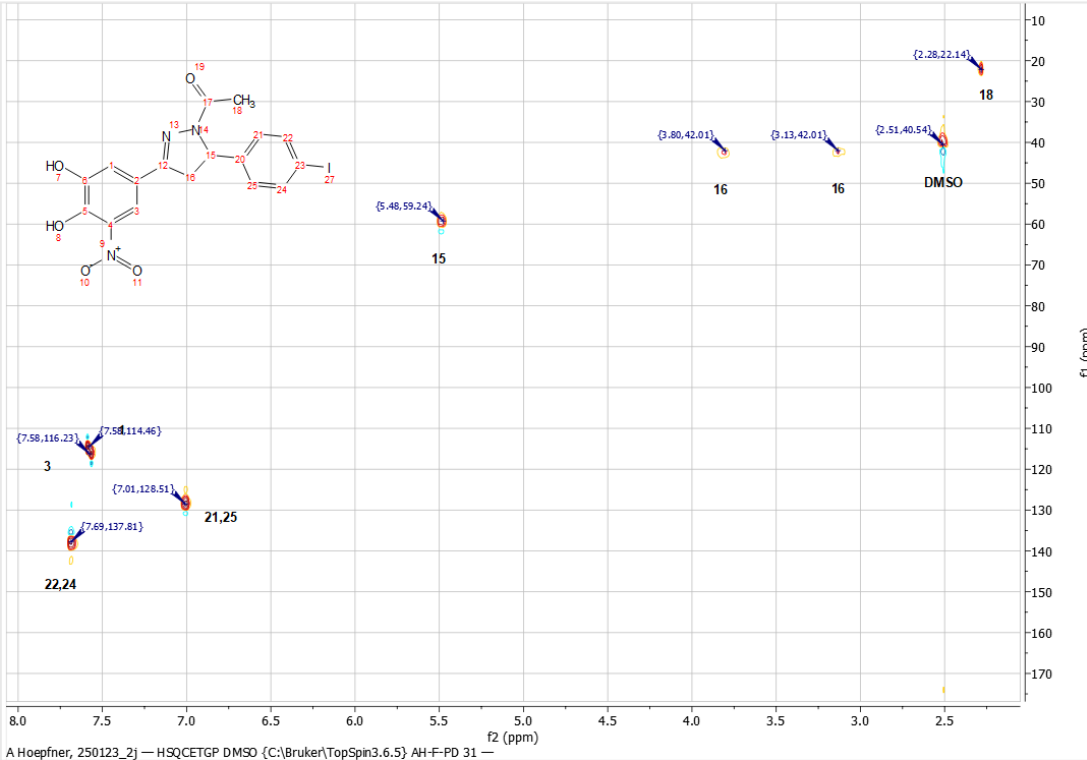
Proton (^1H) NMR spectrum



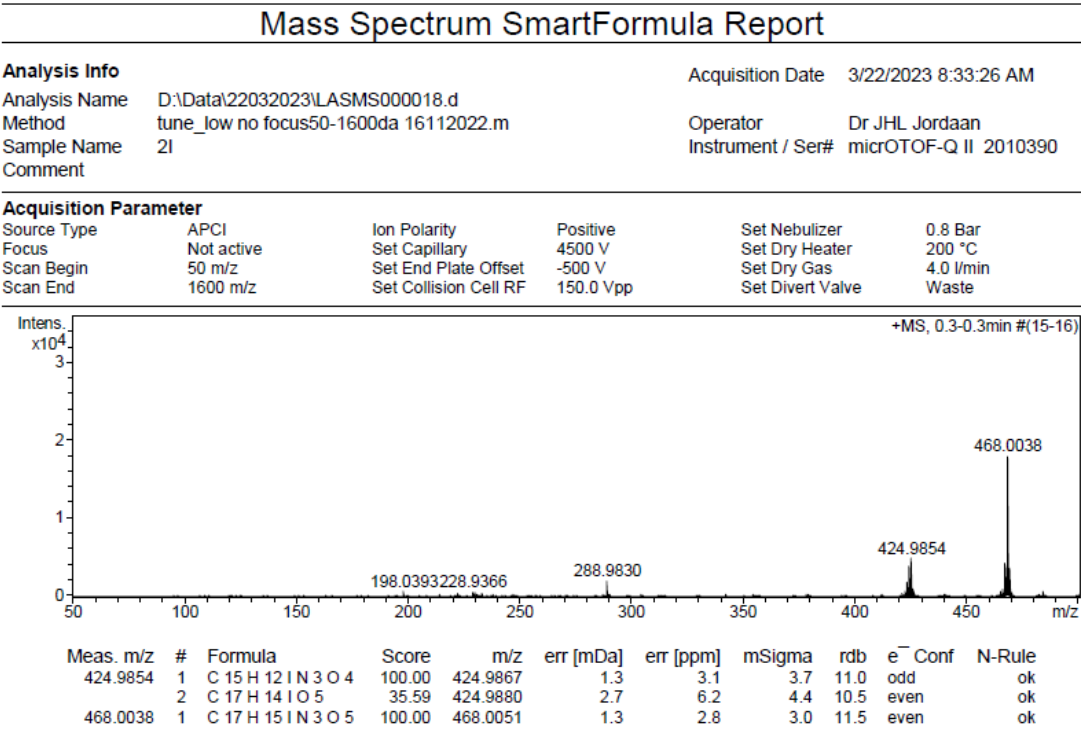
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

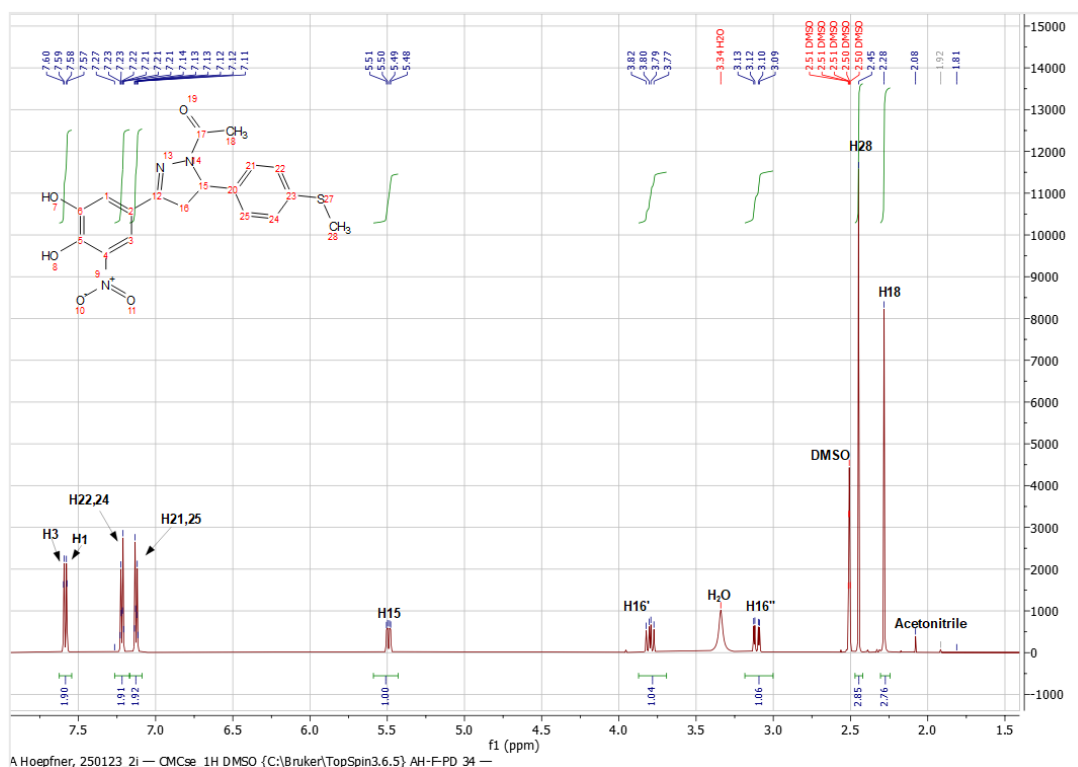


Mass spectrometry (MS) spectrum

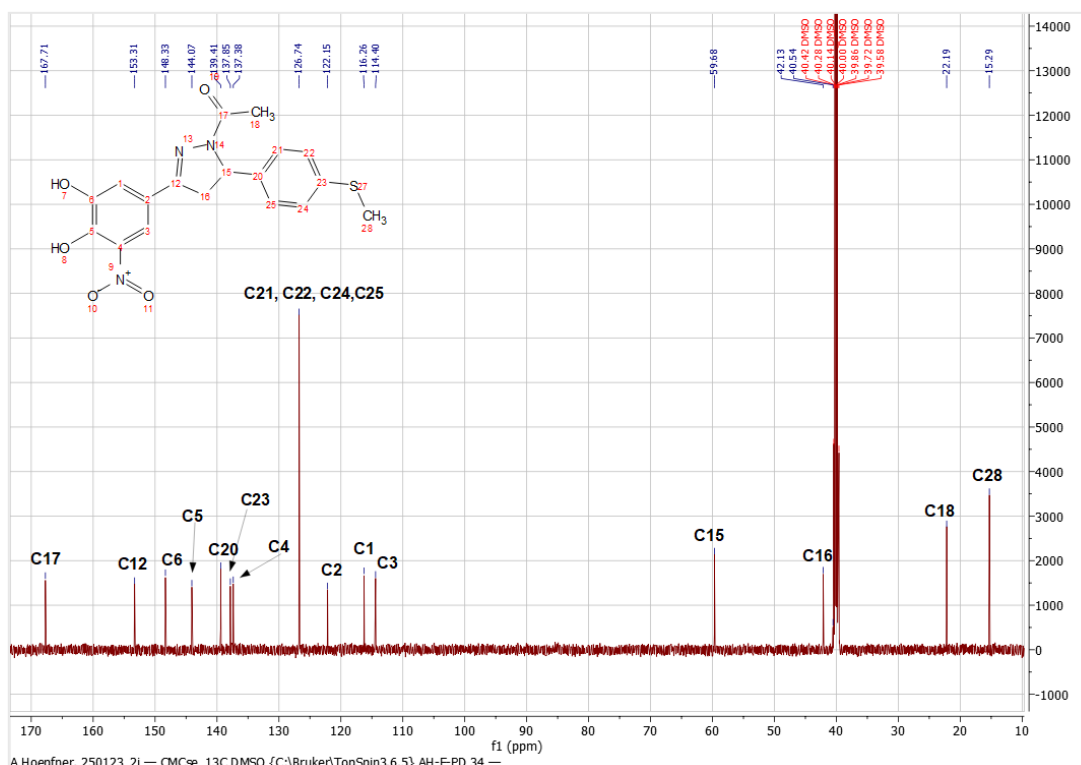


1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-[4-(methylsulfonyl)phenyl]-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4g)

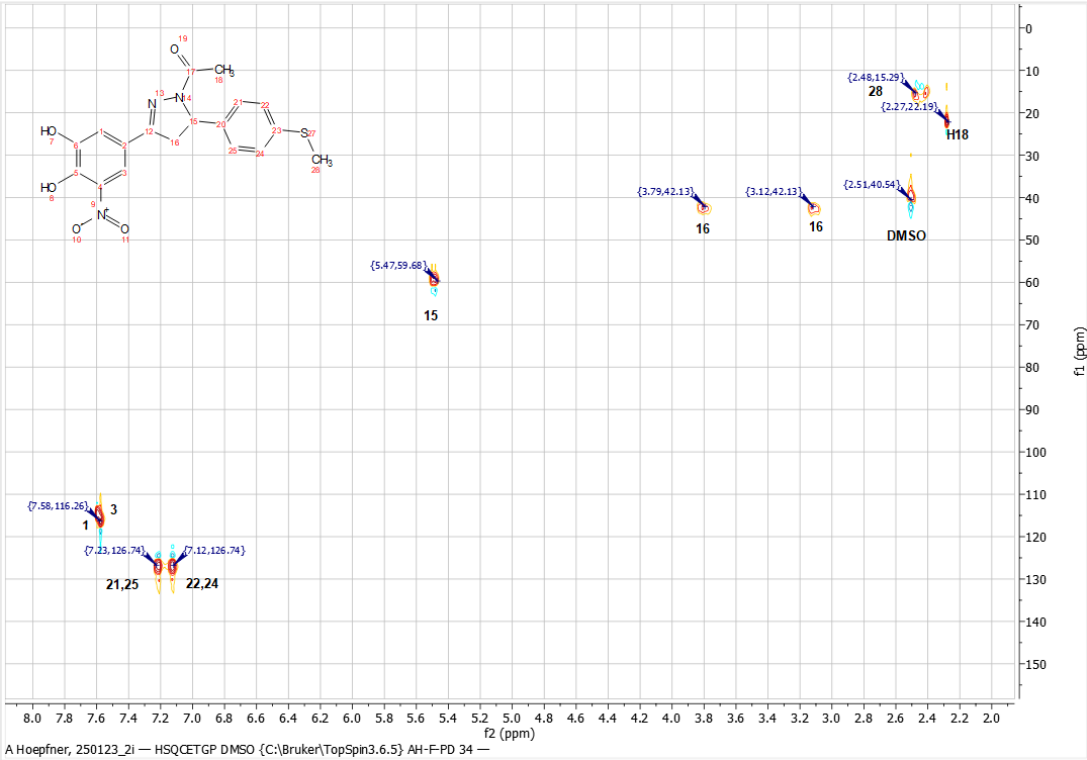
Proton (^1H) NMR spectrum



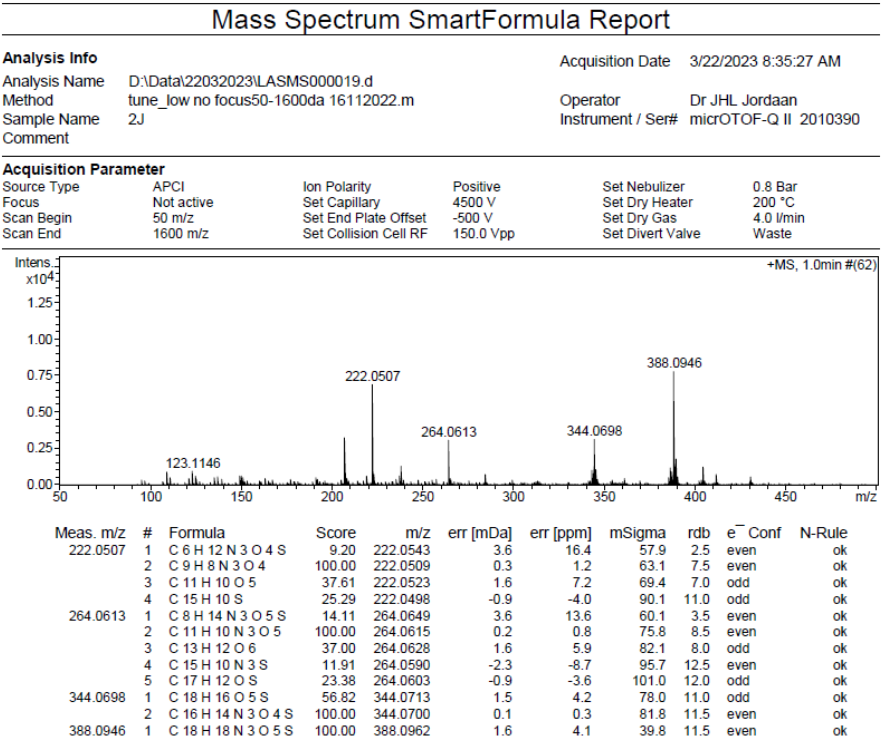
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

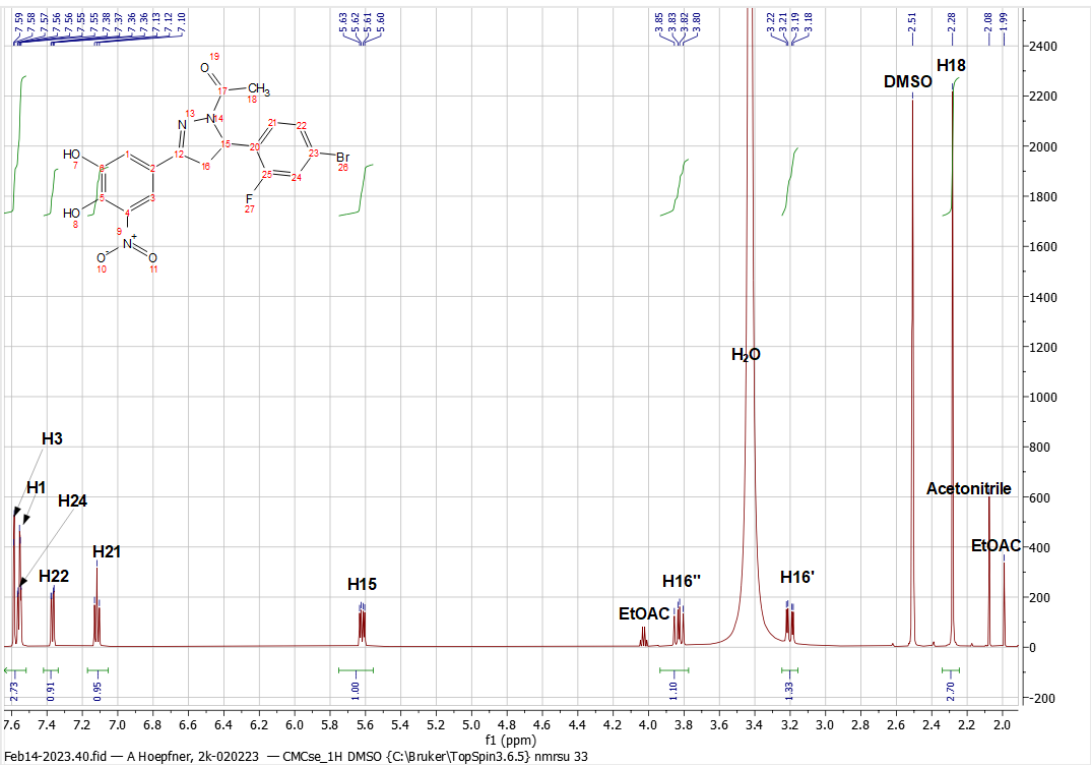


Mass spectrometry (MS) spectrum

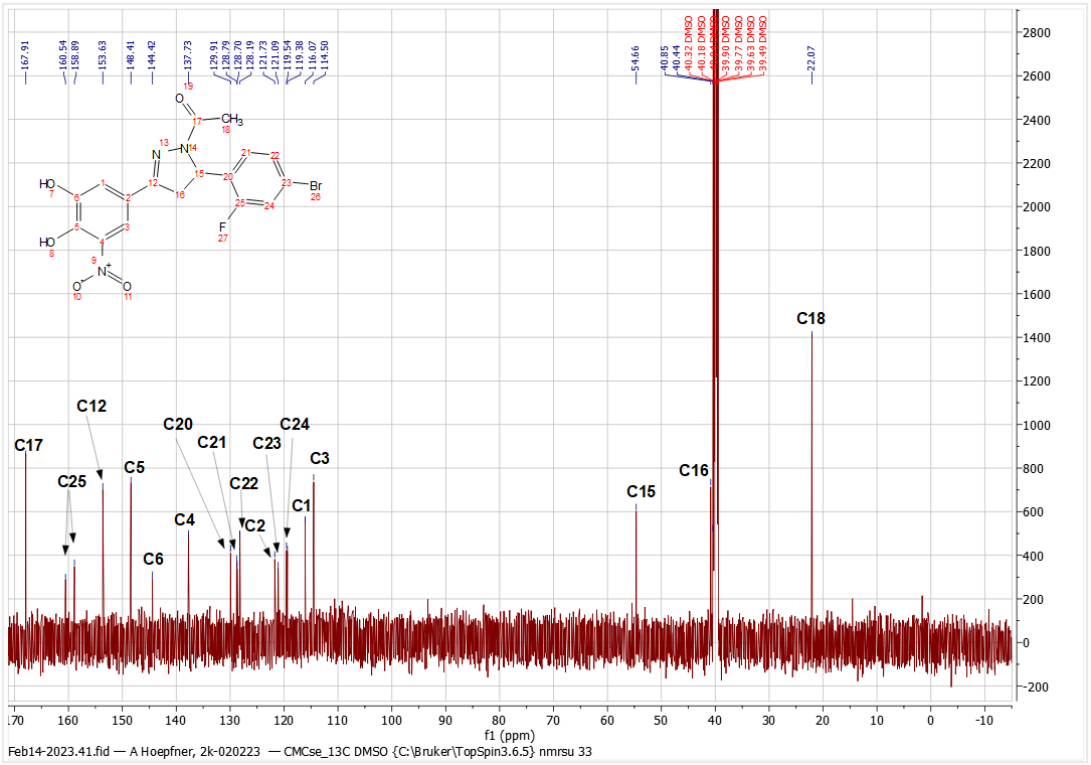


1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-bromo-2-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4h)

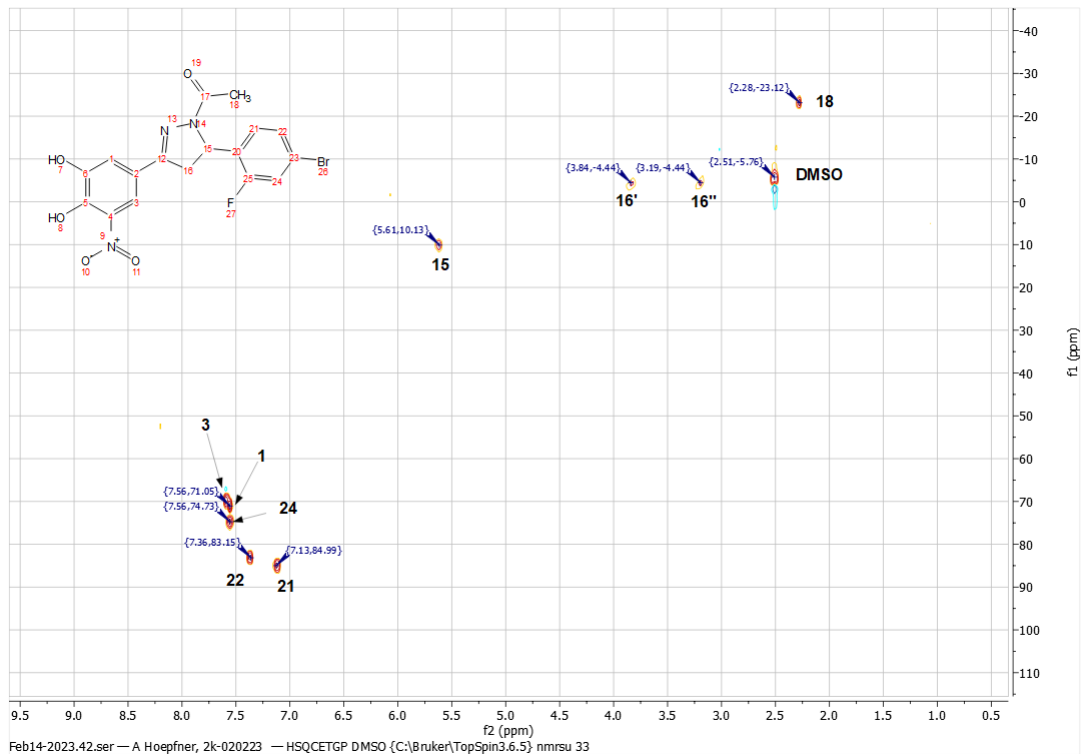
Proton (¹H) NMR spectrum



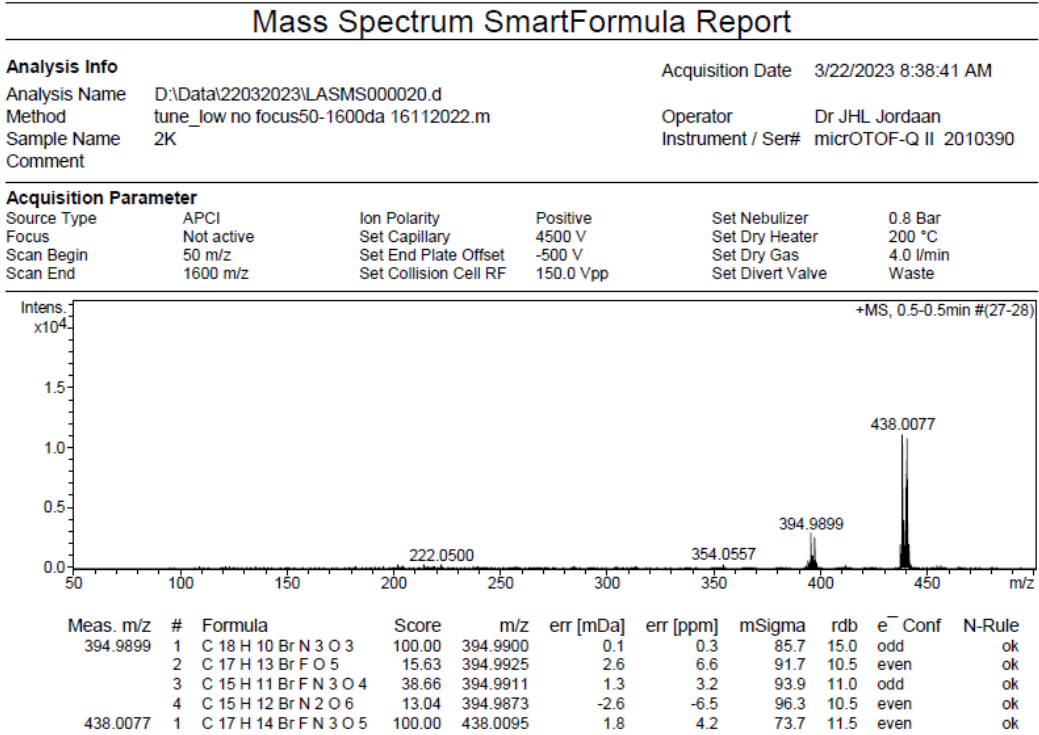
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

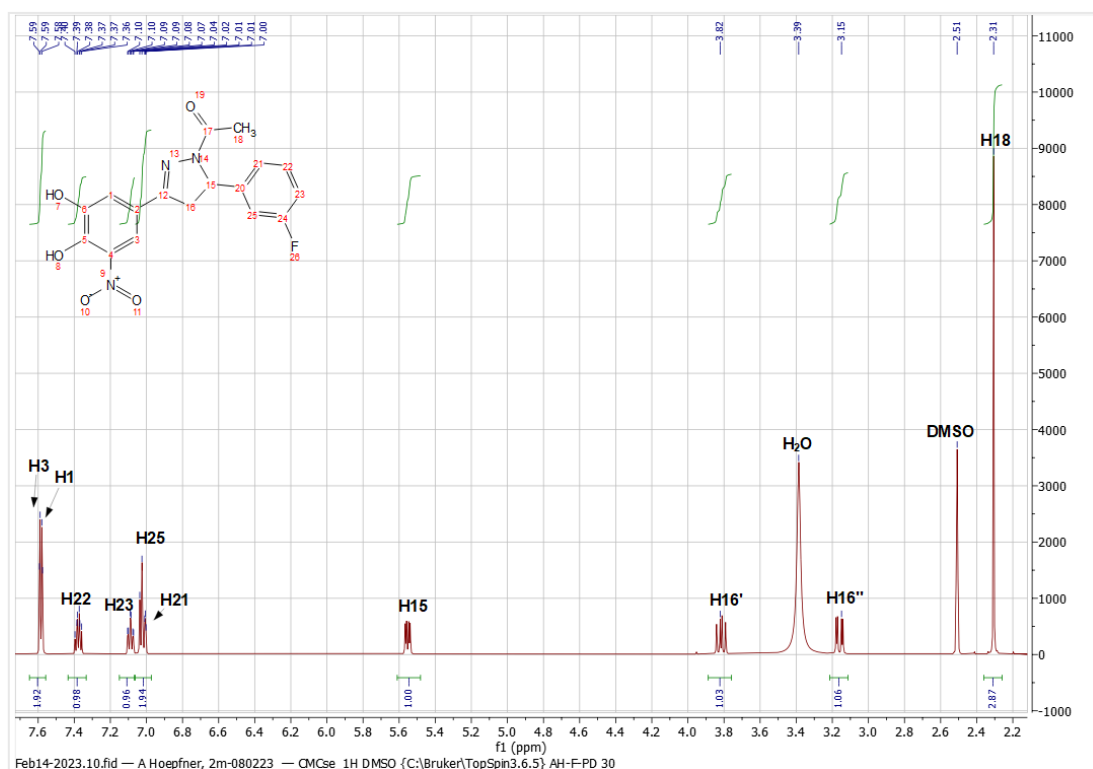


Mass spectrometry (MS) spectrum

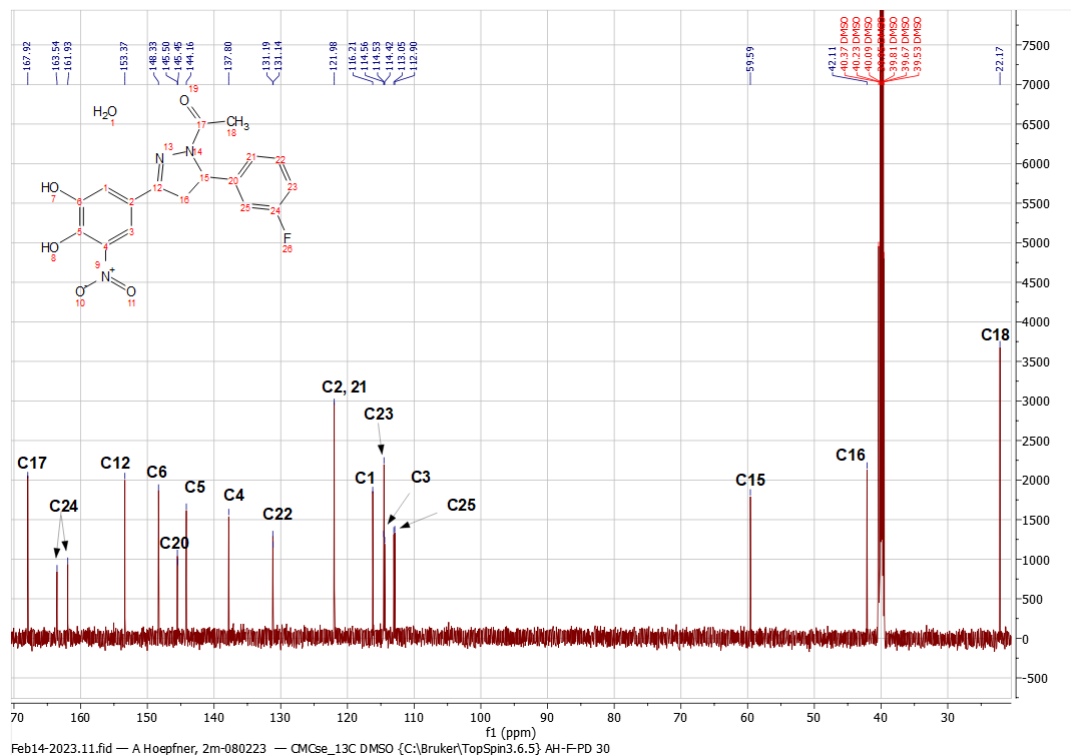


1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(3-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4i)

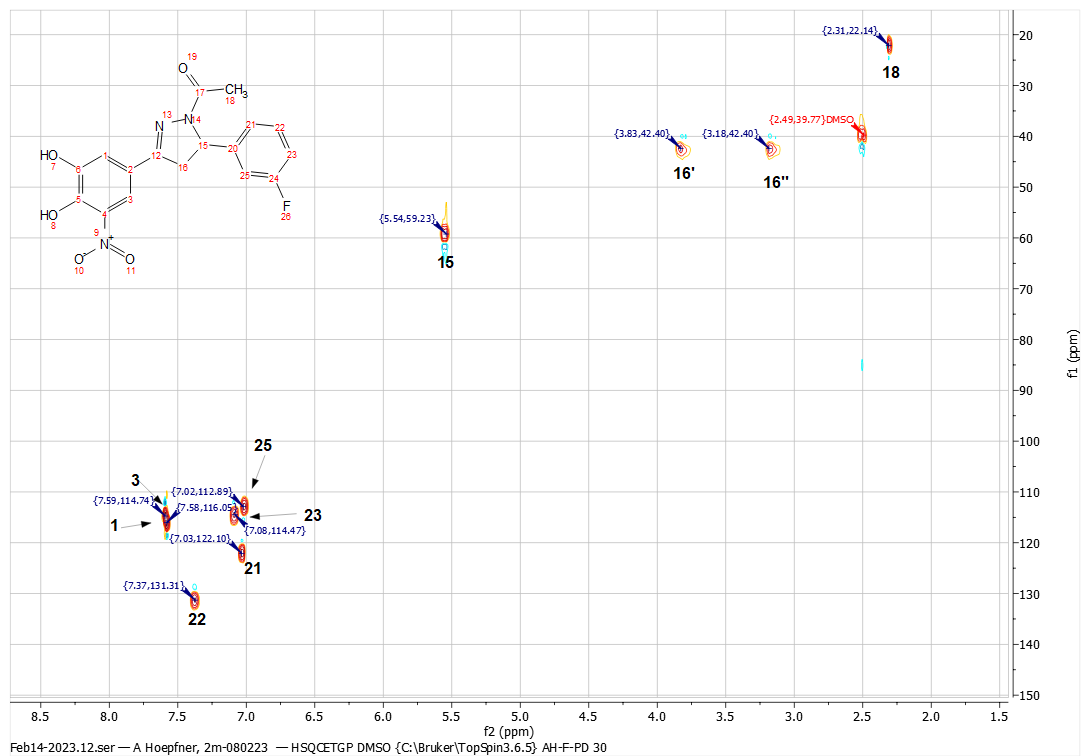
Proton (^1H) NMR spectrum



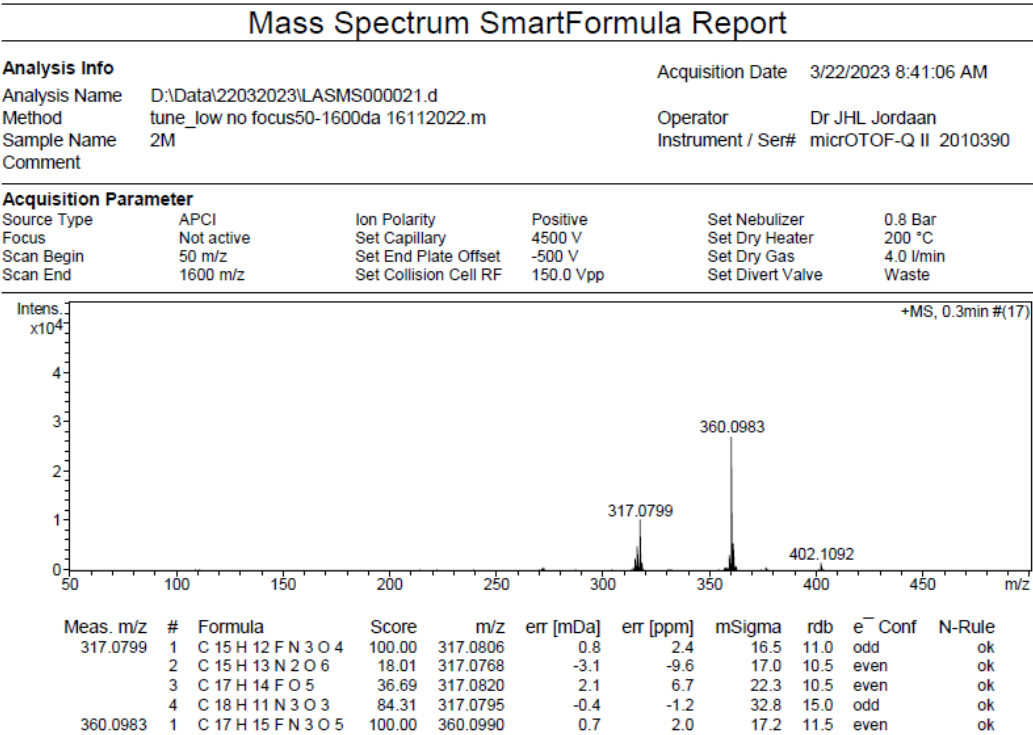
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

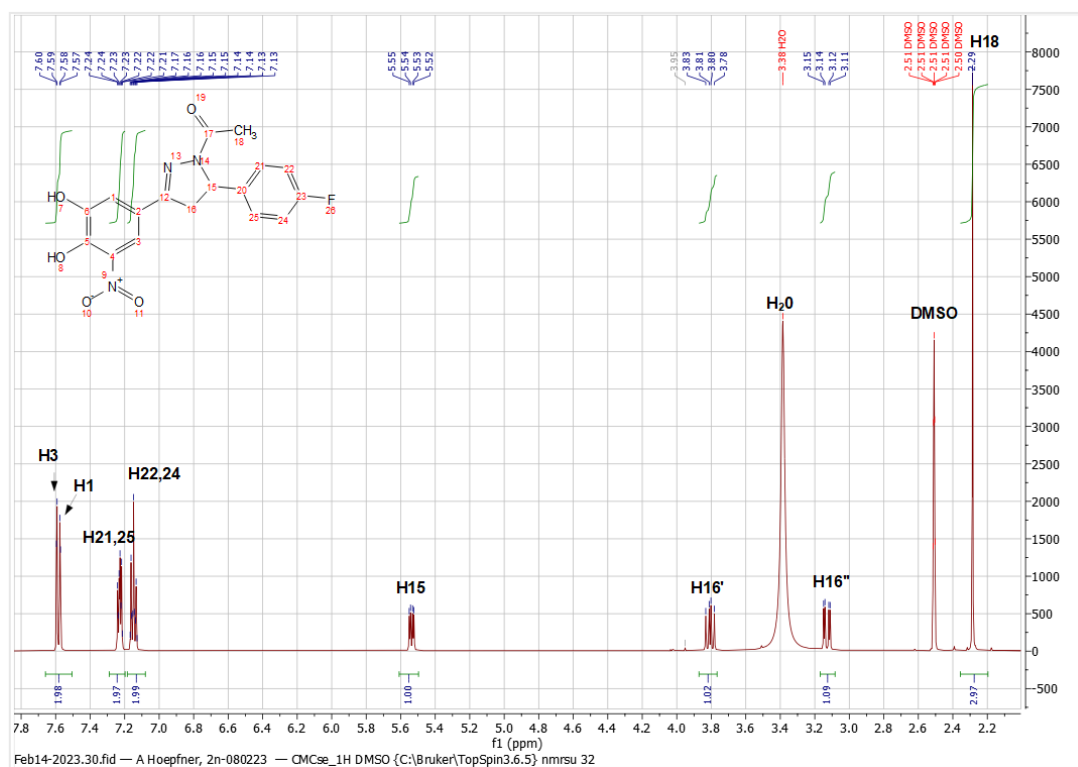


Mass spectrometry (MS) spectrum

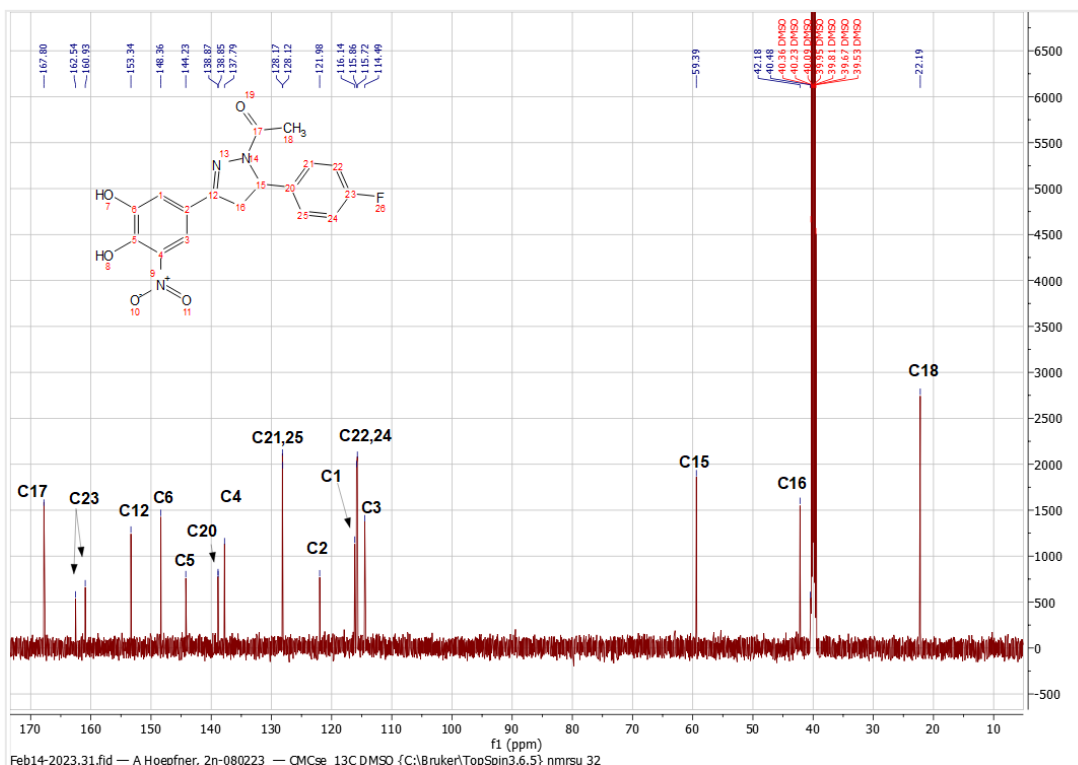


1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4j)

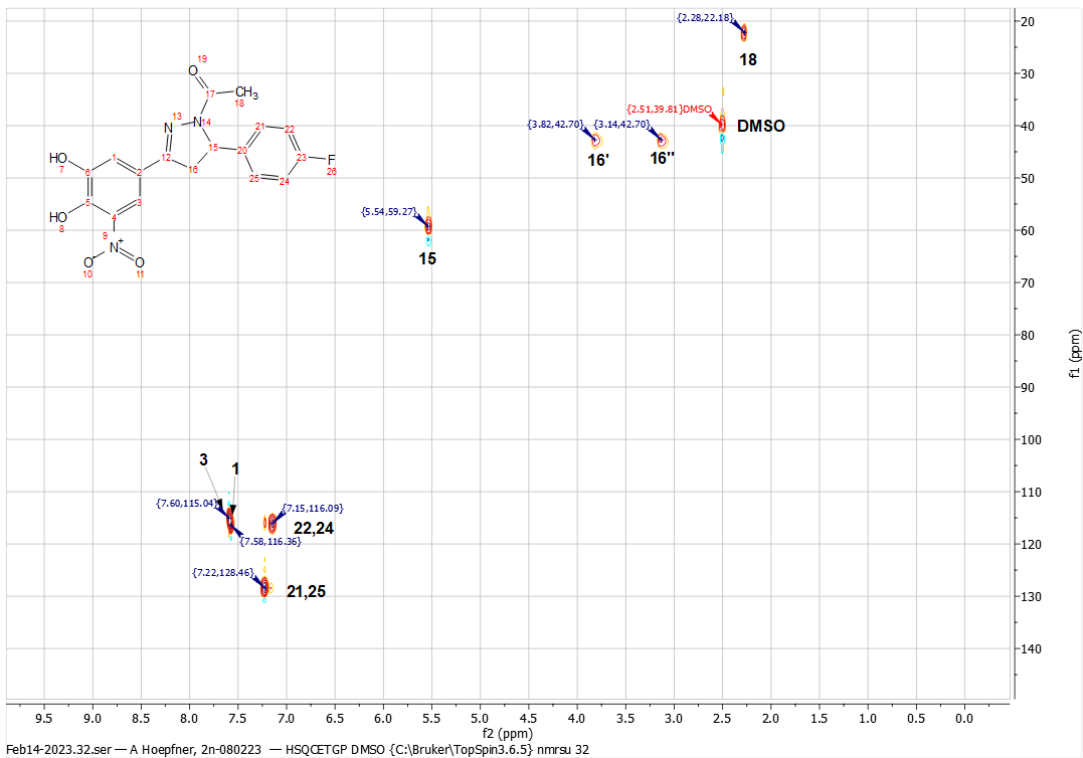
Proton (^1H) NMR spectrum



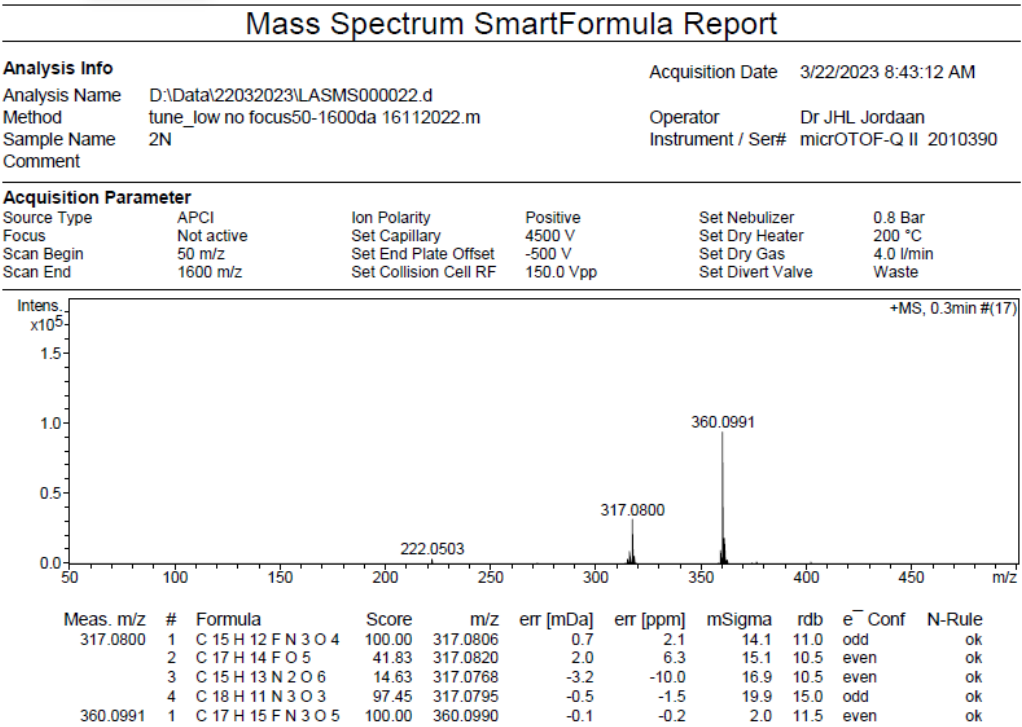
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum



Mass spectrometry (MS) spectrum

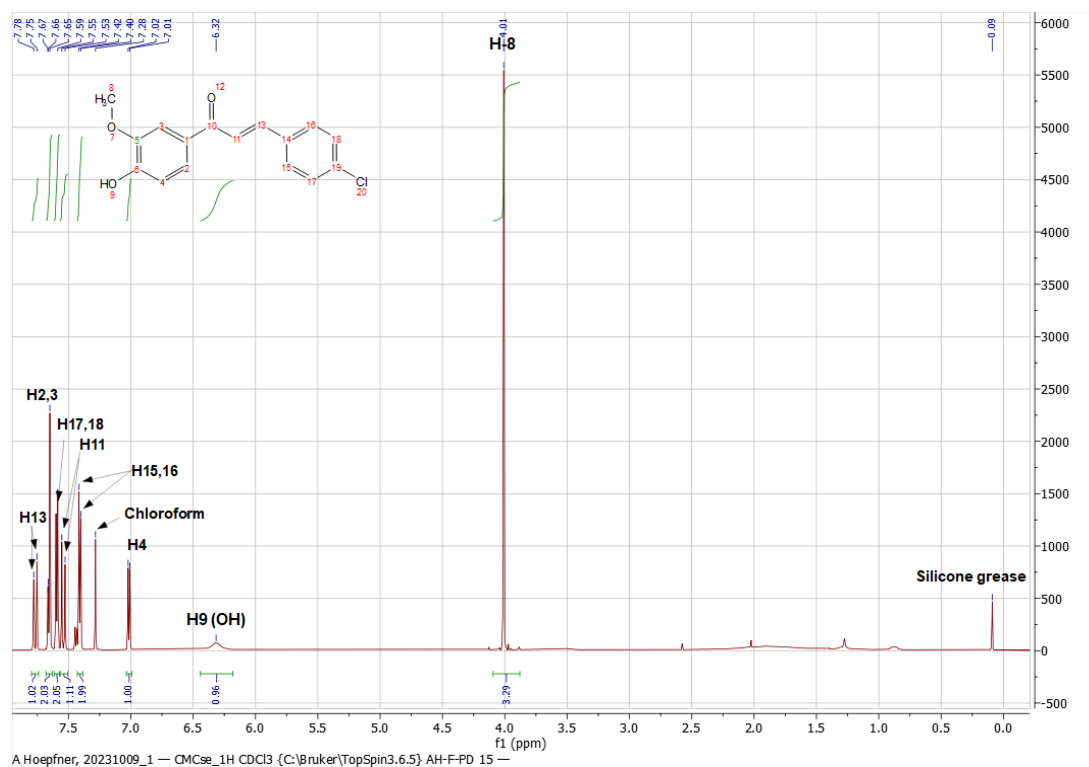


ANNEXURE B

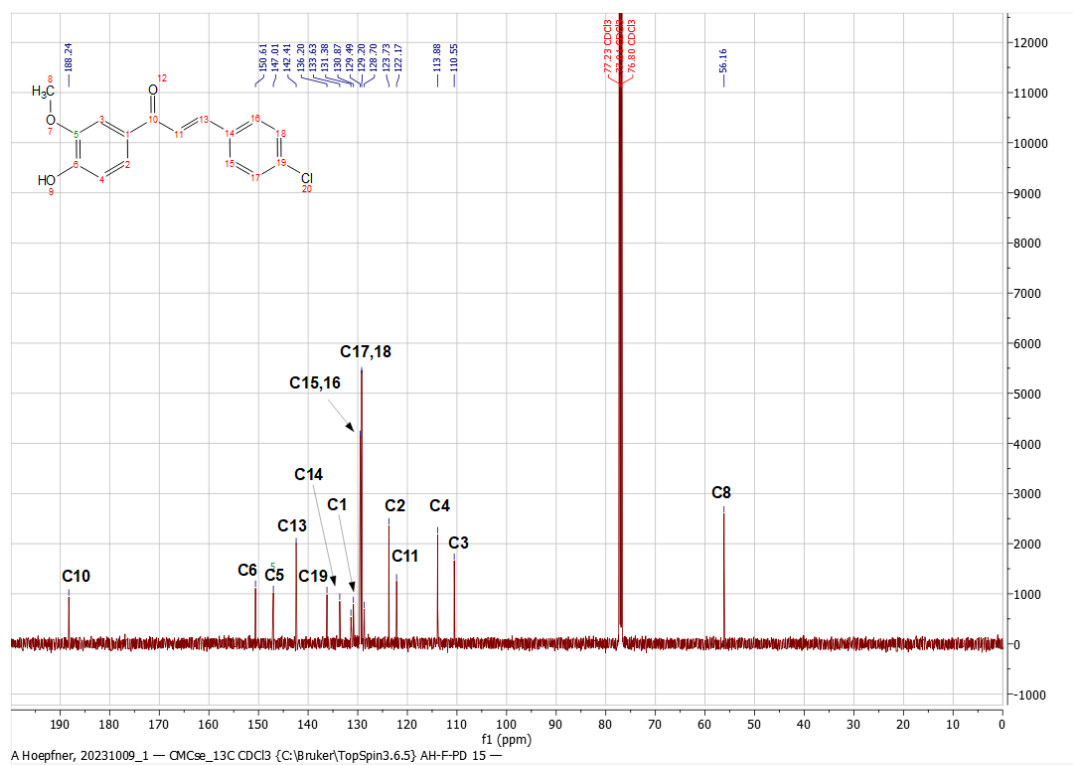
Article 2: Nuclear Magnetic Resonance (^1H , C^{13} and HSQC) and Mass spectrometry (MS) spectra of compounds

(2E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1a)

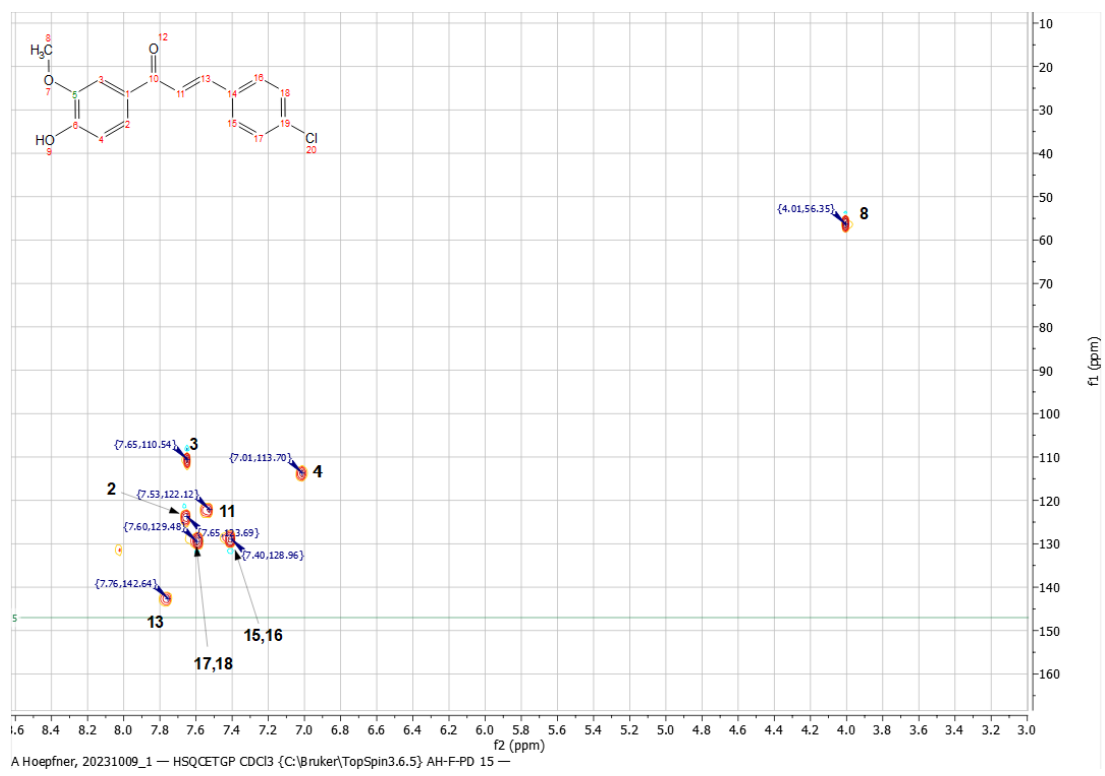
Proton (^1H) NMR spectrum



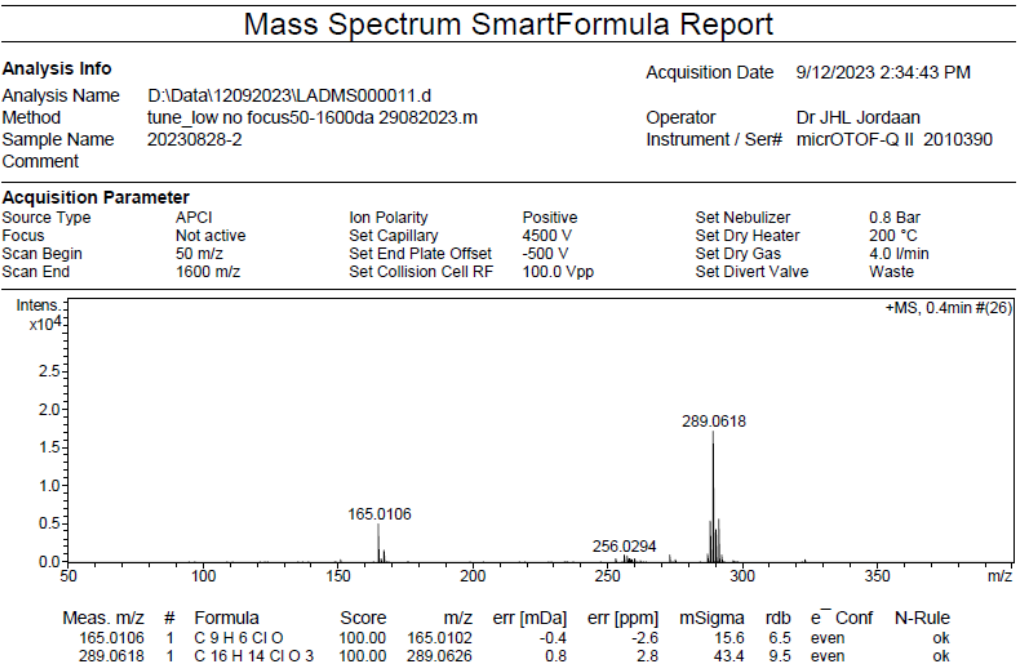
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

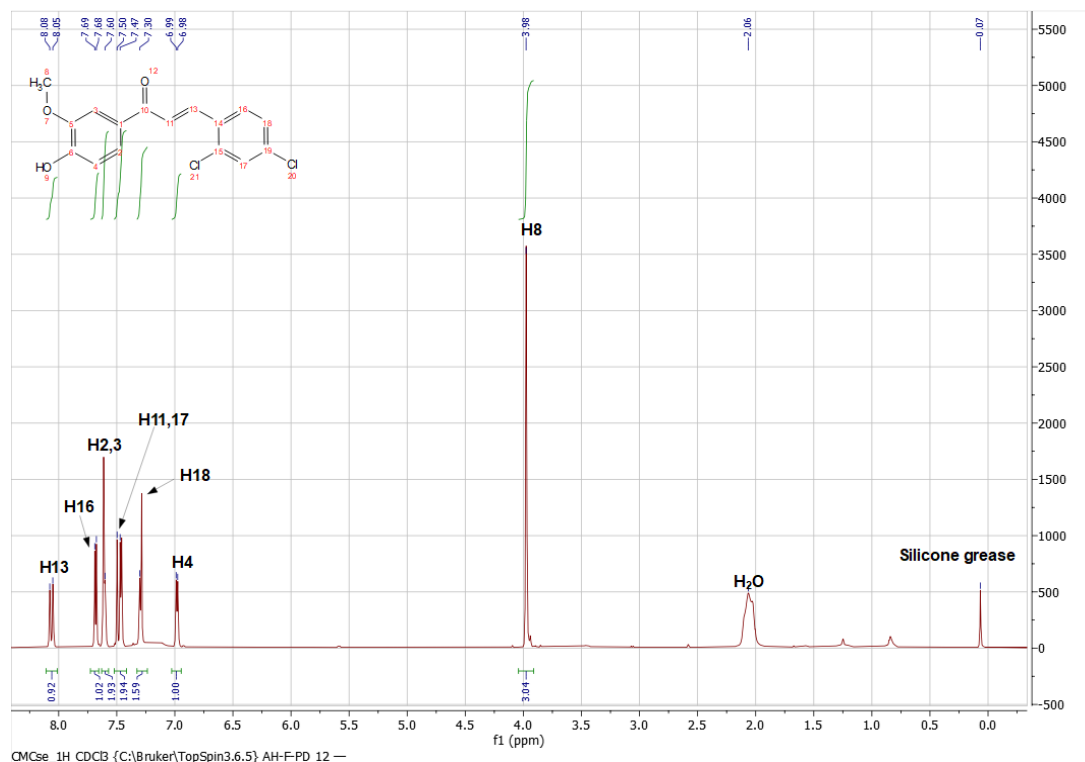


Mass spectrometry (MS) spectrum

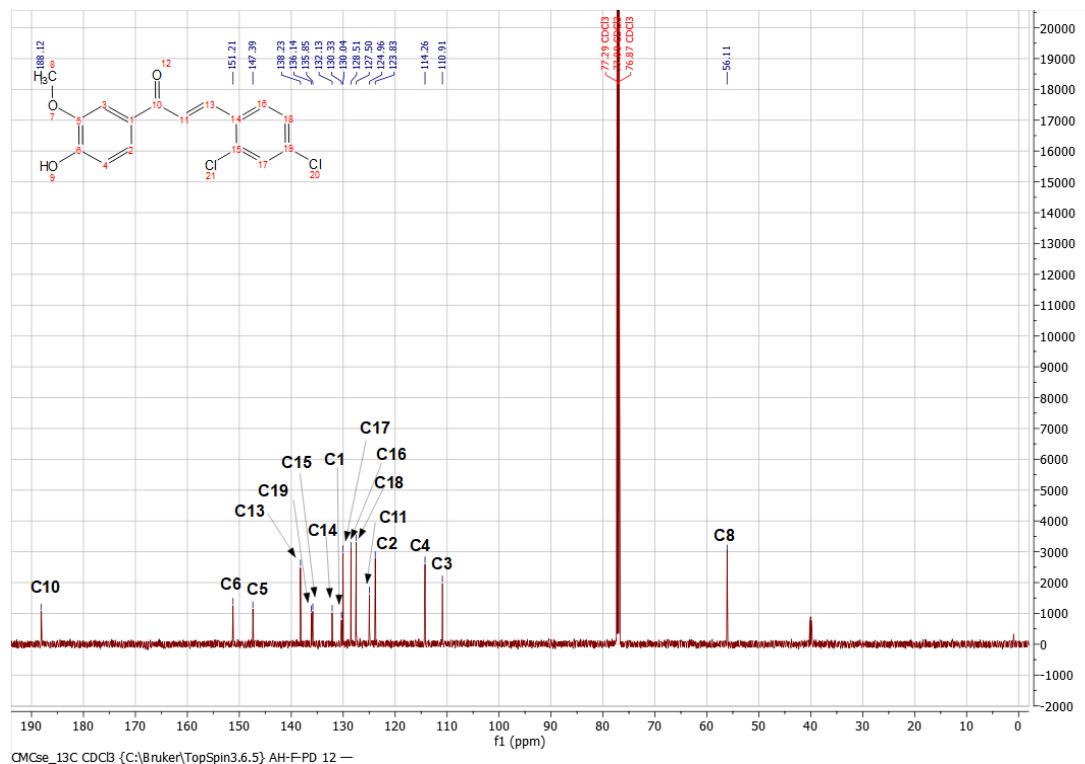


(2E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1b)

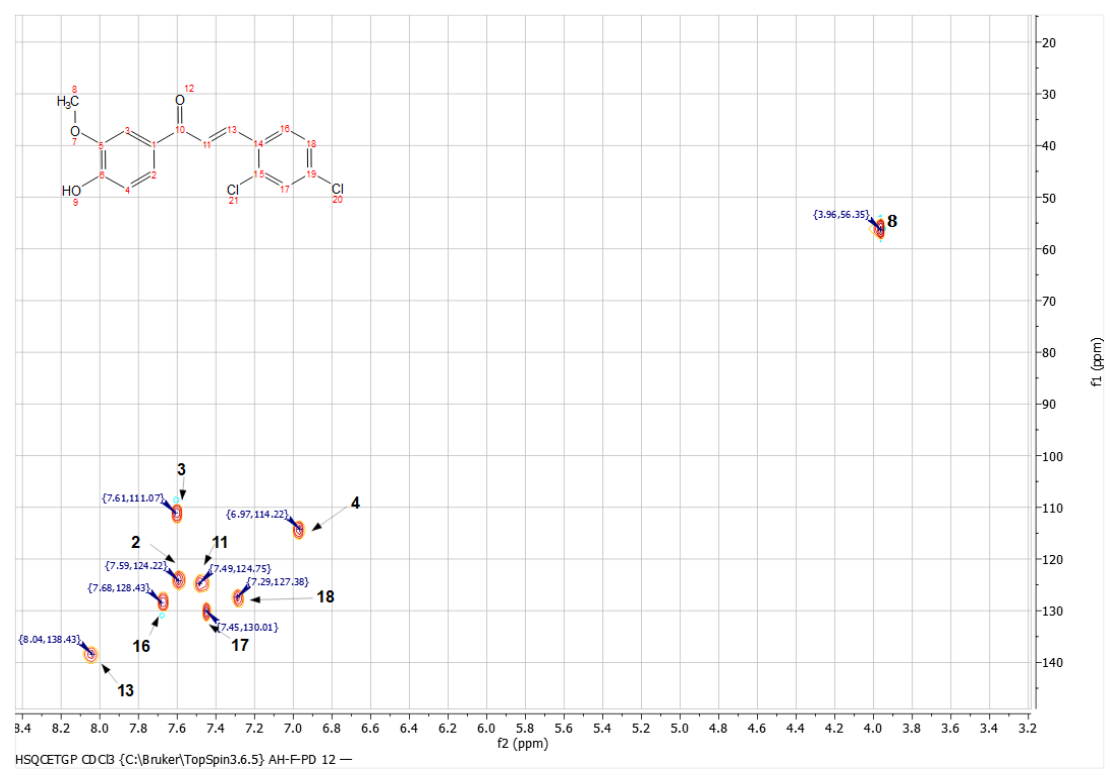
Proton (^1H) NMR spectrum



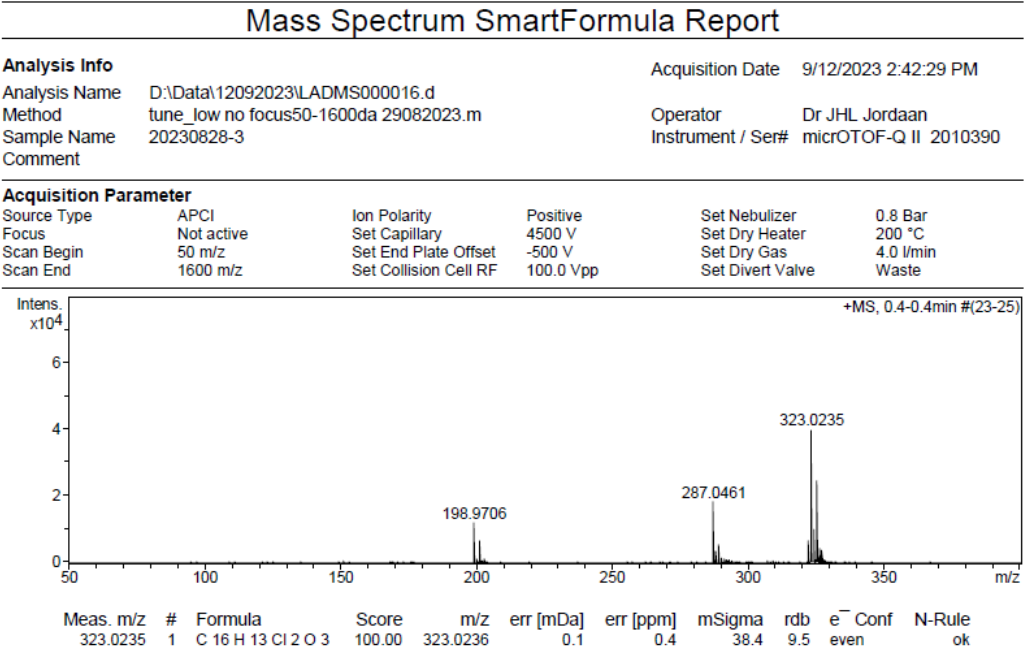
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

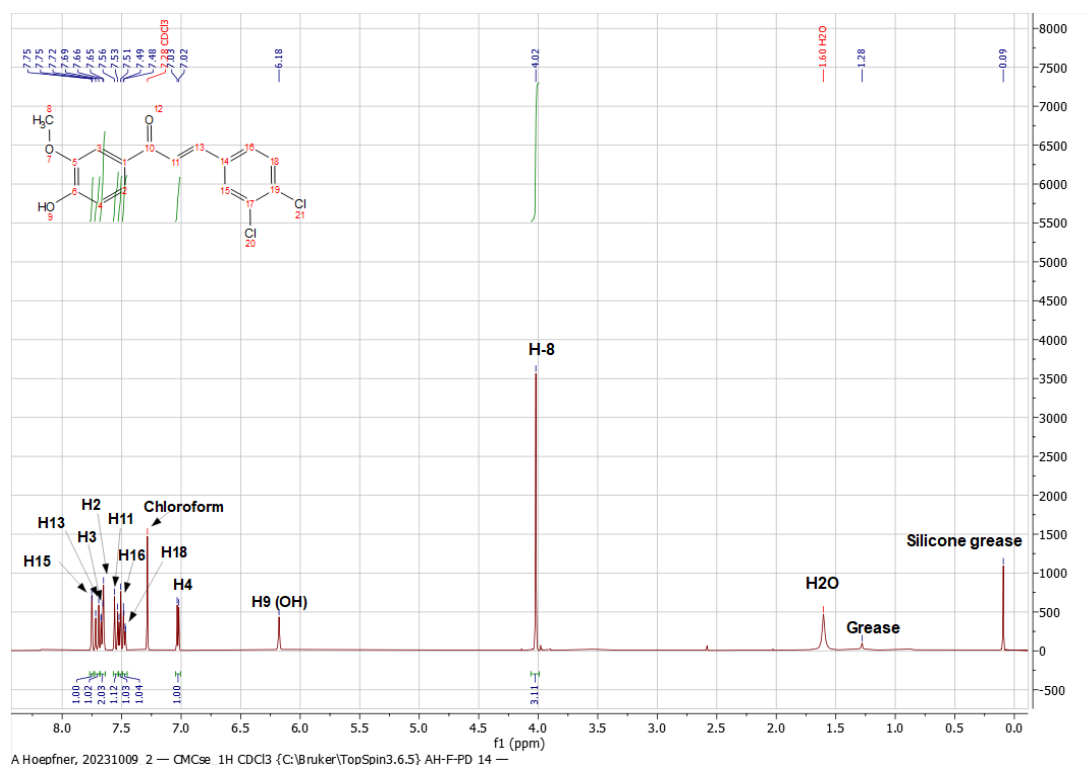


Mass spectrometry (MS) spectrum

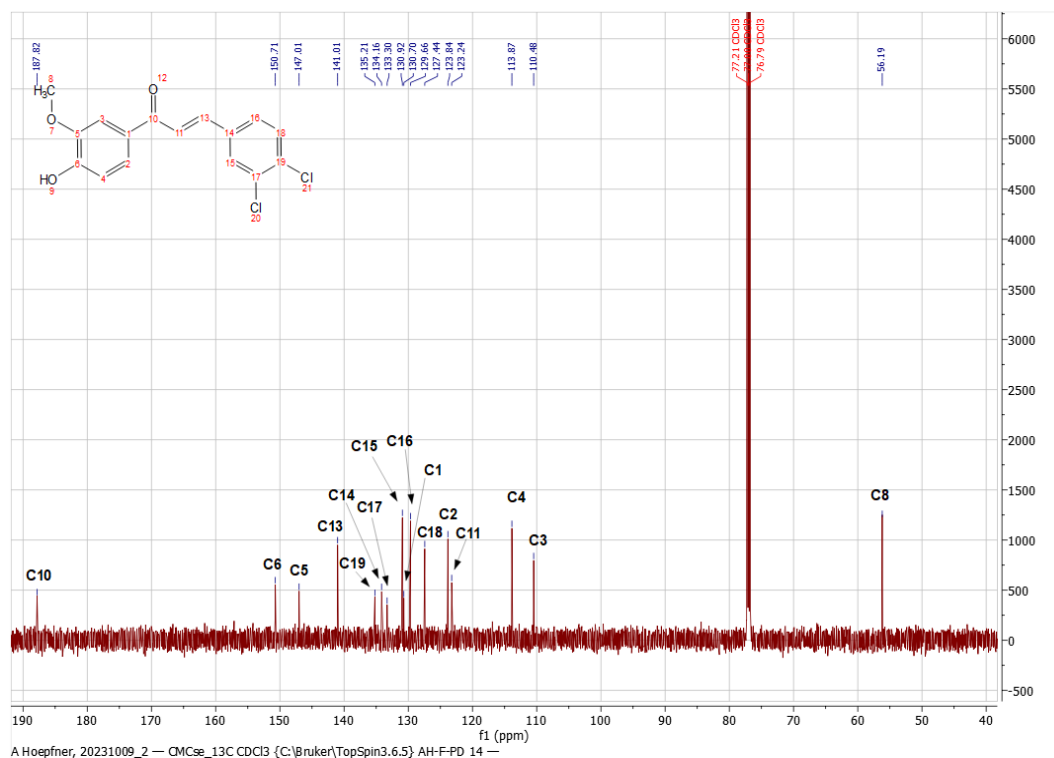


(2E)-3-(3,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1c)

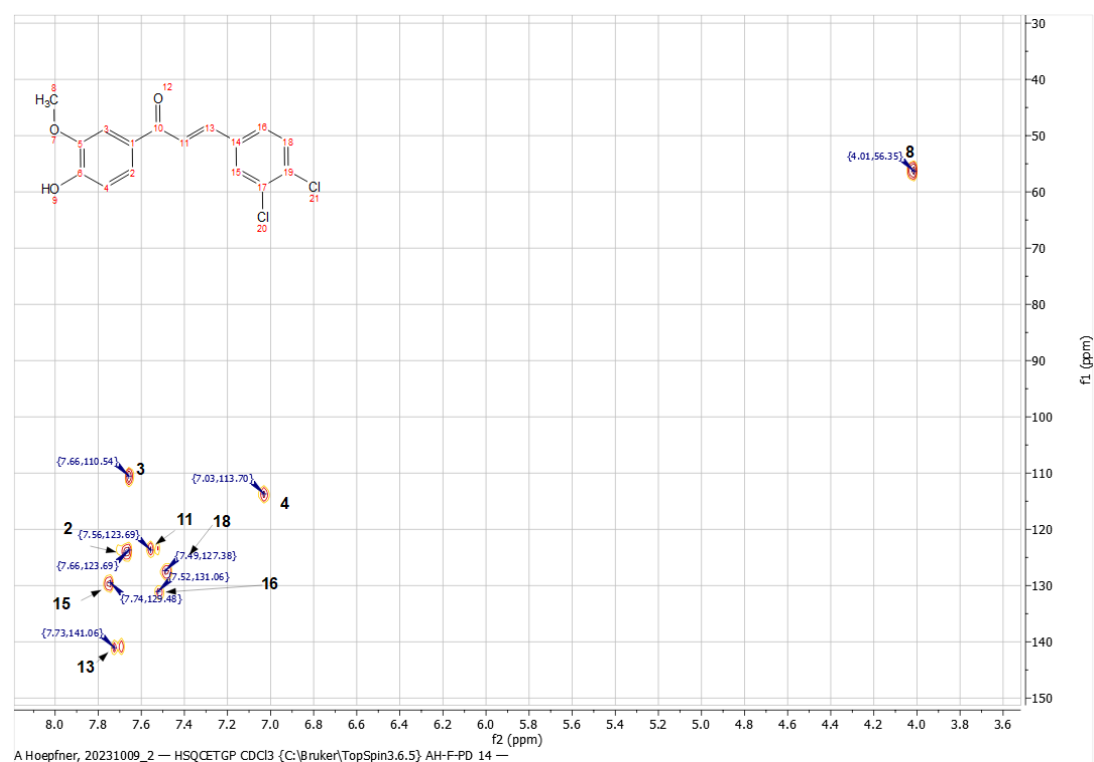
Proton (^1H) NMR spectrum



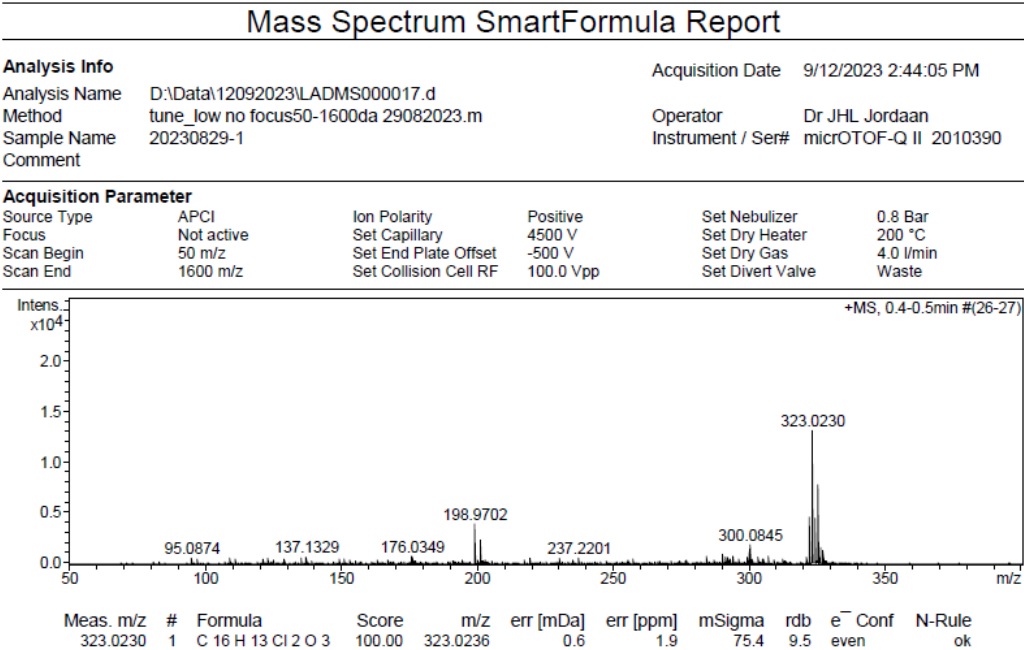
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

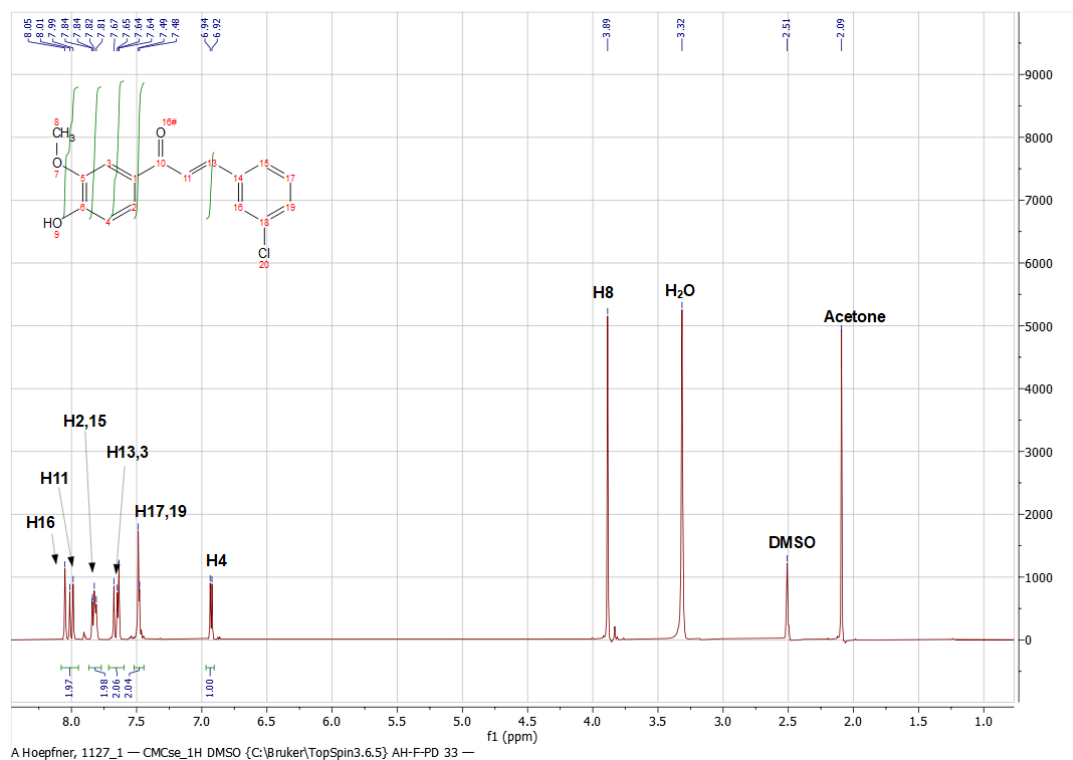


Mass spectrometry (MS) spectrum

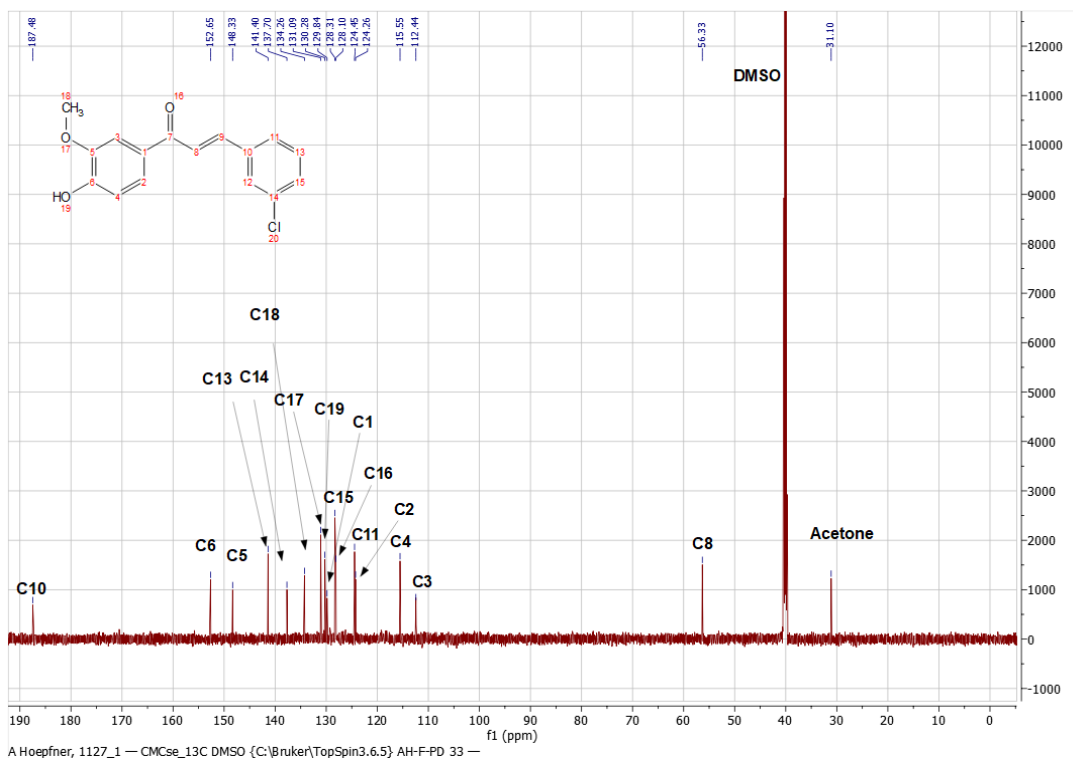


(2E)-3-(3-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1d)

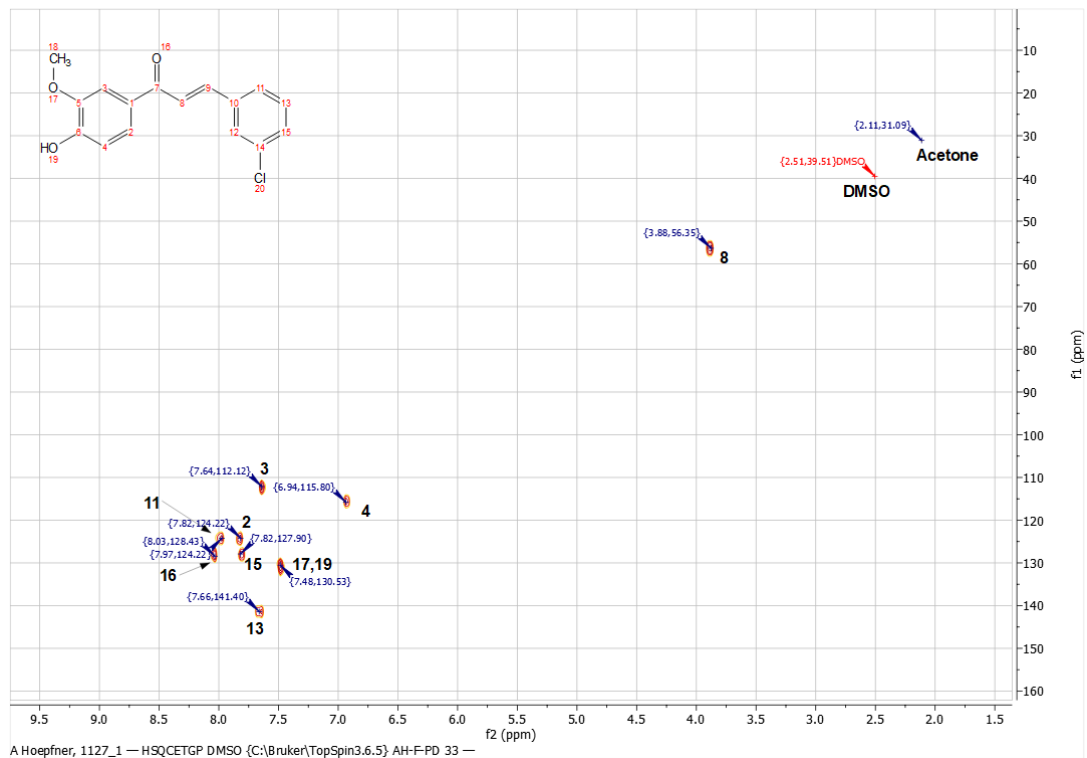
Proton (^1H) NMR spectrum



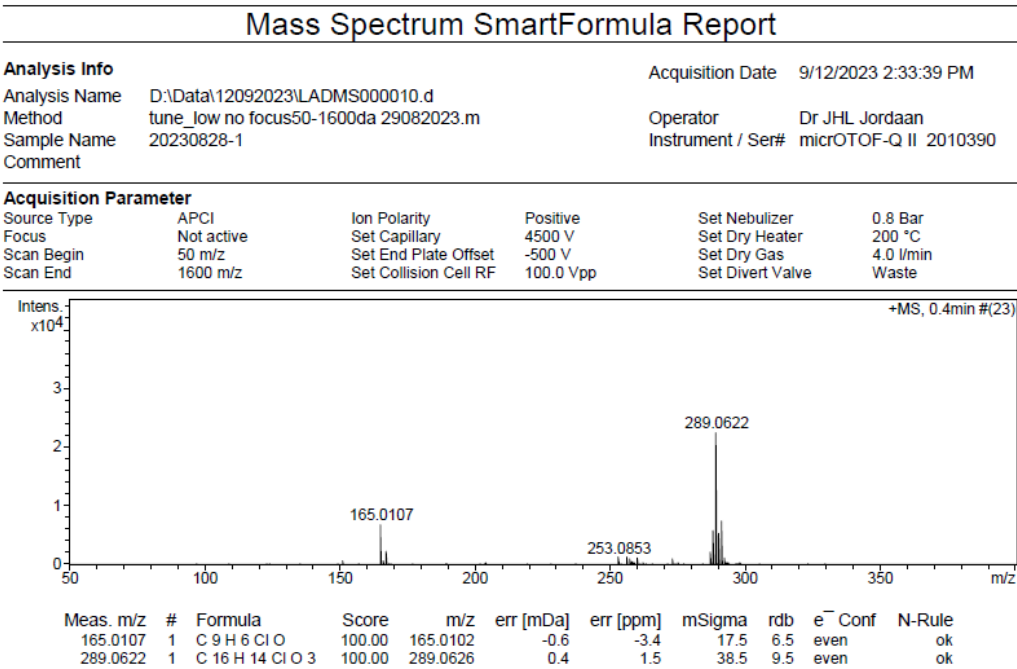
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

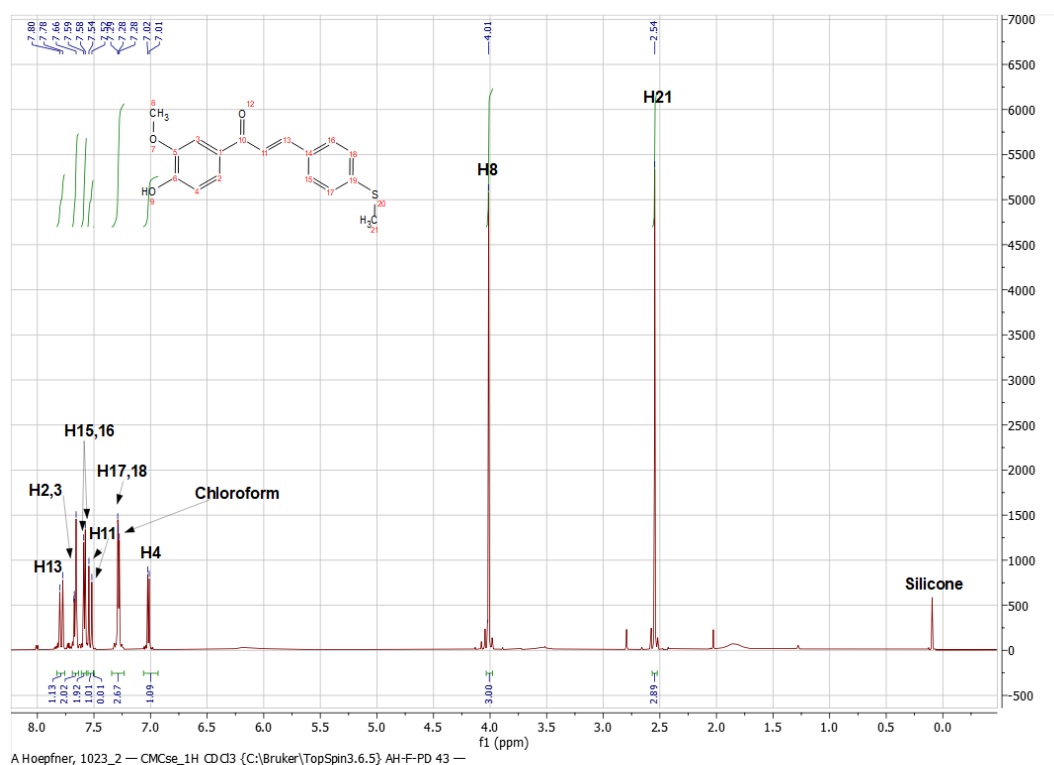


Mass spectrometry (MS) spectrum

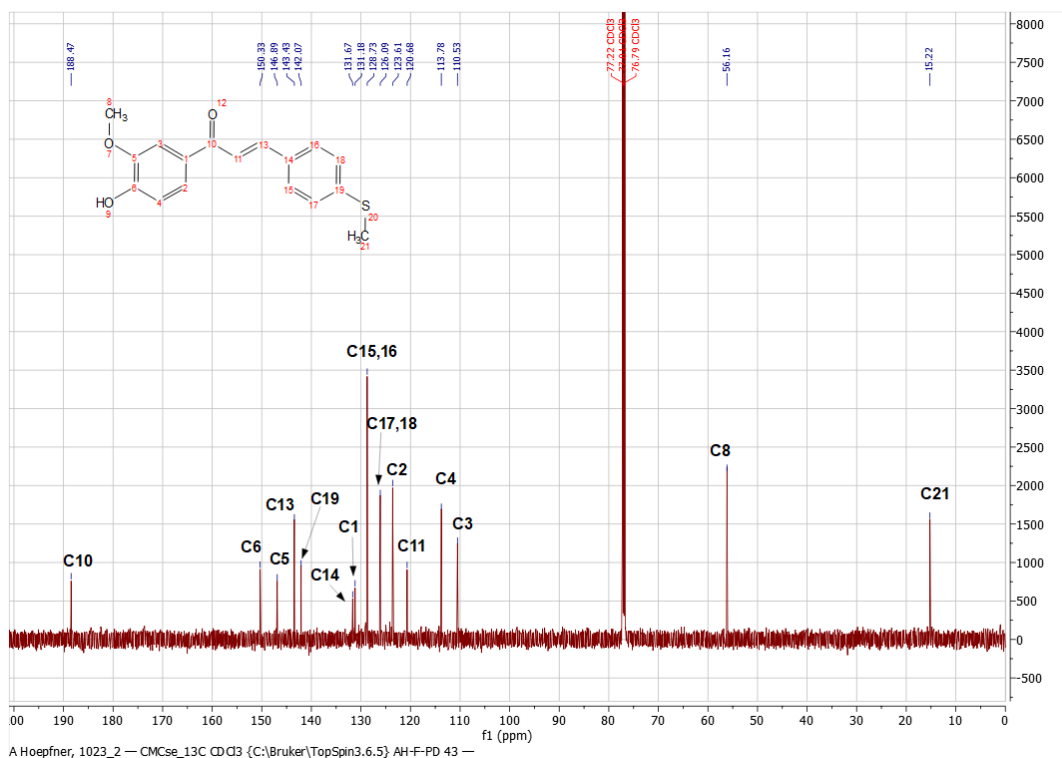


(2E)-1-(4-hydroxy-3-methoxyphenyl)-3-[4-(methylsulfonyl)phenyl]prop-2-en-1-one (1e)

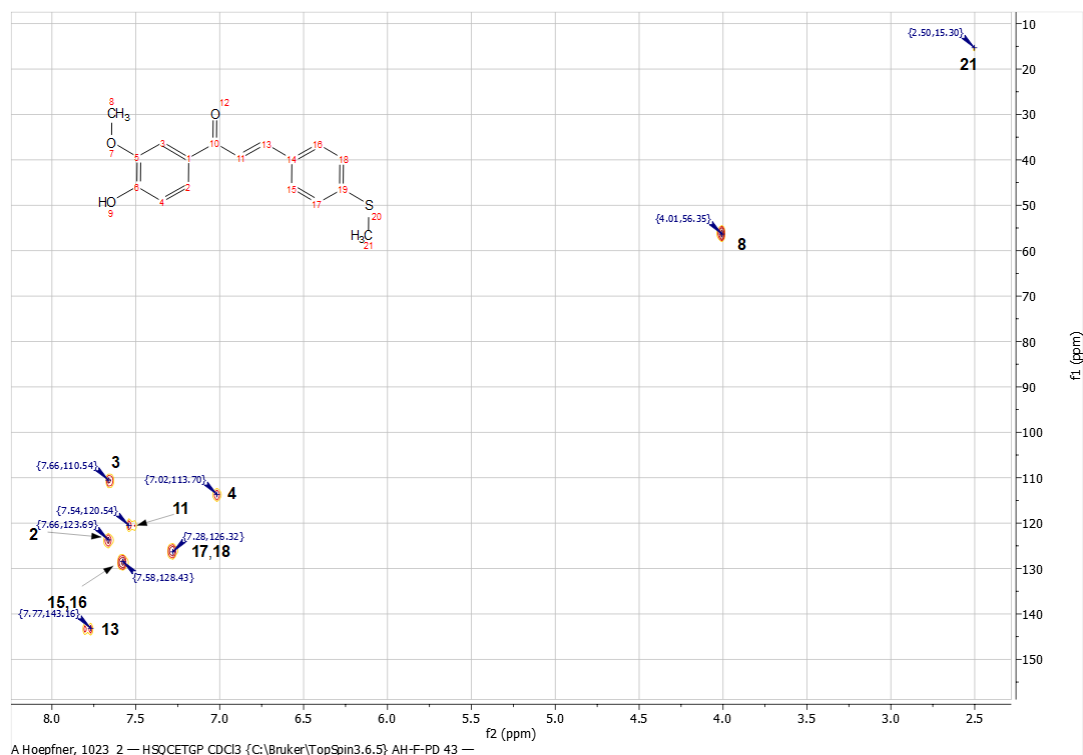
Proton (^1H) NMR spectrum



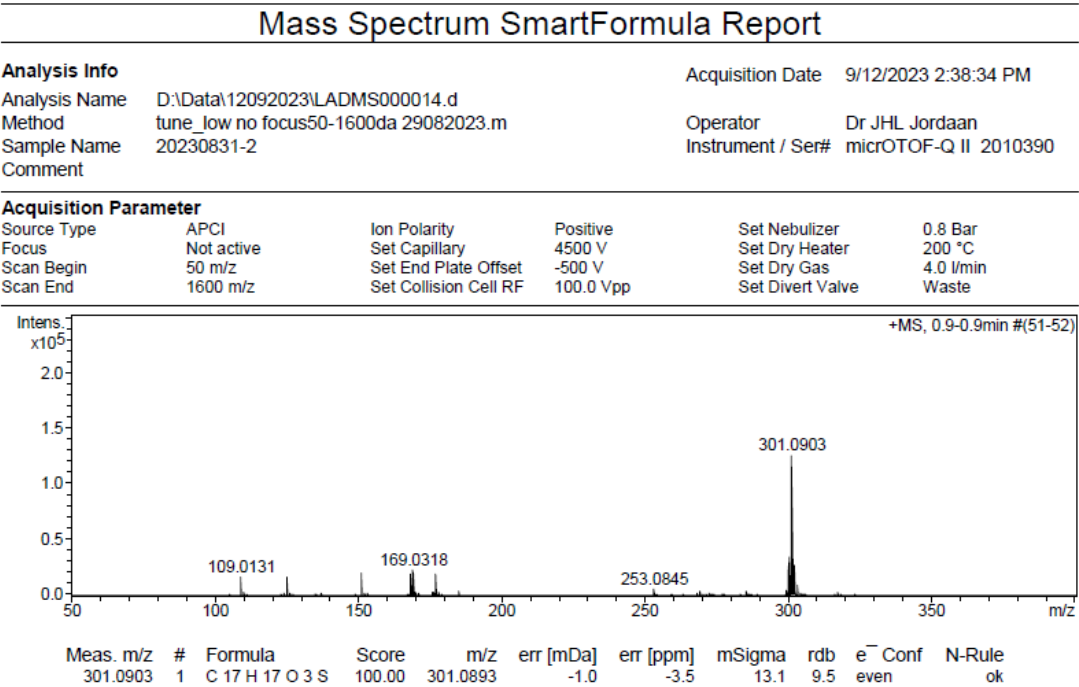
Carbon (^{13}C) NMR spectrum



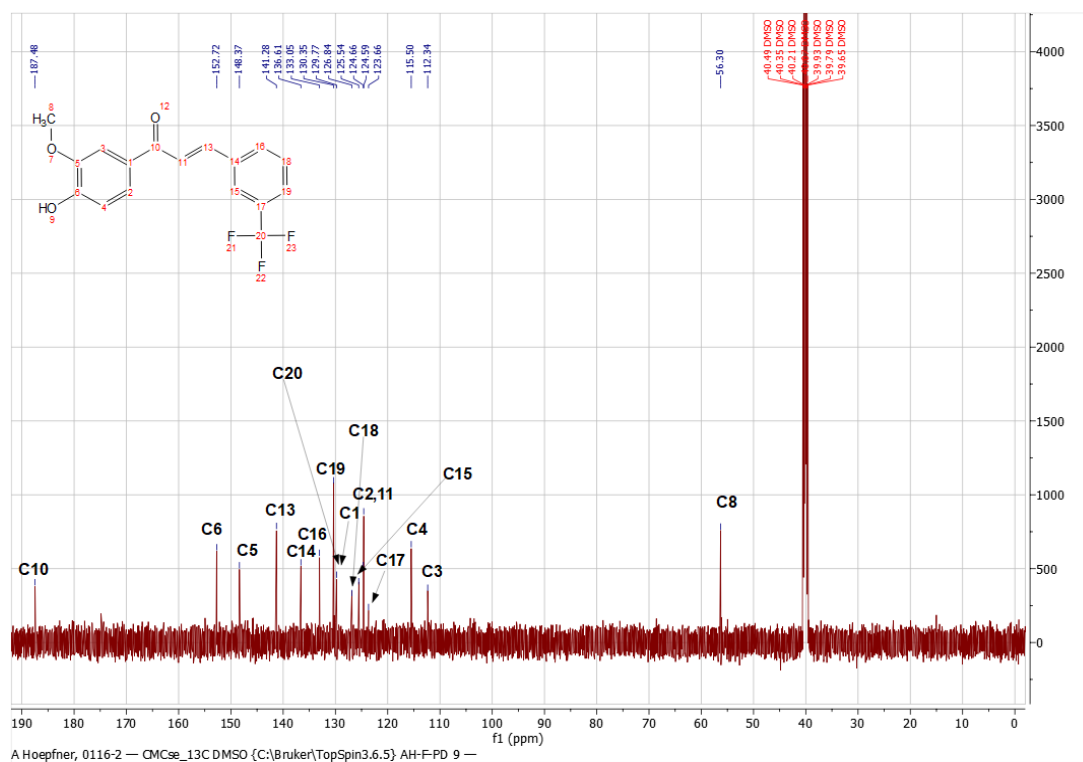
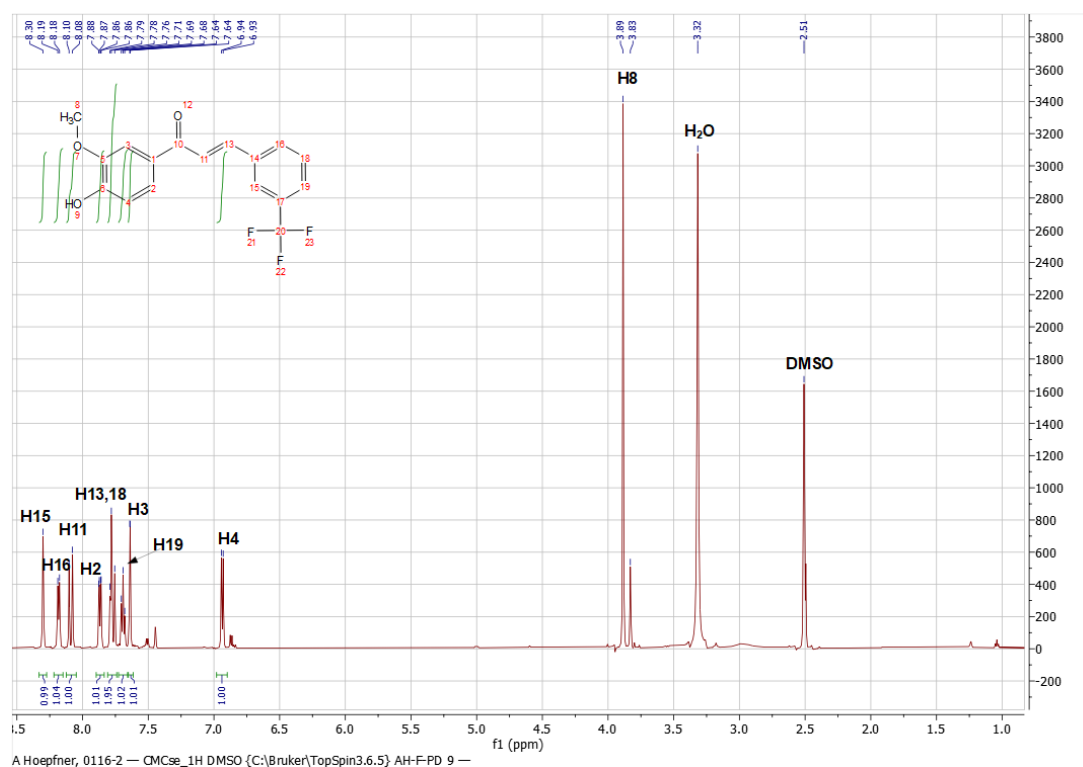
Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum



Mass spectrometry (MS) spectrum



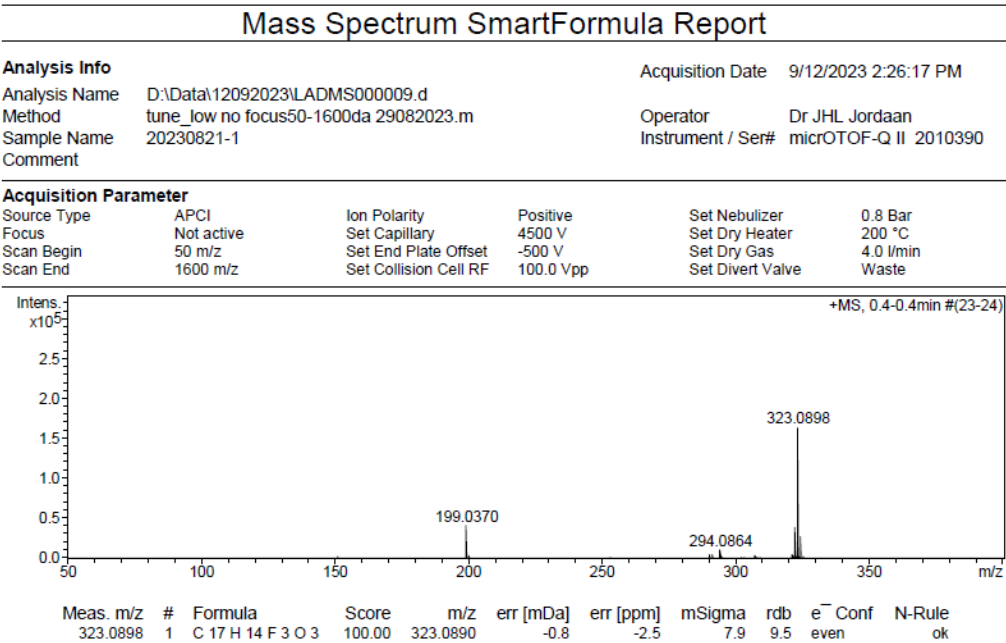
Proton (^1H) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

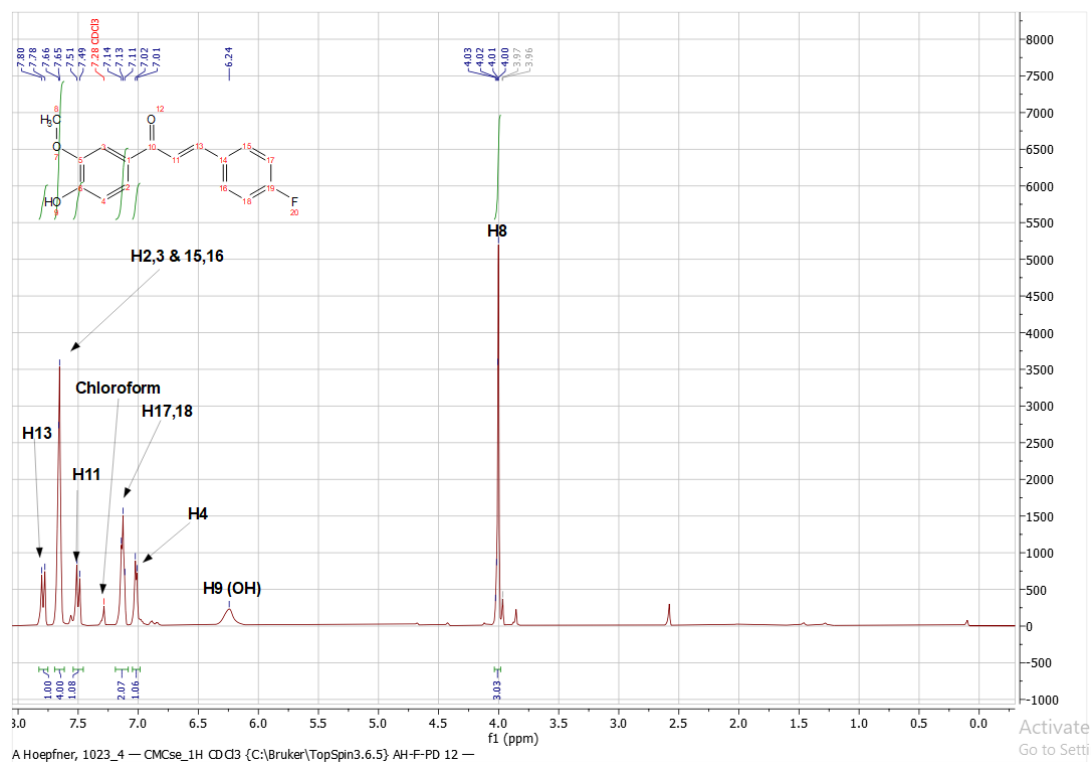


Mass spectrometry (MS) spectrum

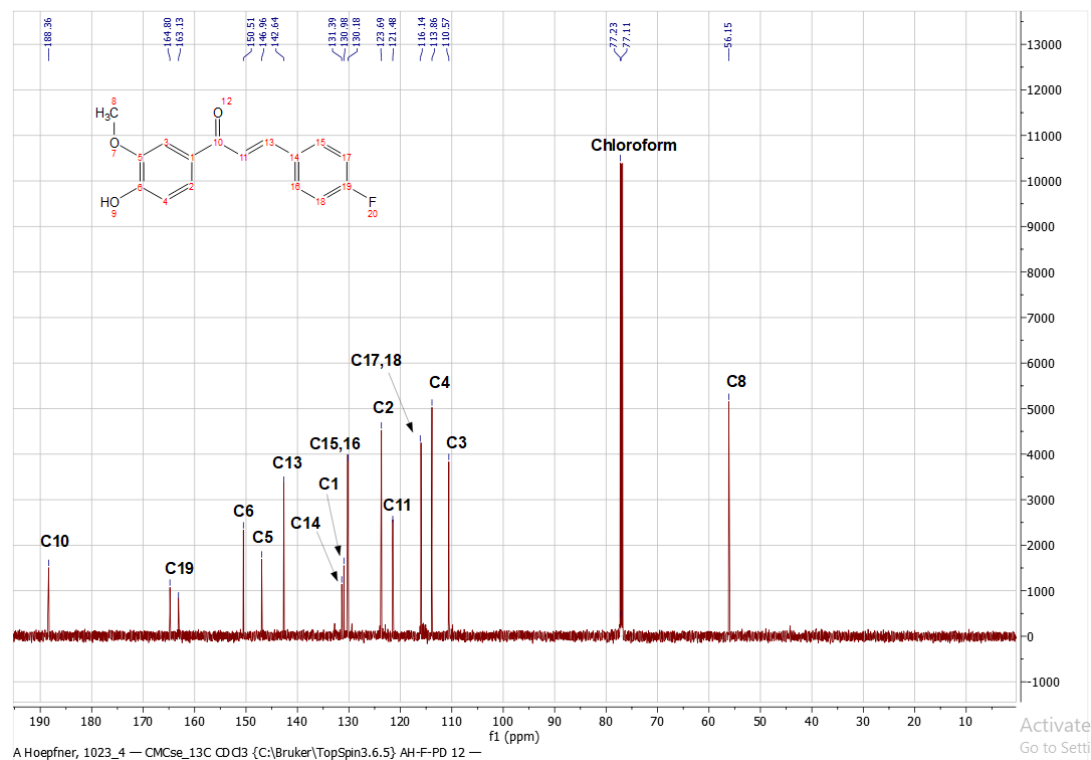


(2E)-3-(4-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1g)

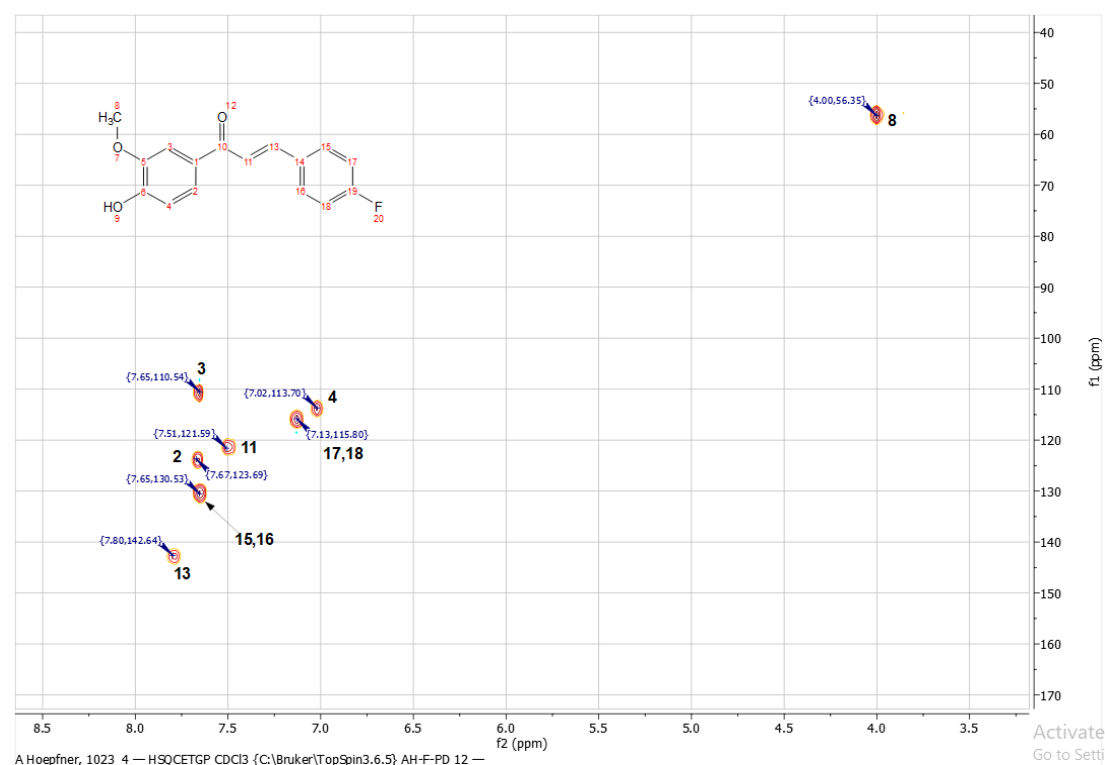
Proton (^1H) NMR spectrum



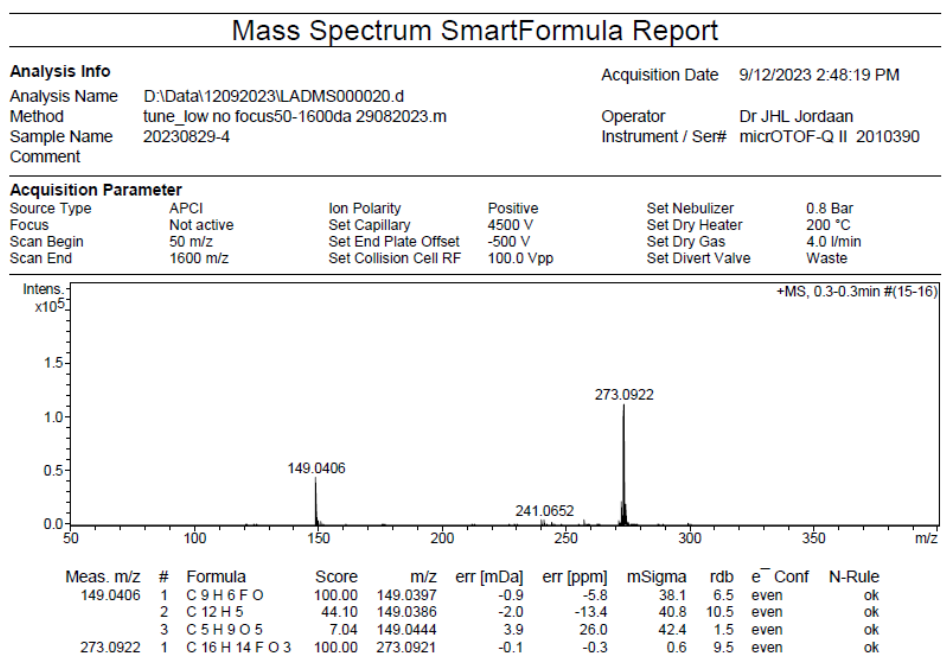
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

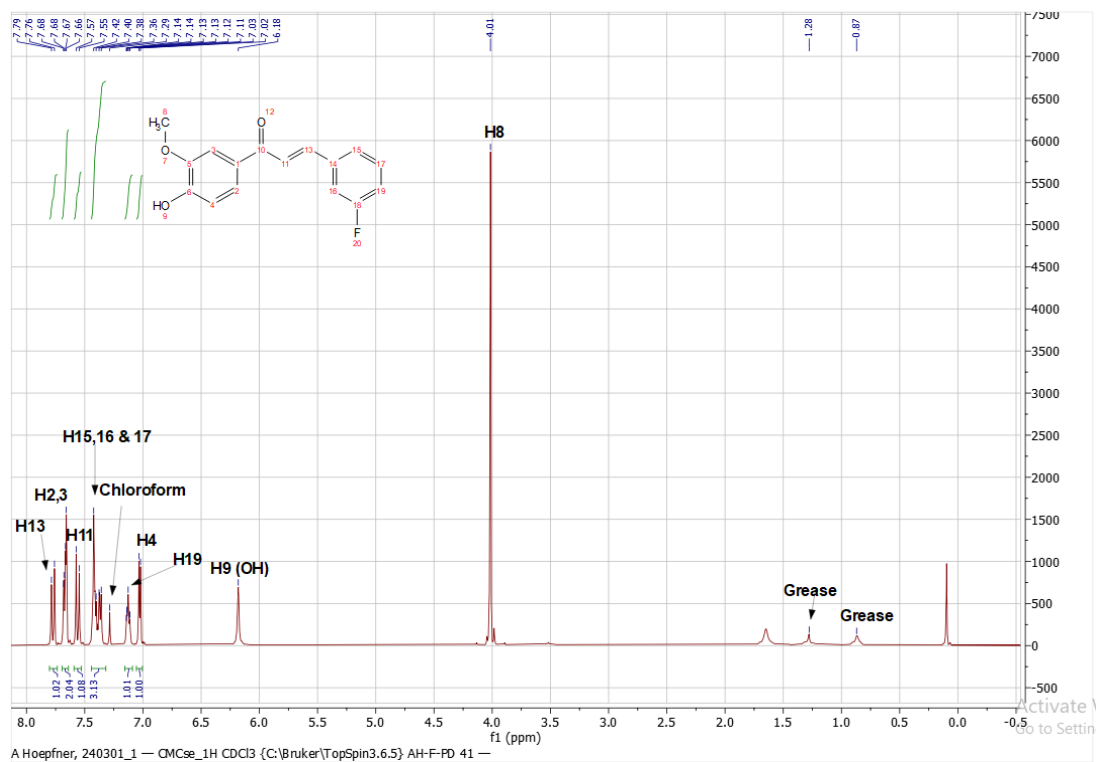


Mass spectrometry (MS) spectrum

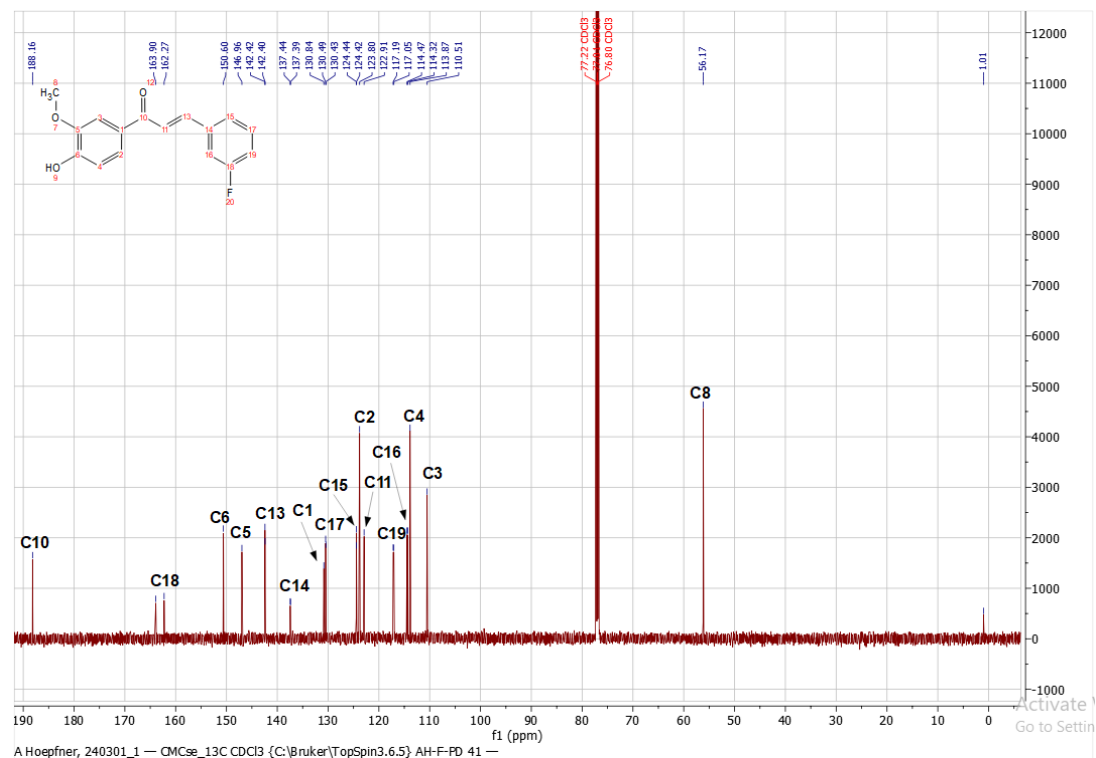


(2E)-3-(3-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1h)

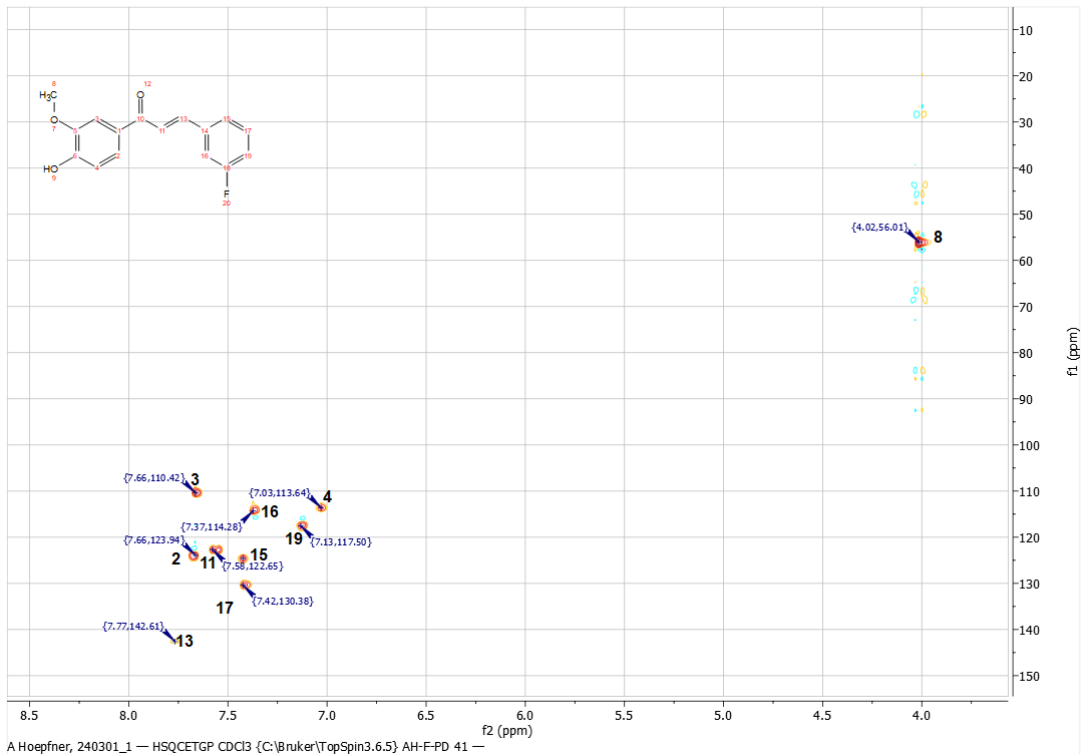
Proton (^1H) NMR spectrum



Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

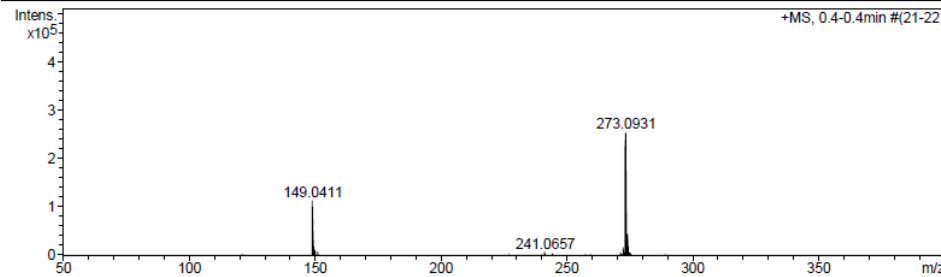


Mass spectrometry (MS) spectrum

Mass Spectrum SmartFormula Report

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Sample Name	20230829-3		
Comment	UNMARKED SAMPLE NO NAME		

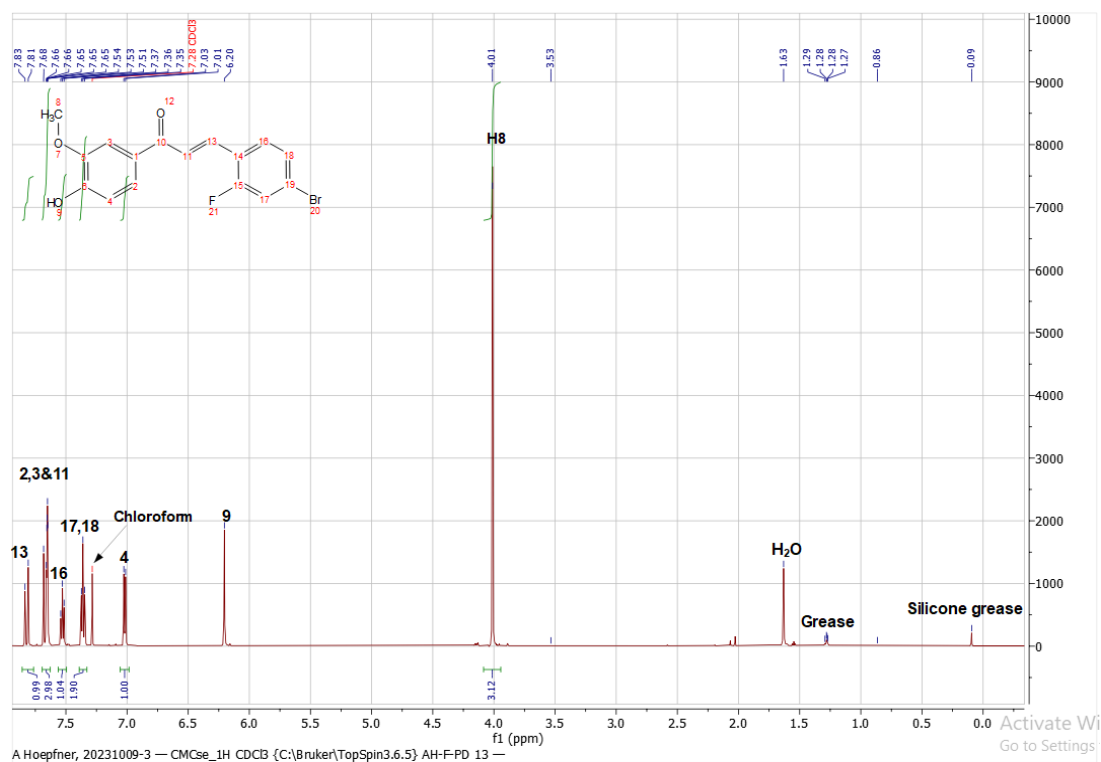
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Focus	Not active	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan Begin	50 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste
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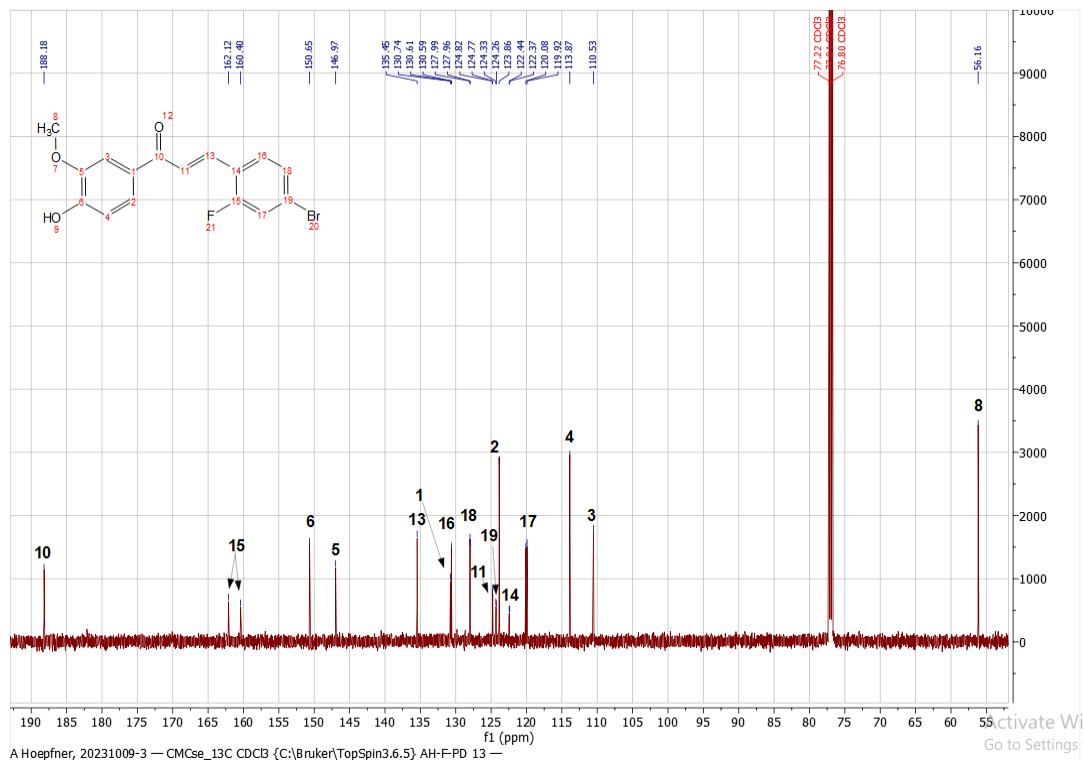
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	2	C 12 H 5	39.27	149.0386	-2.5	-16.7	36.3	10.5	even	ok
	3	C 5 H 9 O 5	15.69	149.0444	3.4	22.7	38.9	1.5	even	ok
273.0931	1	C 16 H 14 F O 3	100.00	273.0921	-0.9	-3.5	2.5	9.5	even	ok

(2E)-3-(4-bromo-2-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1i)

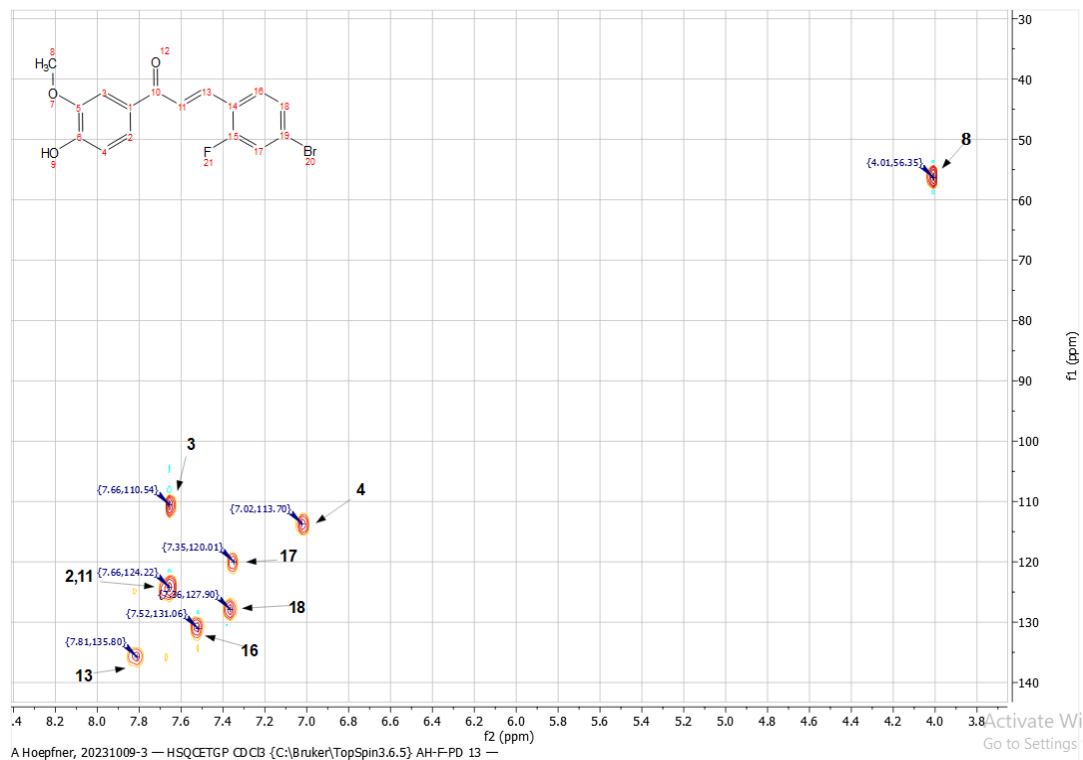
Proton (^1H) NMR spectrum



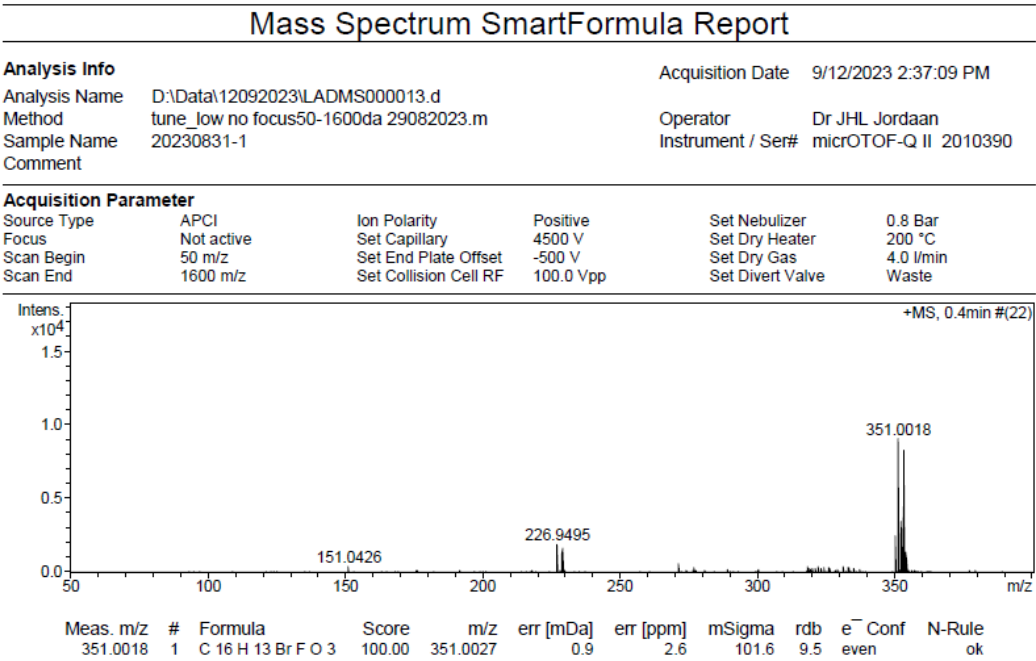
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

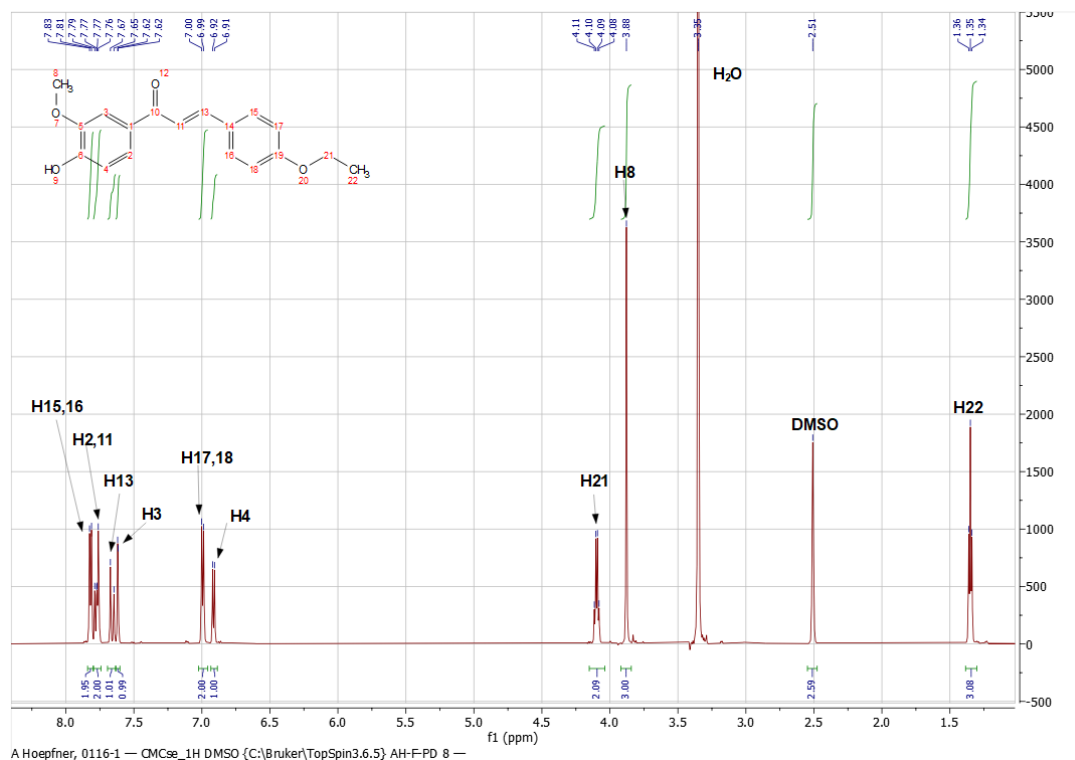


Mass spectrometry (MS) spectrum

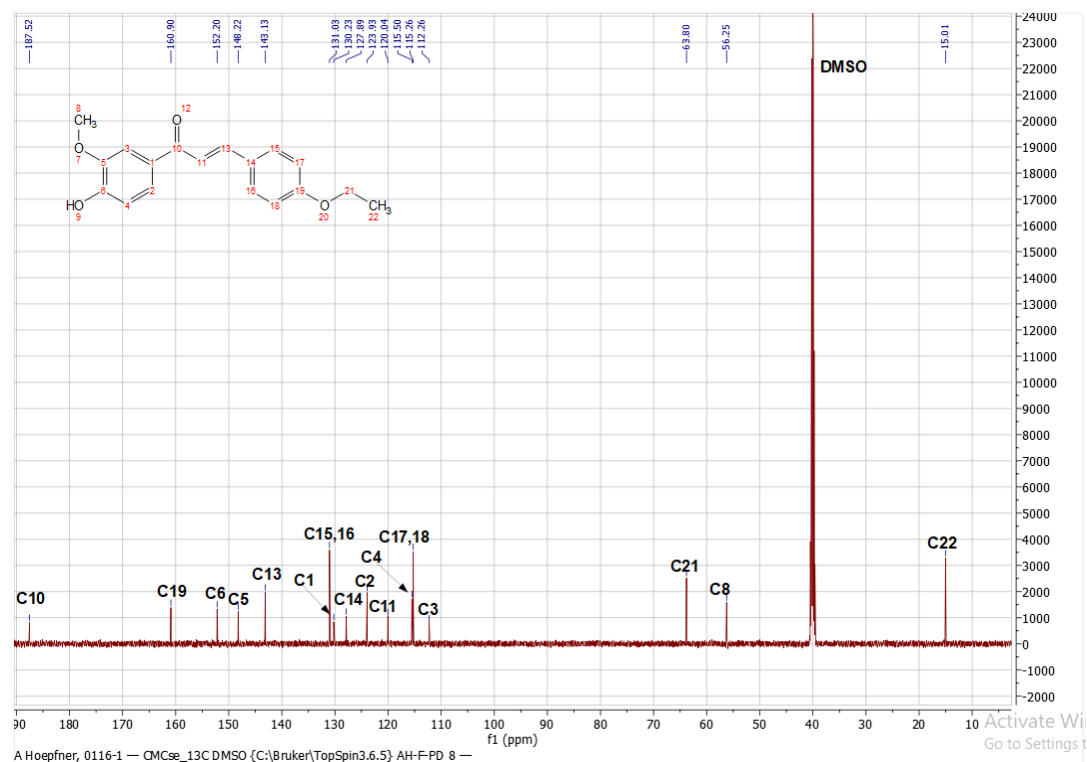


(2E)-3-(4-ethoxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1j)

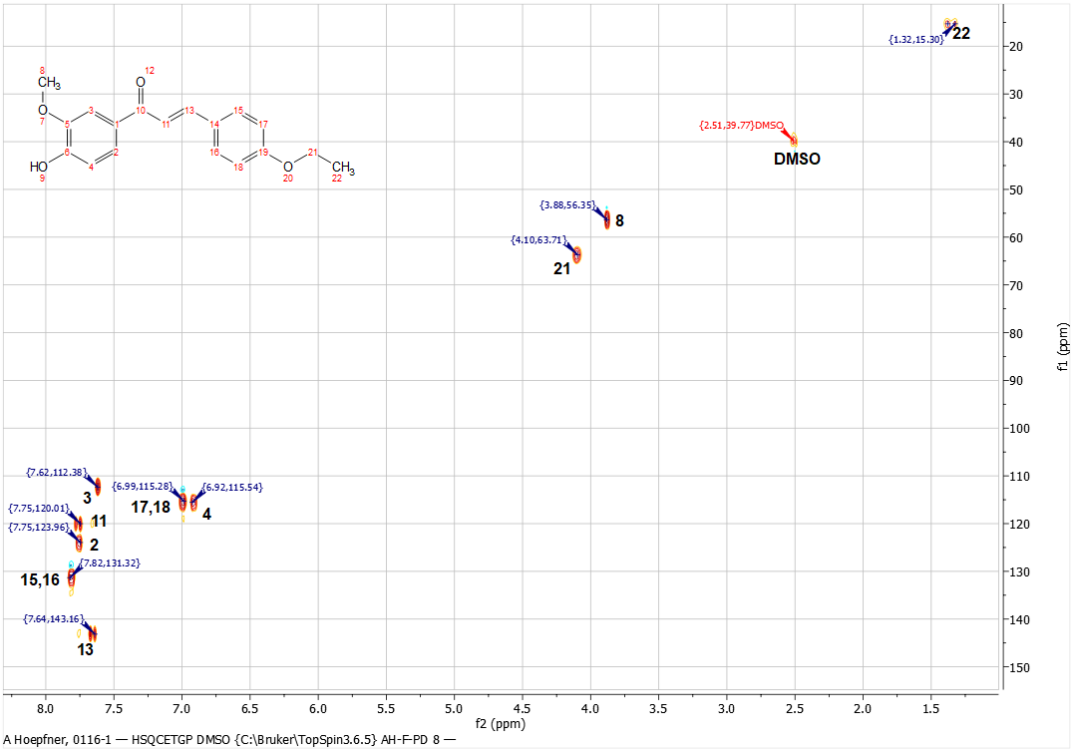
Proton (^1H) NMR spectrum



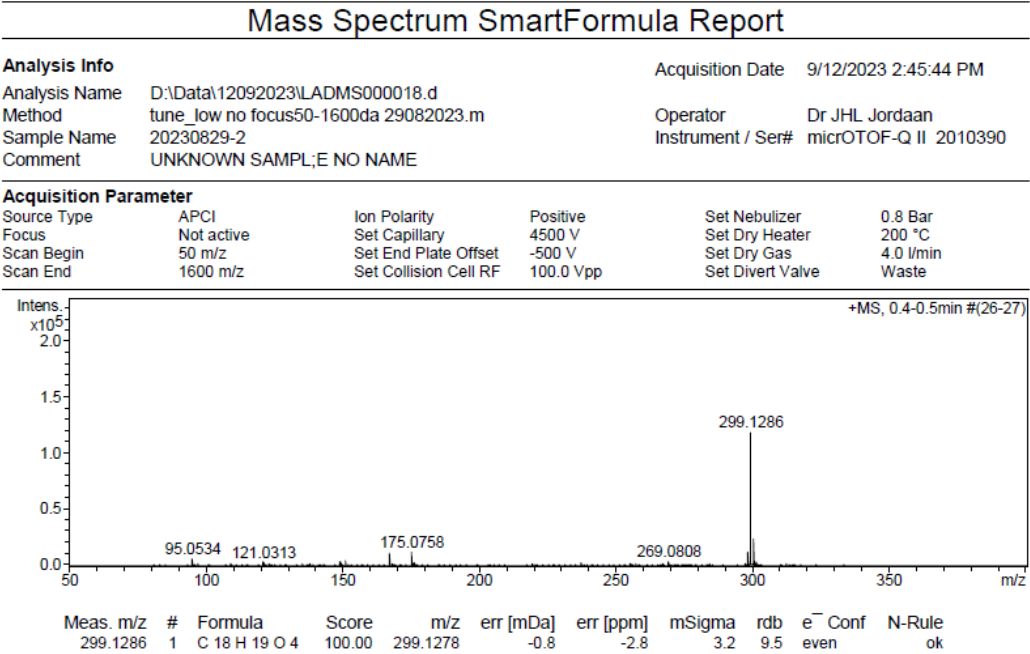
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

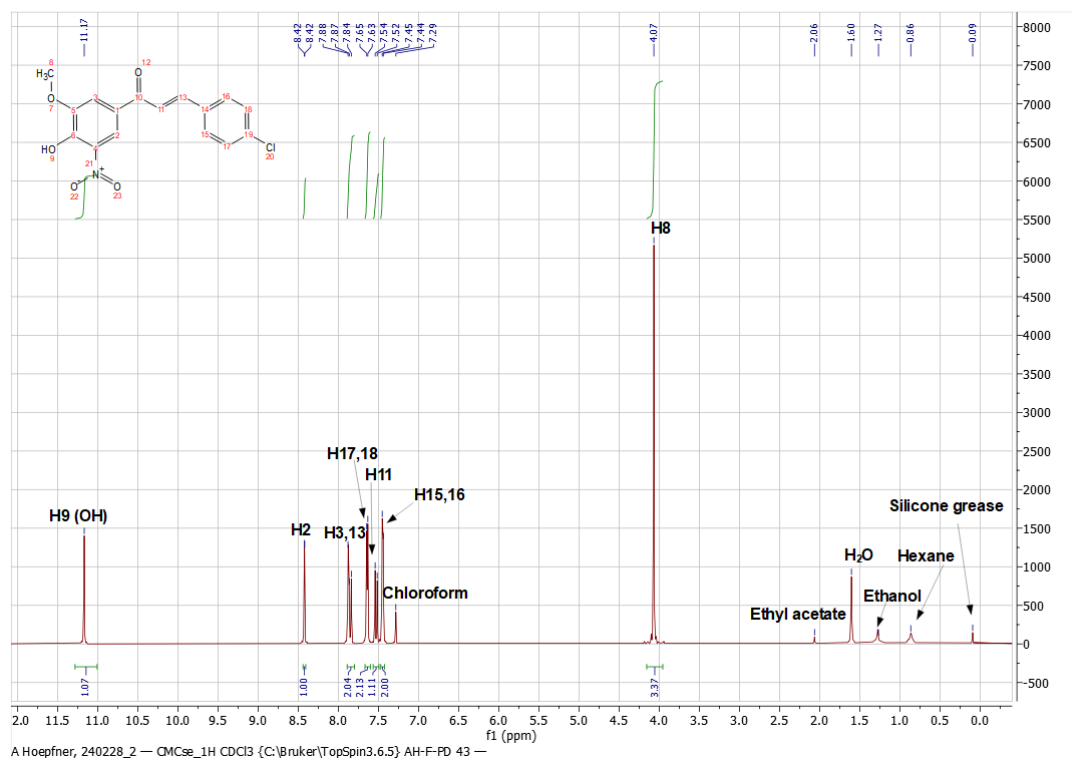


Mass spectrometry (MS) spectrum

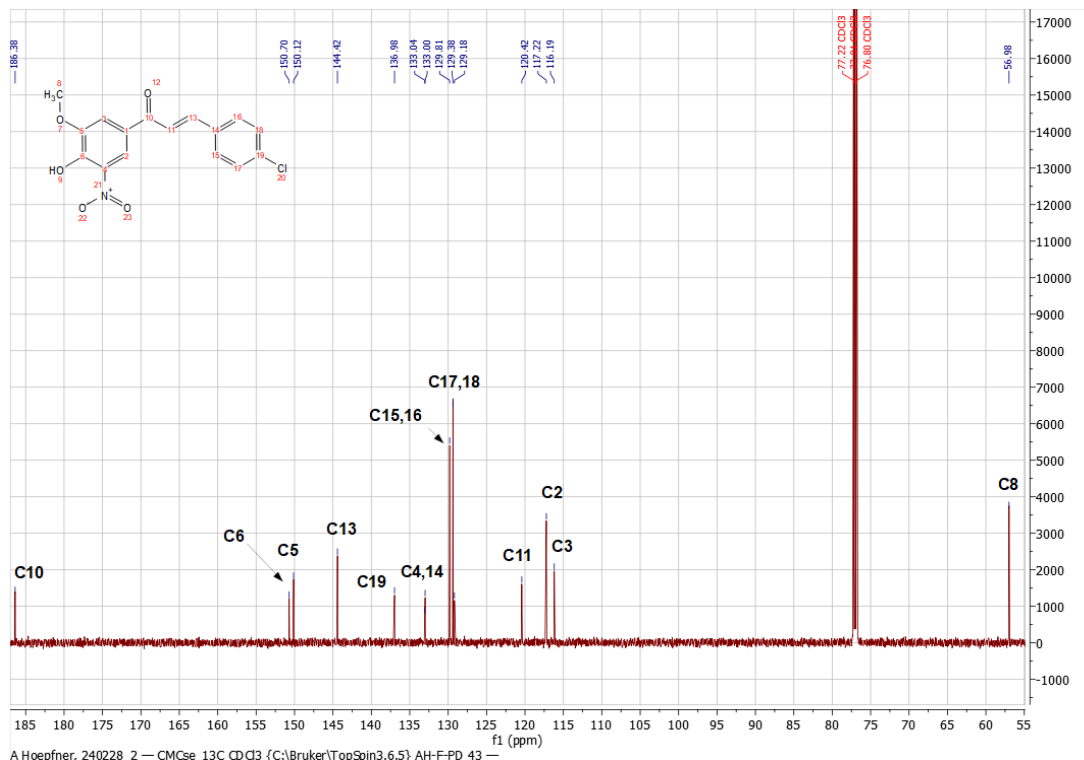


(2E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2a)

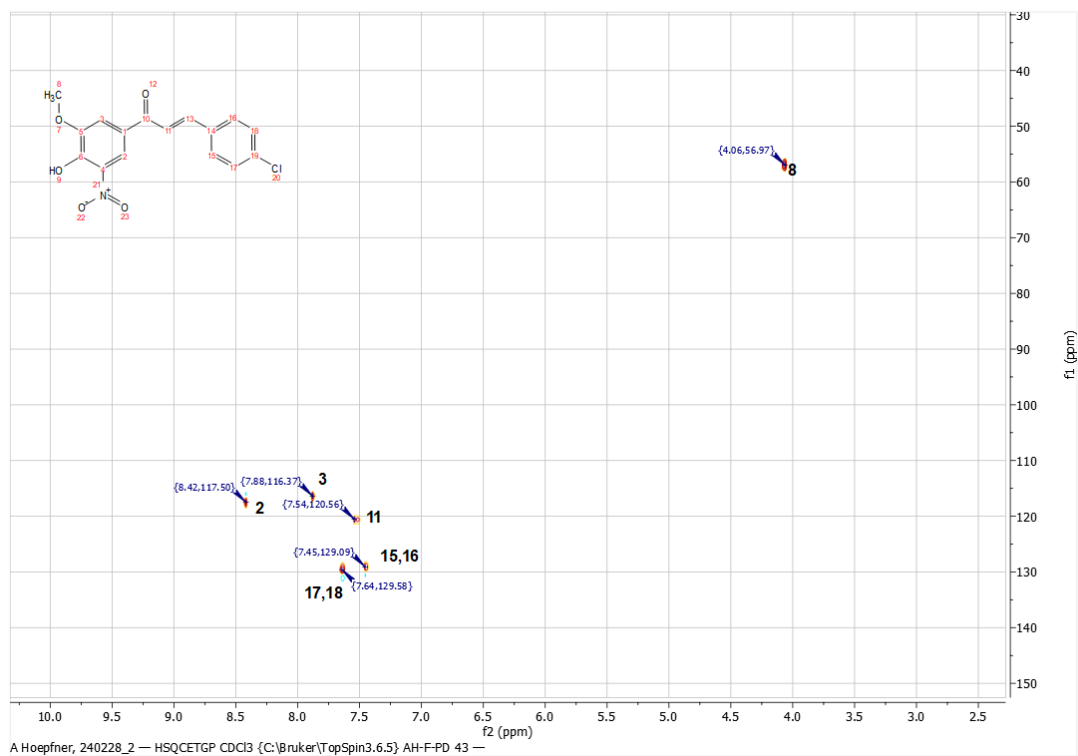
Proton (^1H) NMR spectrum



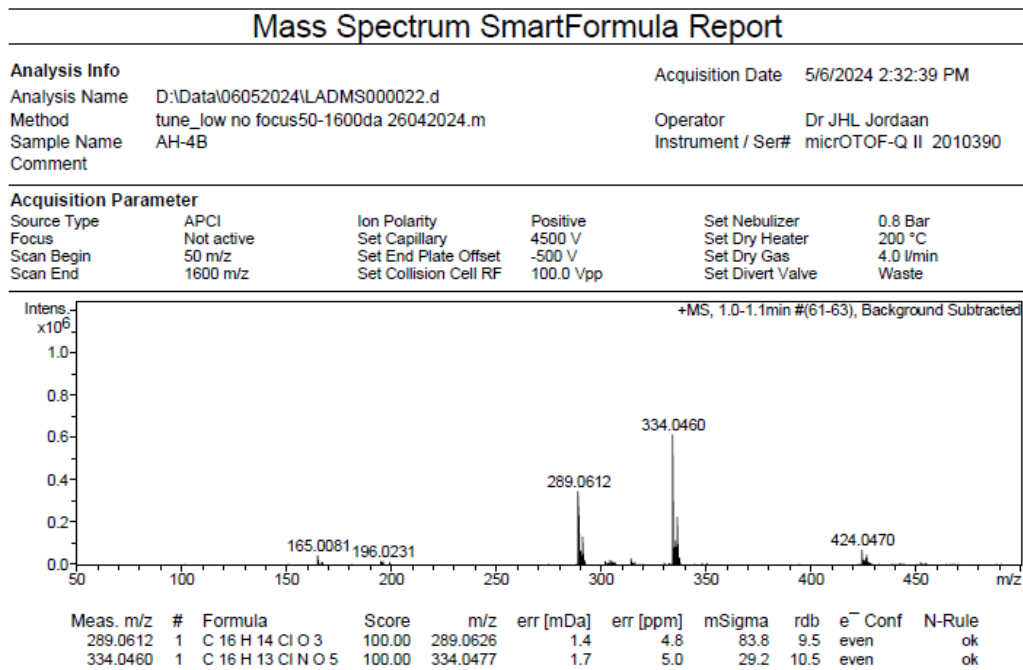
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

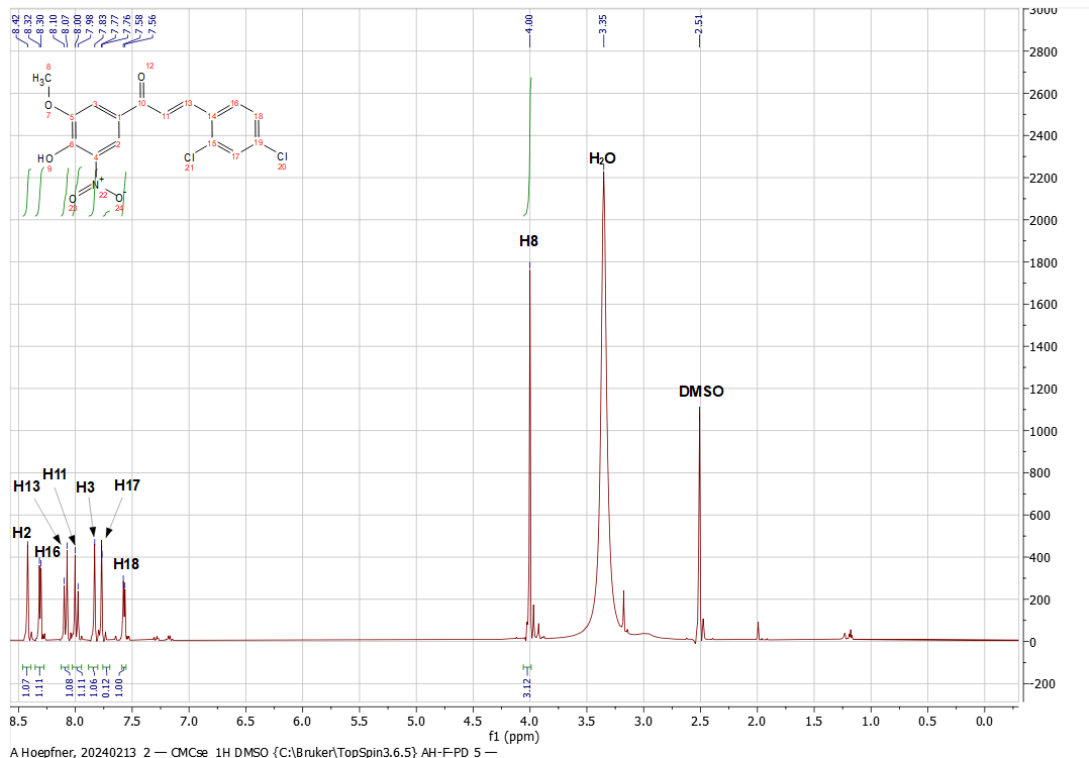


Mass spectrometry (MS) spectrum

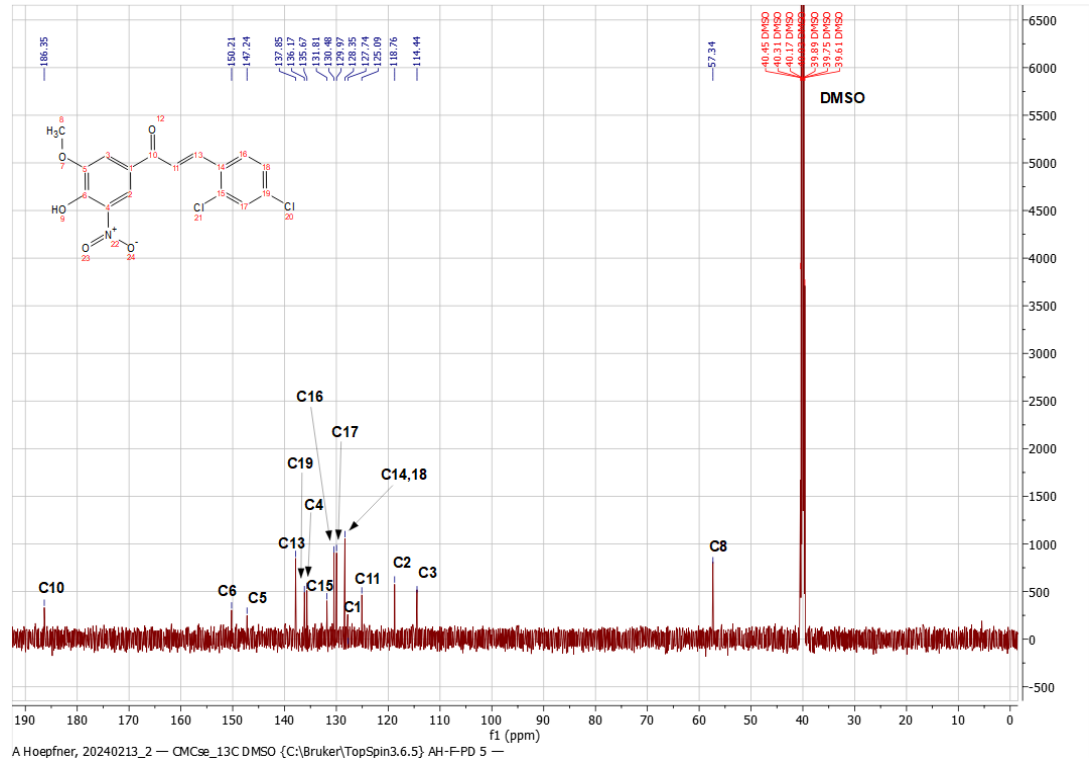


(2E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one
(2b)

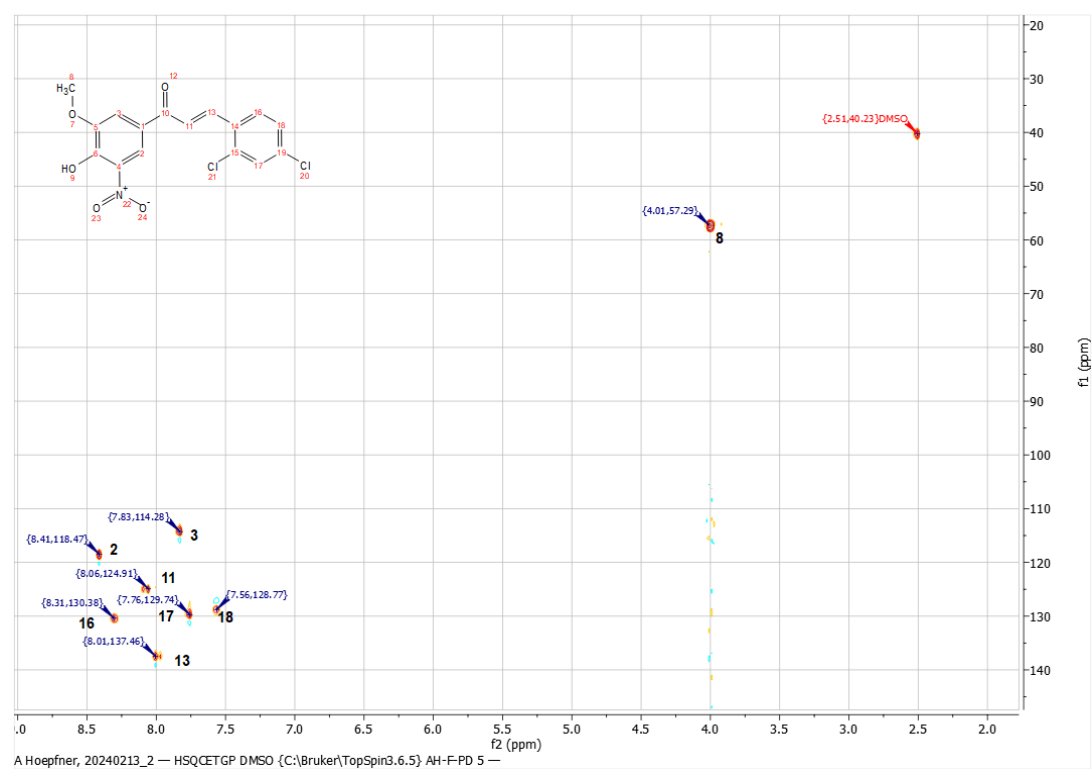
Proton (¹H) NMR spectrum



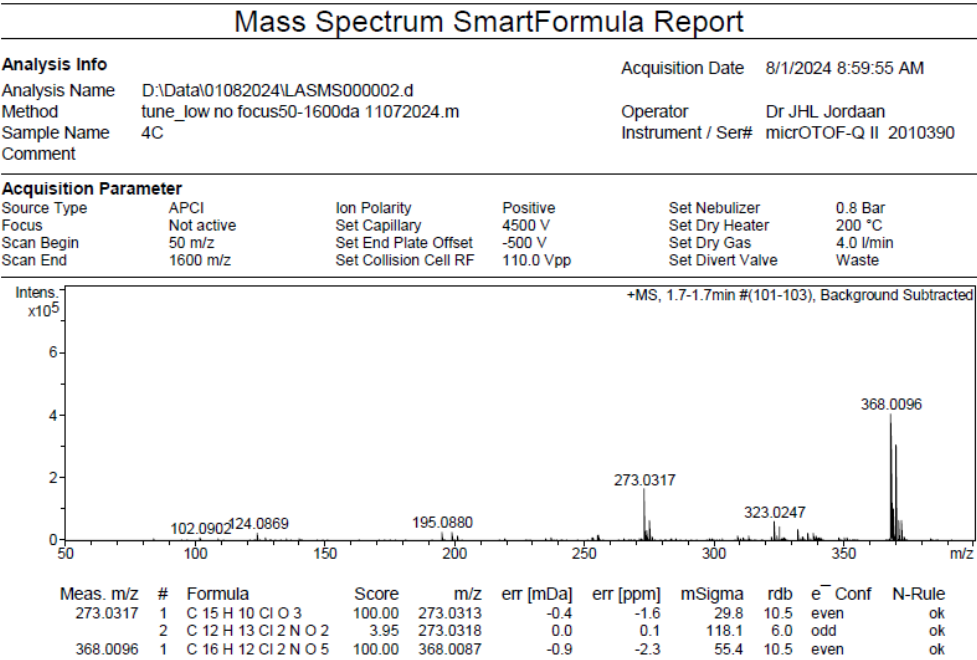
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

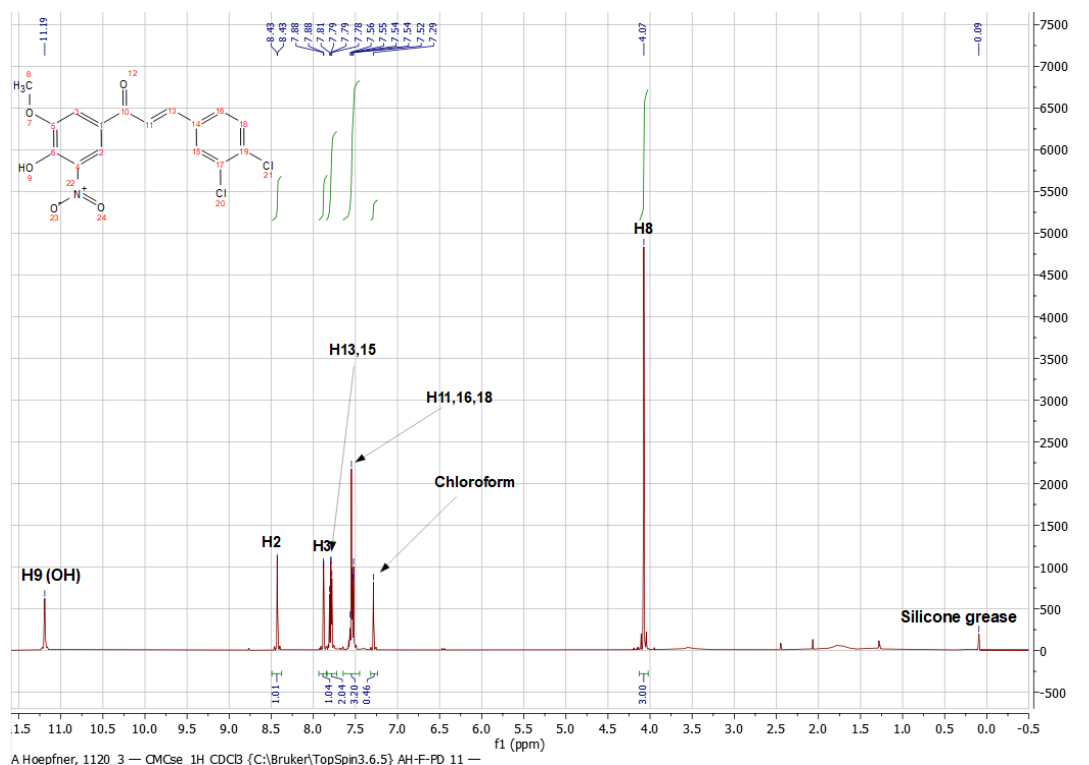


Mass spectrometry (MS) spectrum

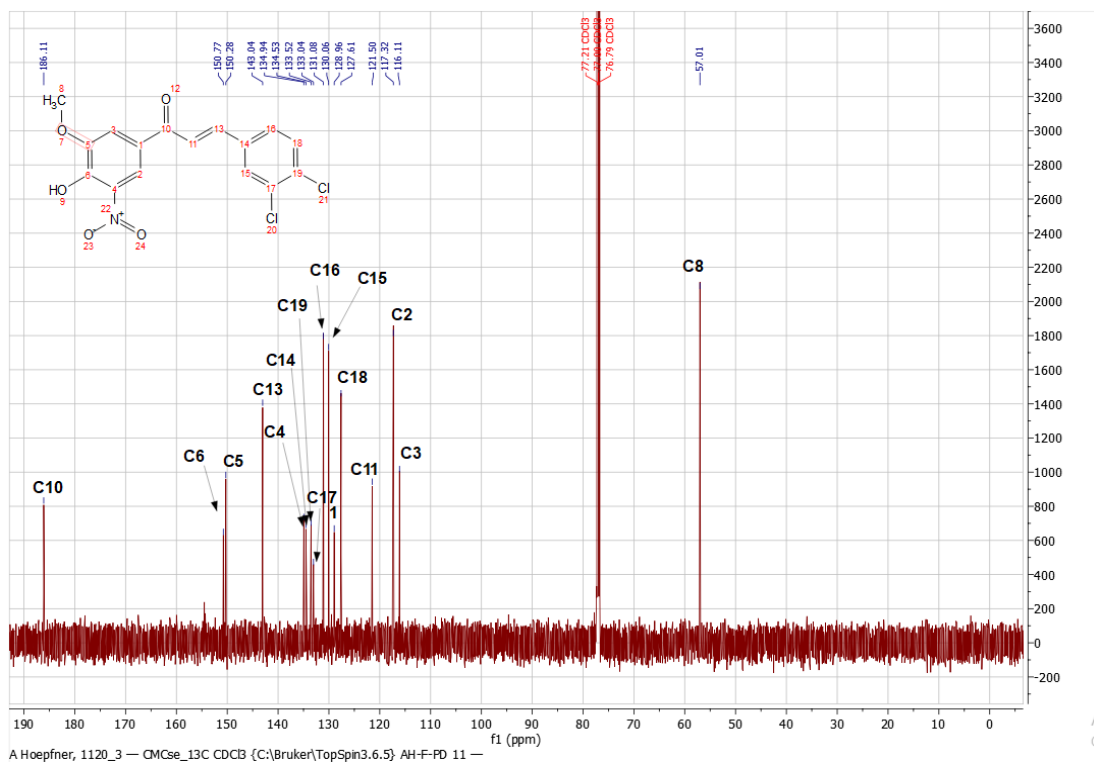


(2E)-3-(3,4-dichlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one
(2c)

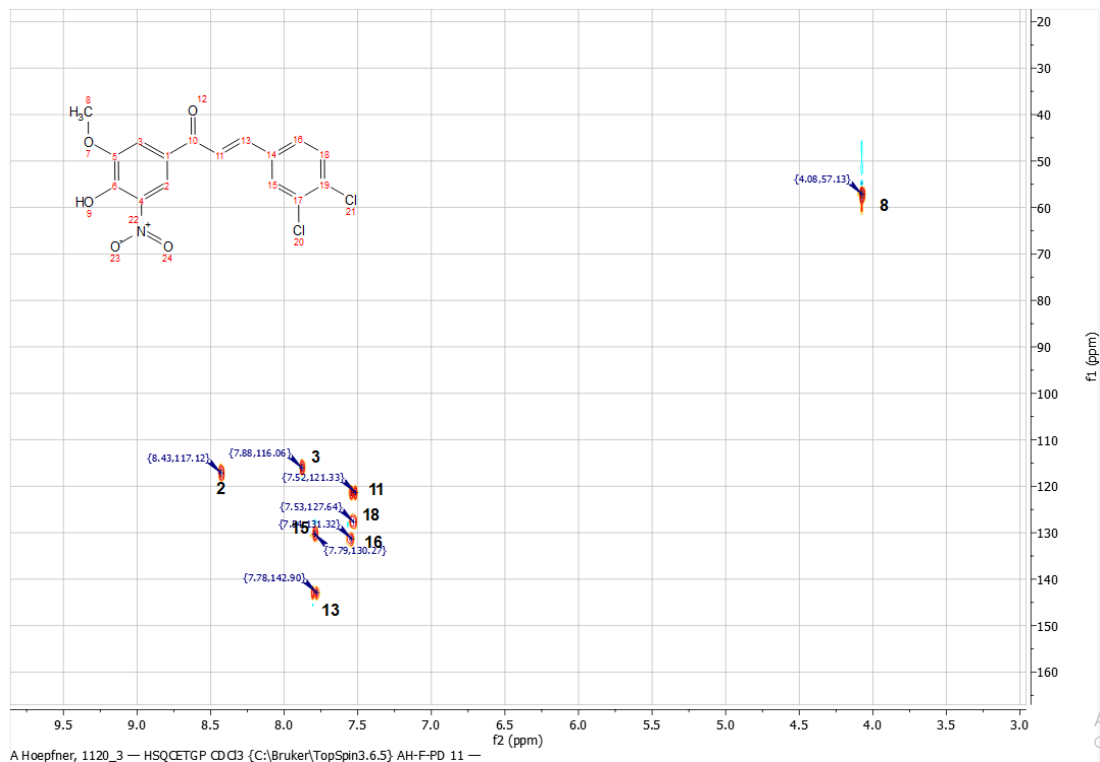
Proton (^1H) NMR spectrum



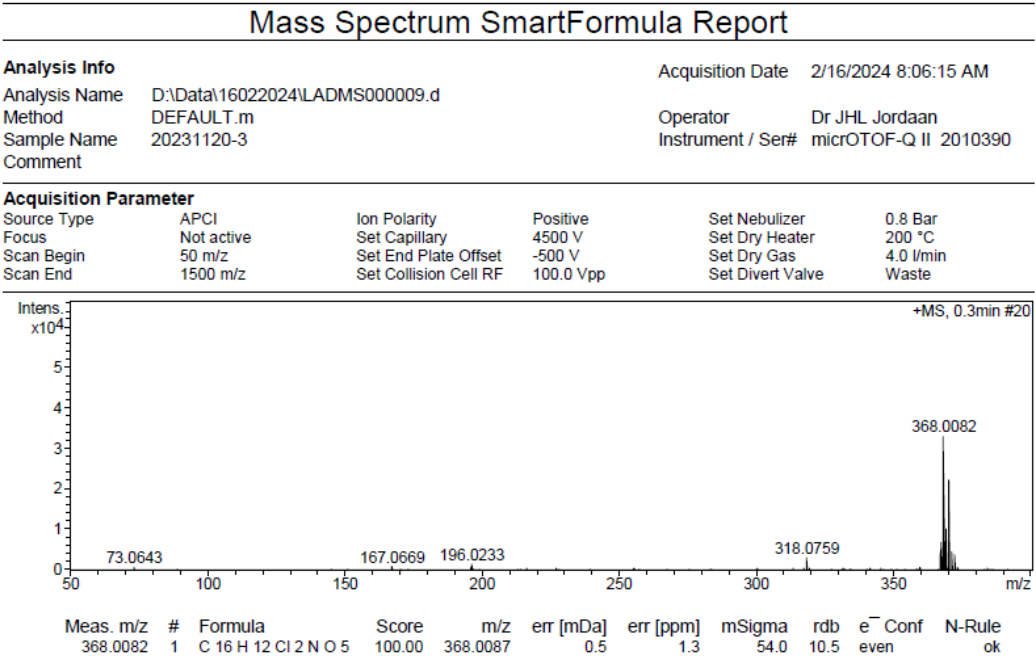
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

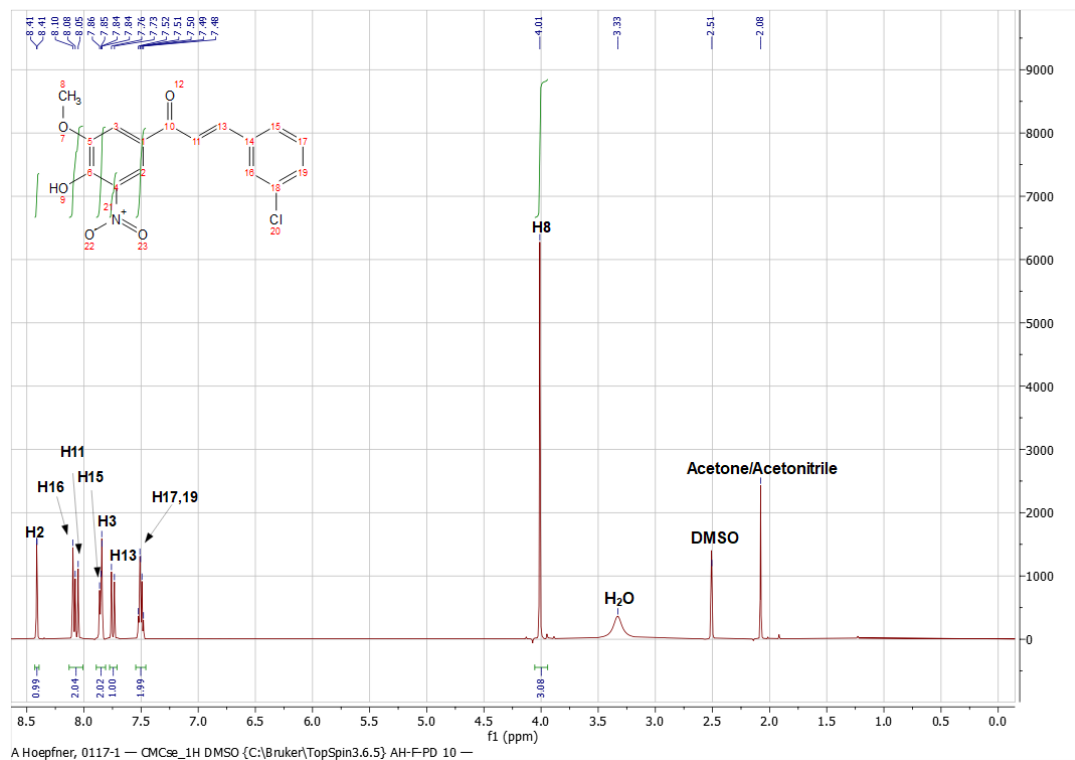


Mass spectrometry (MS) spectrum

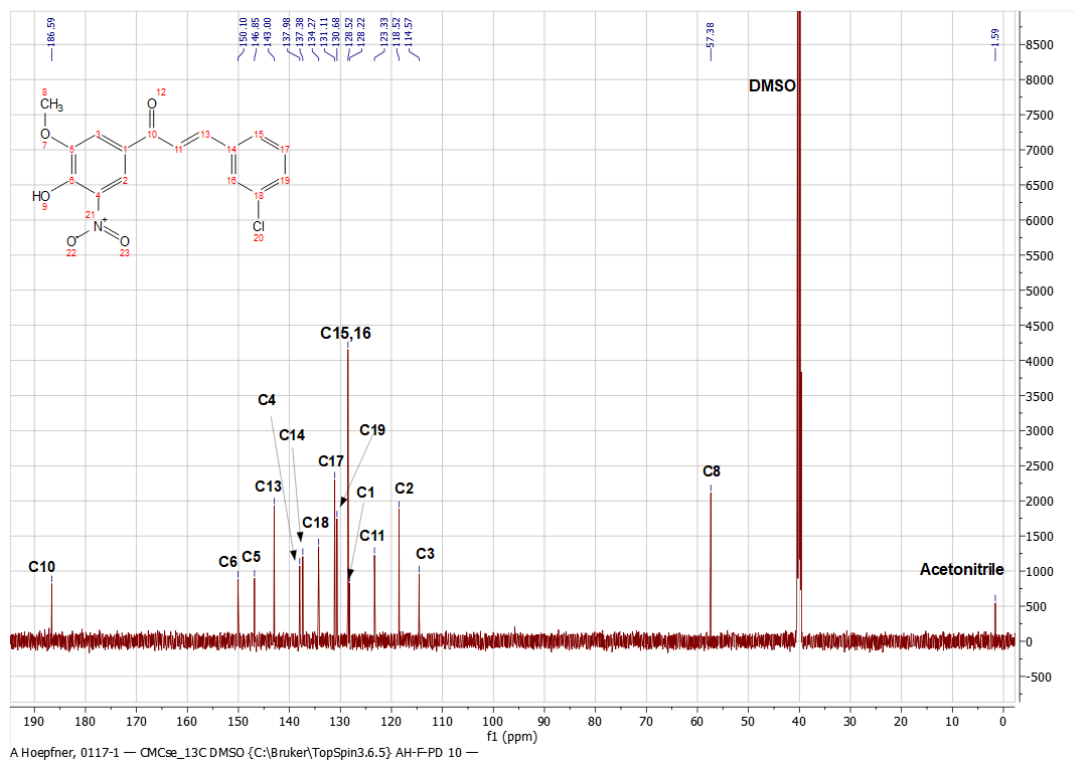


(2E)-3-(3-chlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2d)

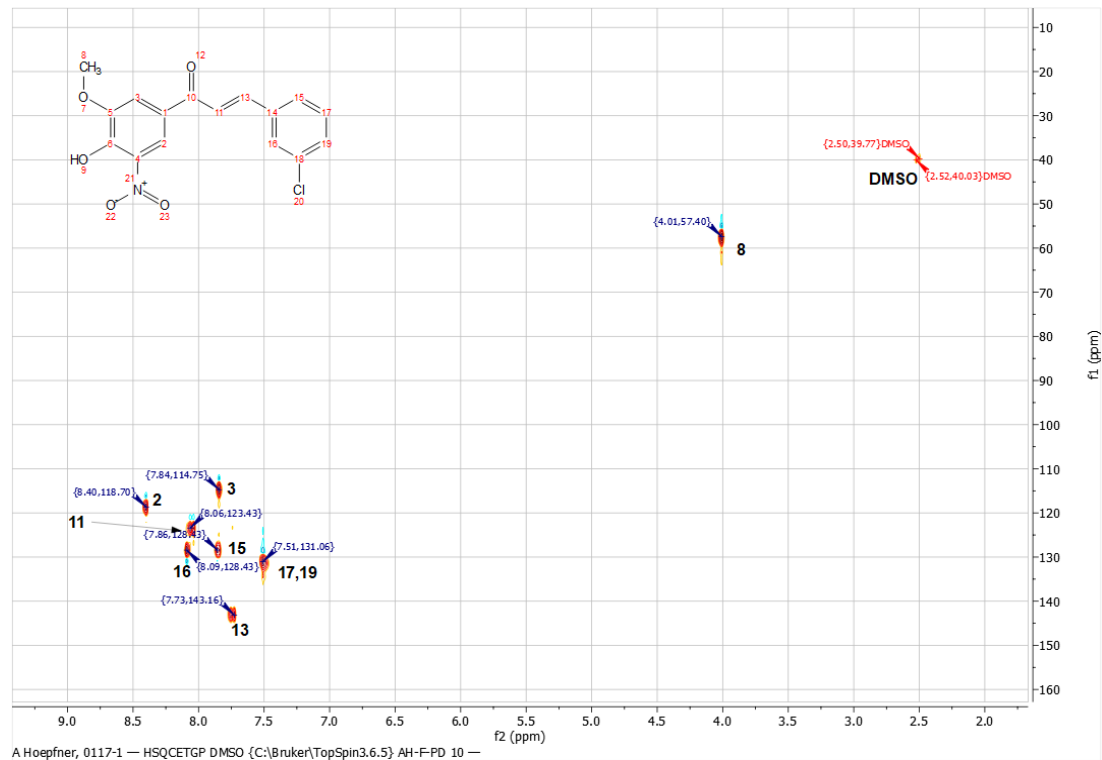
Proton (^1H) NMR spectrum



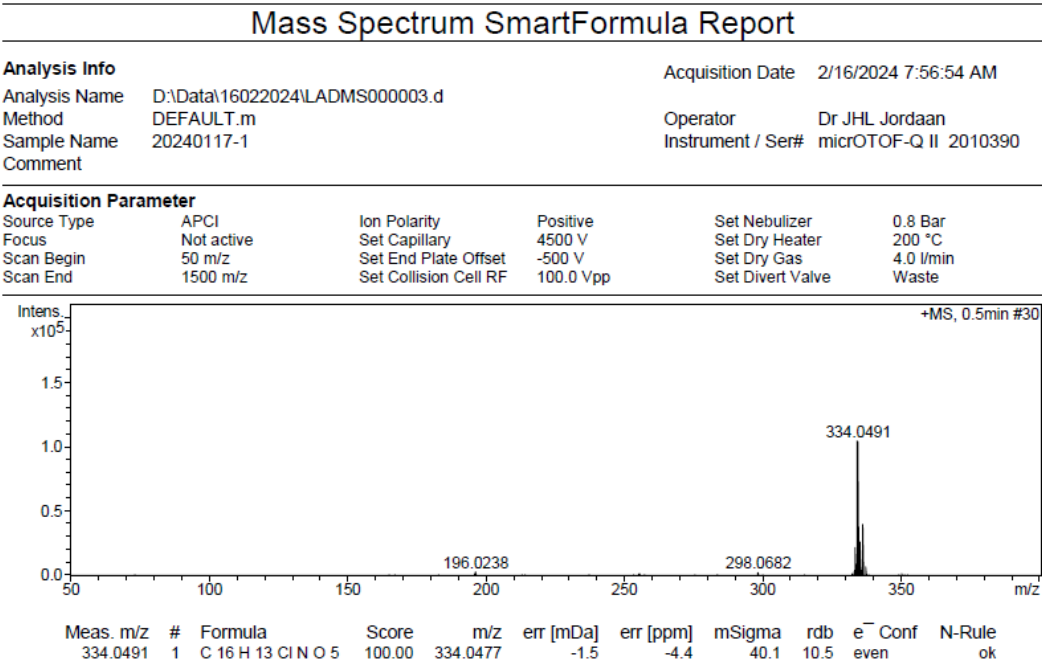
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

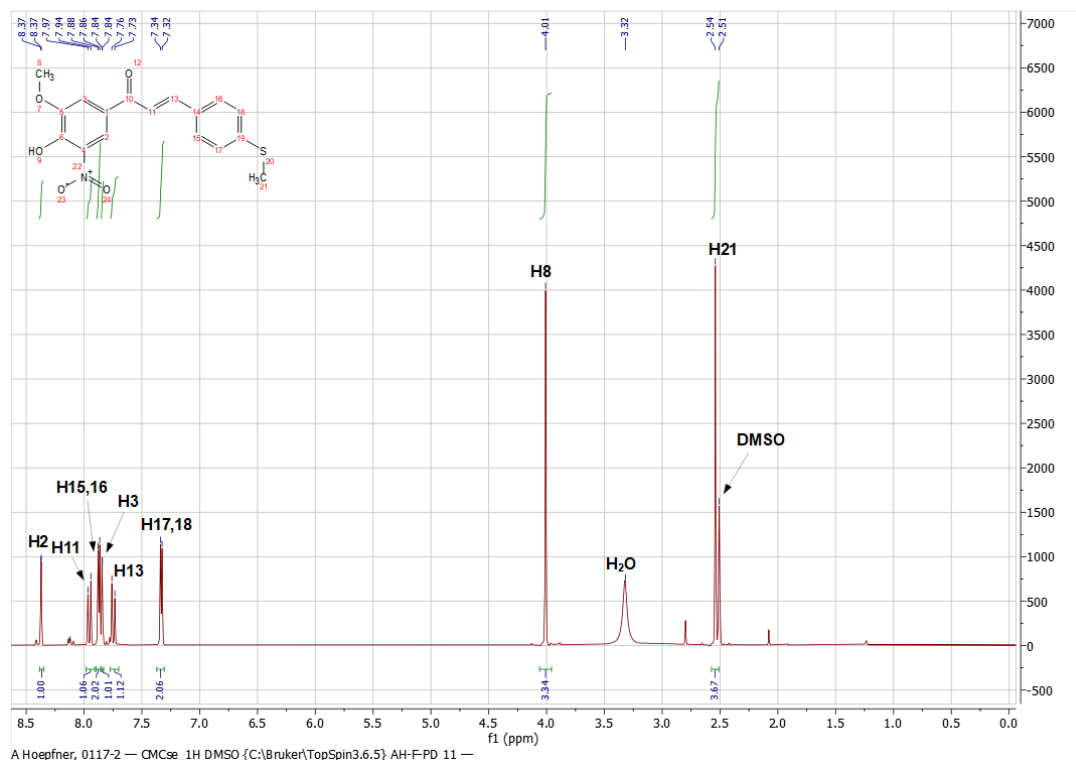


Mass spectrometry (MS) spectrum

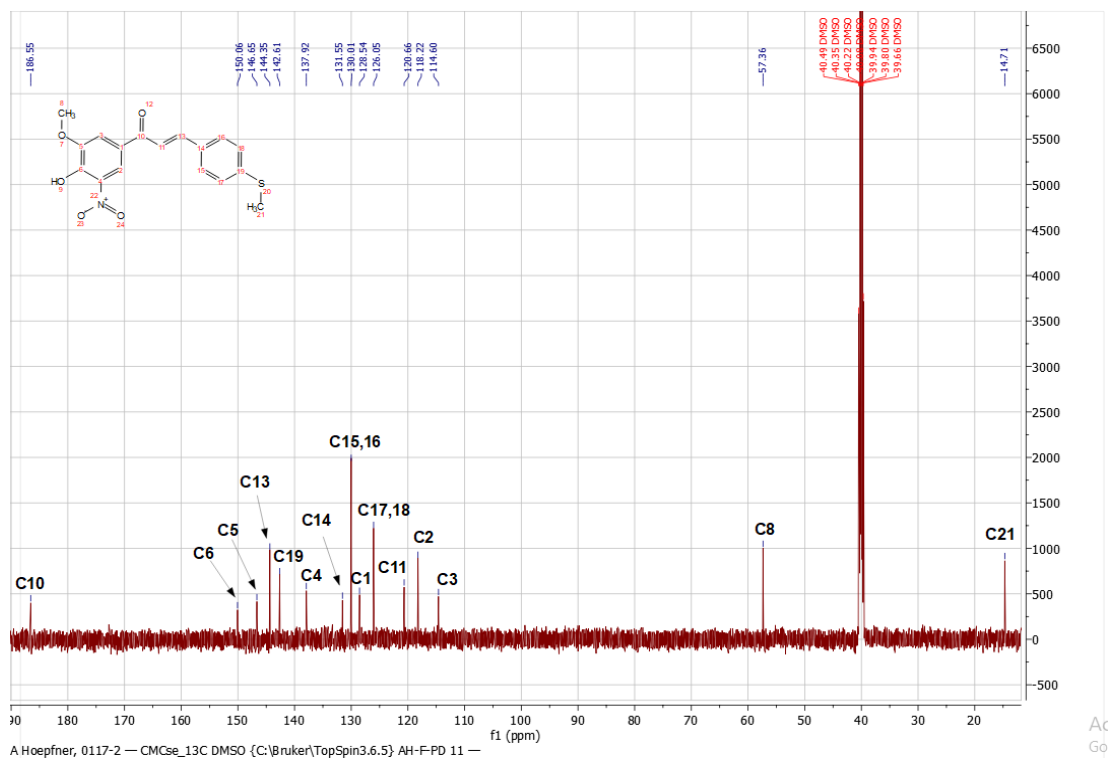


(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-[4-(methylsulfonyl)phenyl]prop-2-en-1-one (2e)

Proton (^1H) NMR spectrum



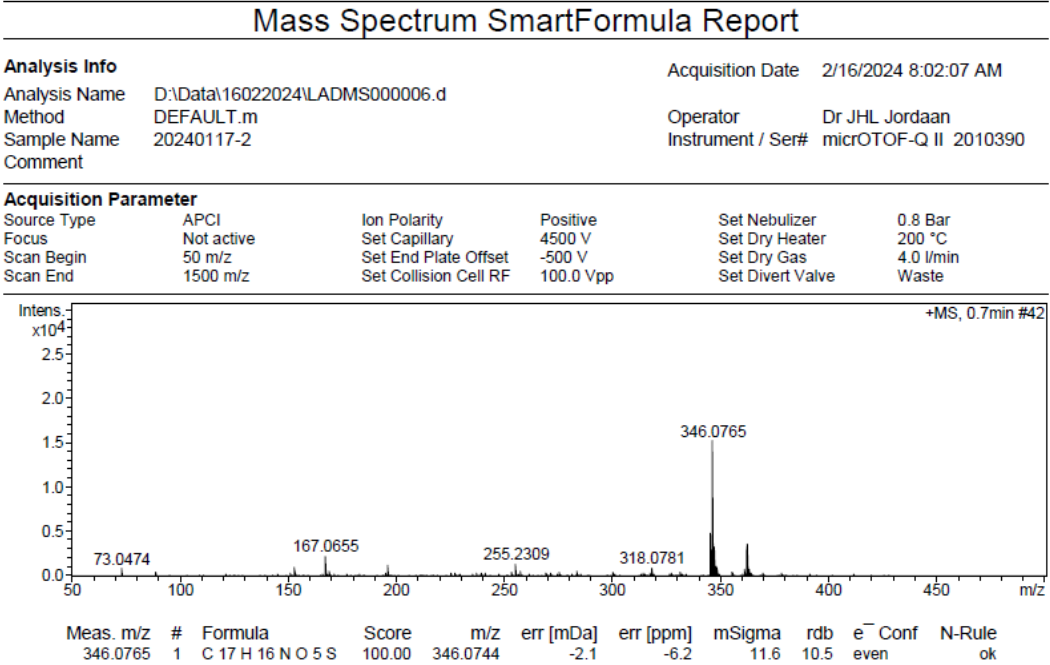
Carbon (^{13}C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

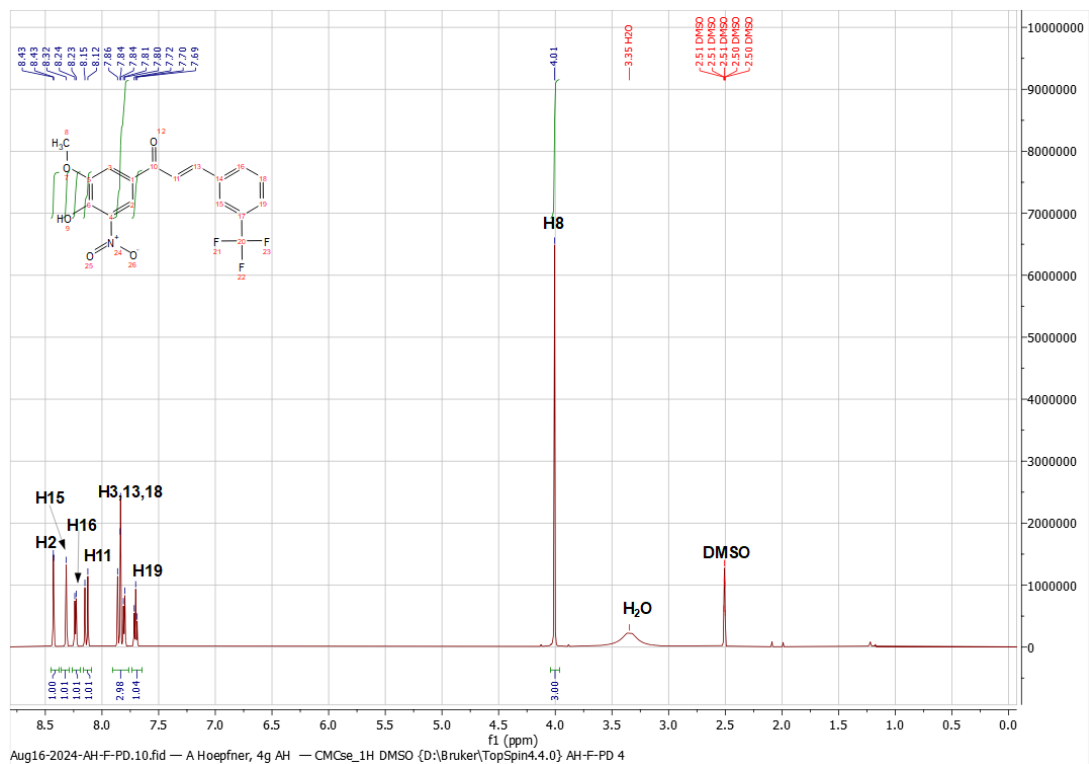


Mass spectrometry (MS) spectrum

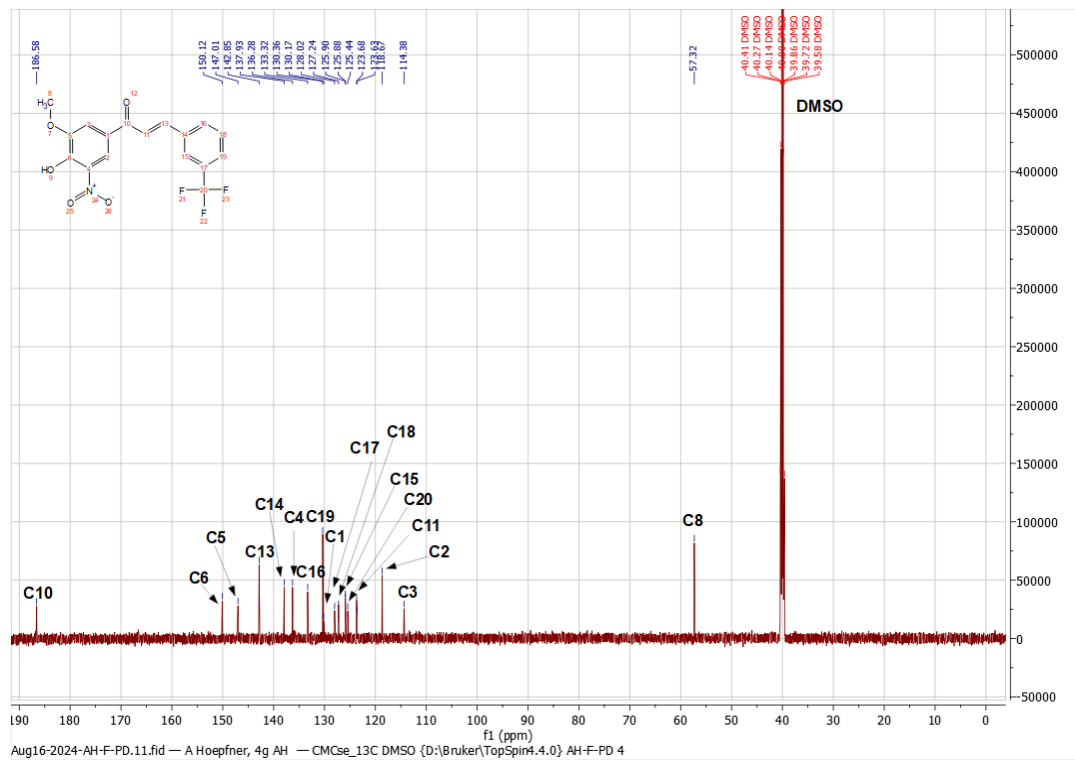


(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (2f)

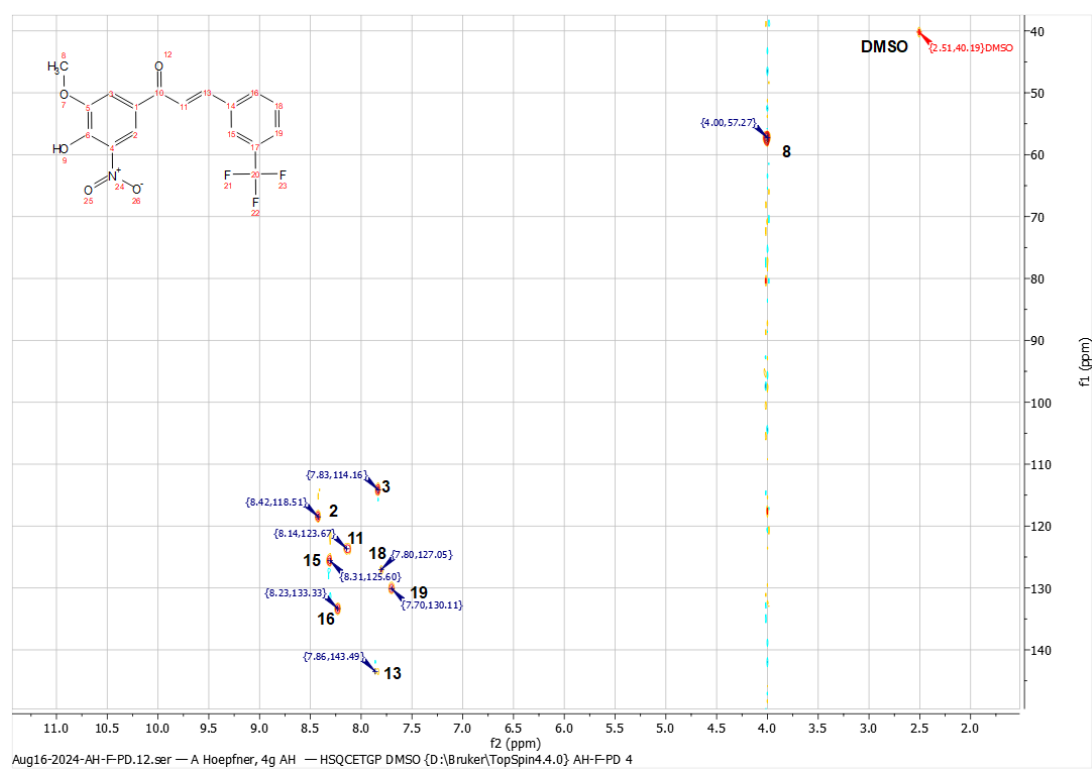
Proton (¹H) NMR spectrum



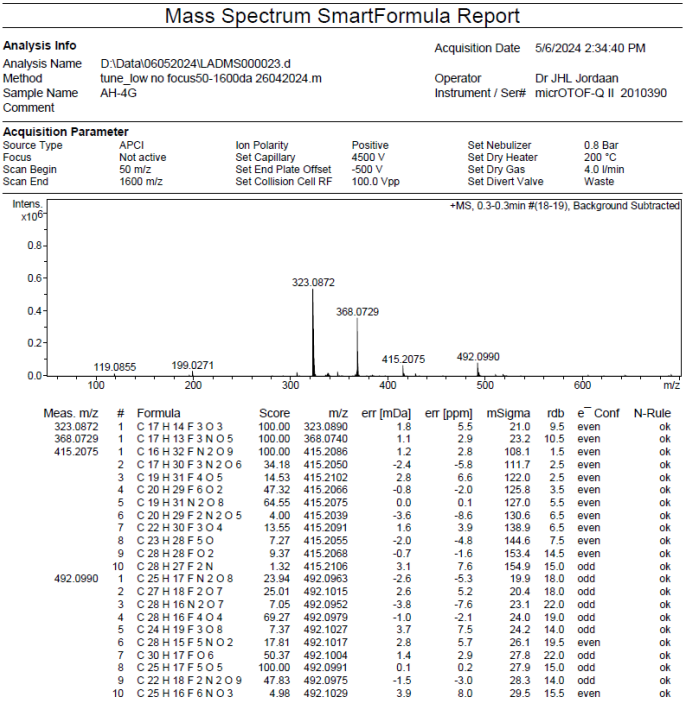
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum

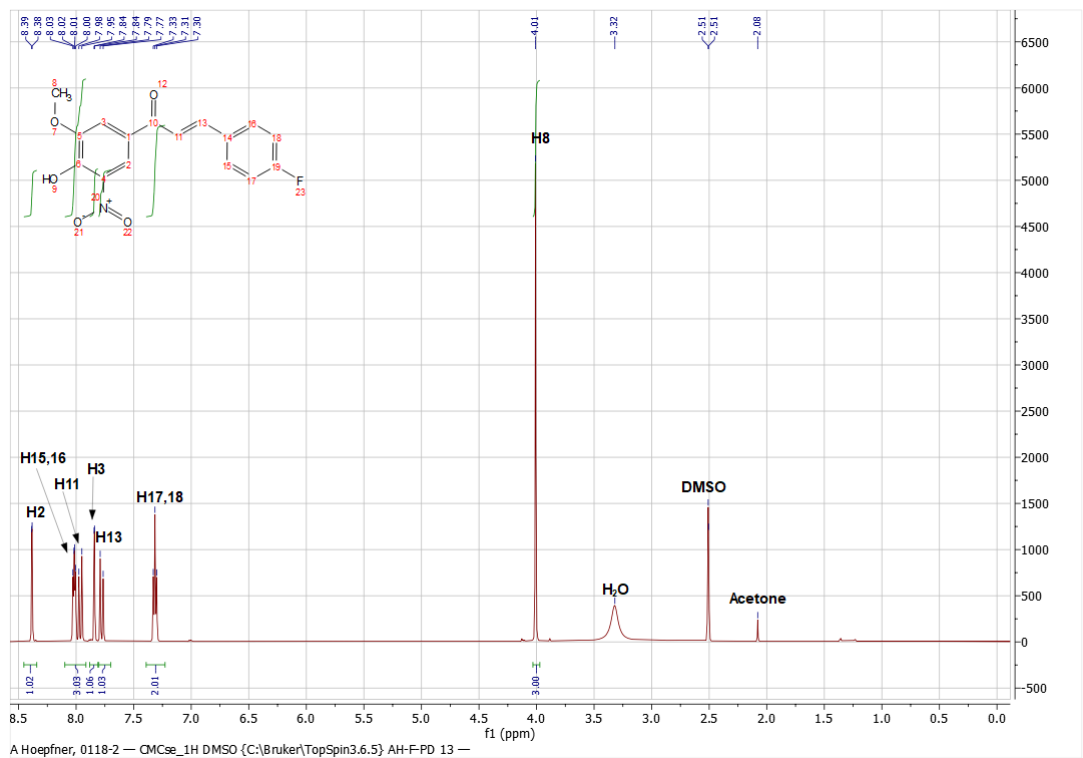


Mass spectrometry (MS) spectrum

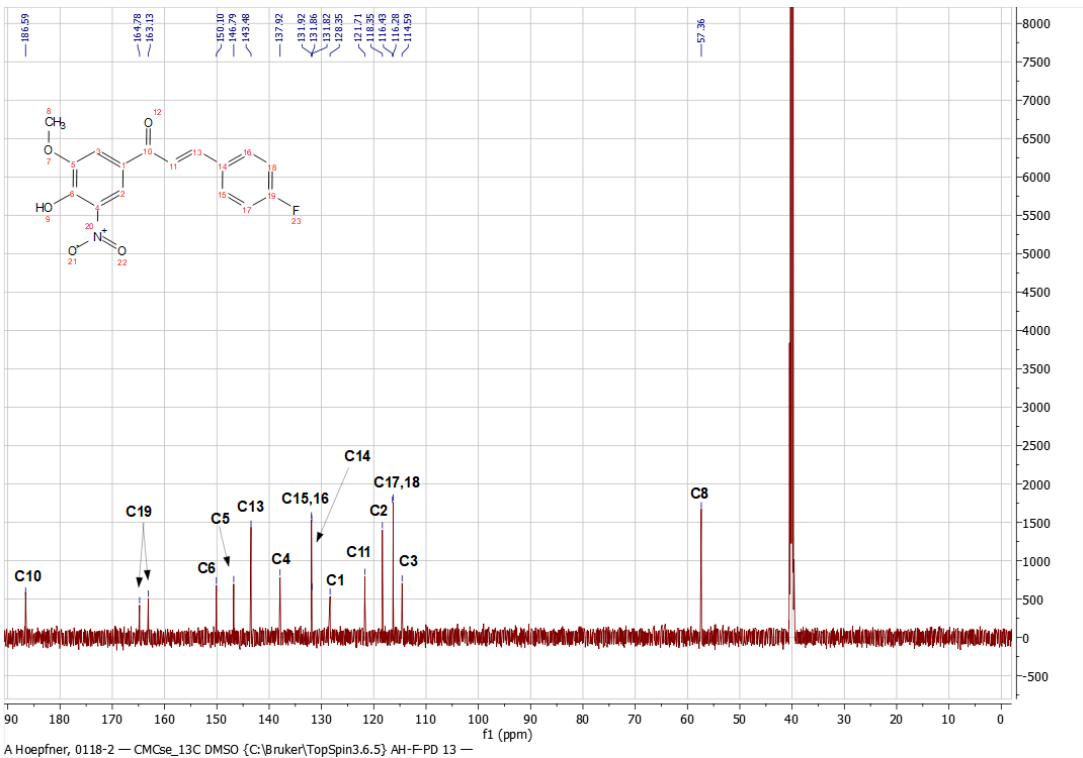


(2E)-3-(4-fluorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2g)

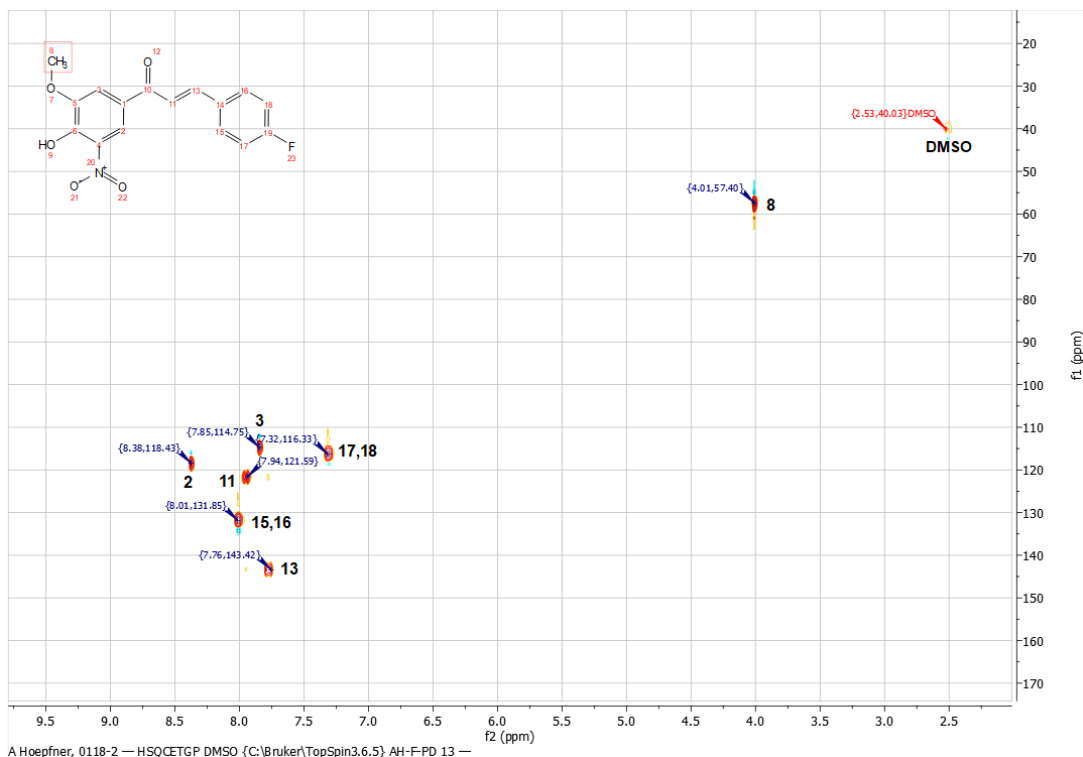
Proton (¹H) NMR spectrum



Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum



Mass spectrometry (MS) spectrum

Mass Spectrum SmartFormula Report

Analysis Info		Acquisition Date	
Analysis Name	D:\Data\16022024\LADMS000007.d	2/16/2024 8:03:13 AM	
Method	DEFAULT.m	Operator	Dr JHL Jordaan
Sample Name	20240118-2	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter					
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

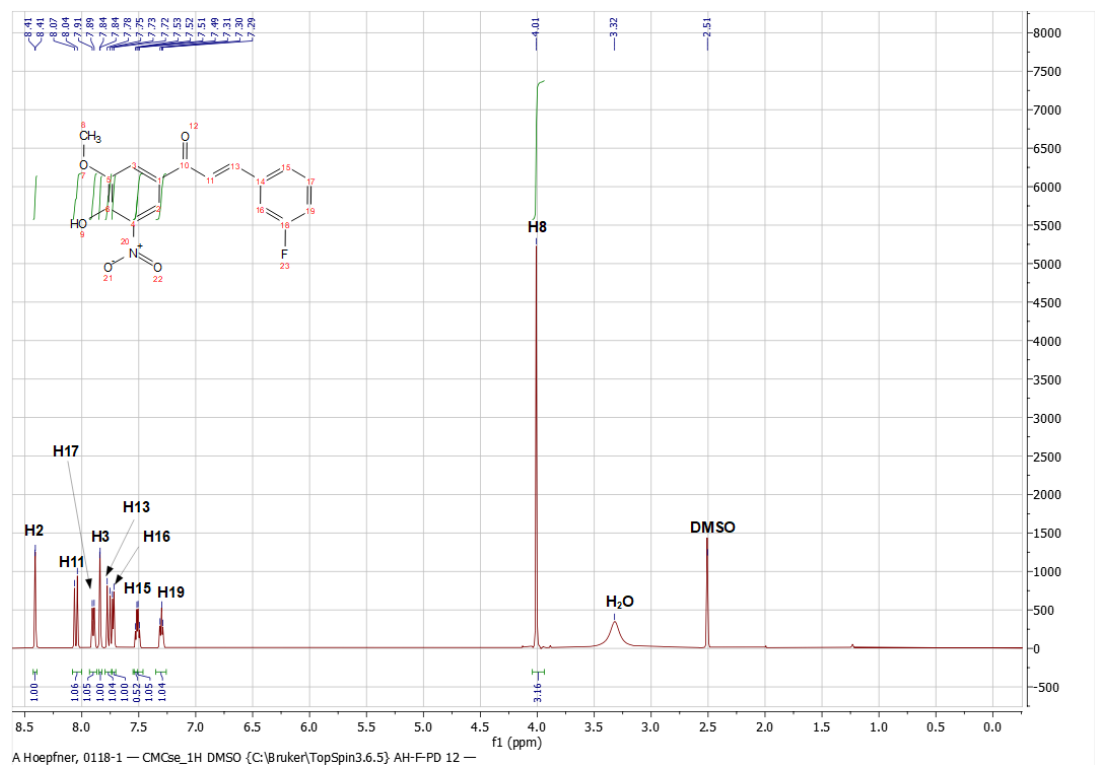
Mass Spectrum Data (Estimated from Plot):

m/z	Intensity (x10 ⁴)
167.0699	~0.2
196.0238	~0.2
255.2312	~0.5
283.2614	~0.5
318.0780	~4.5

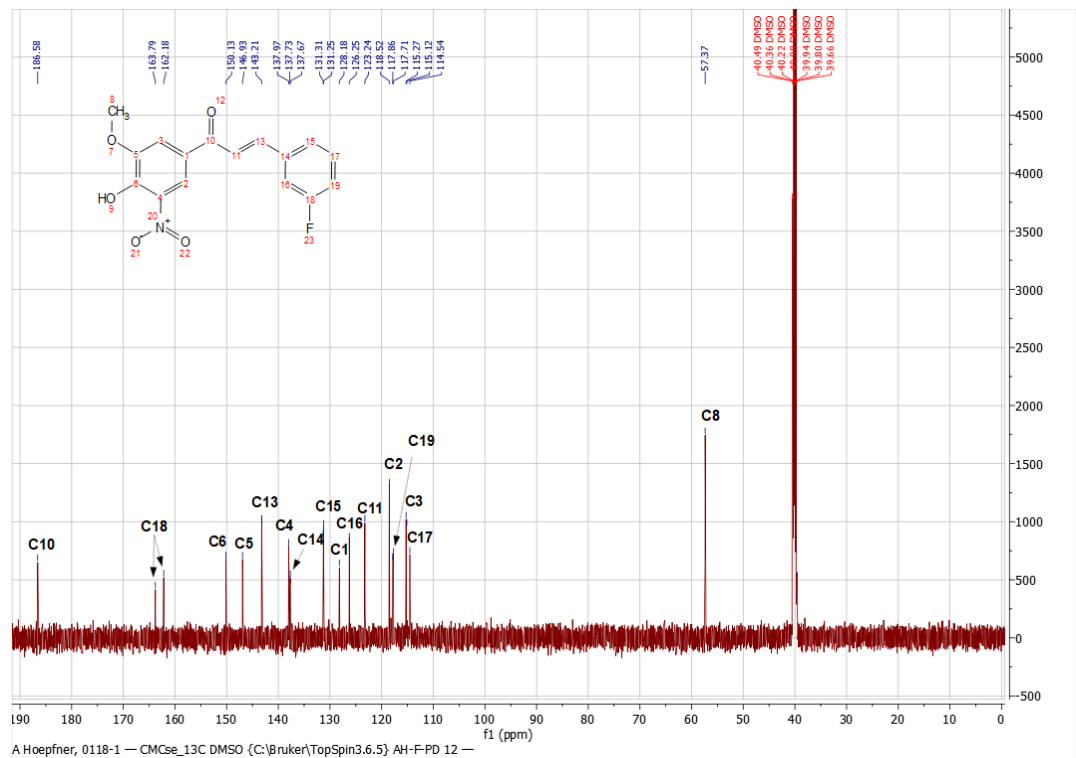
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdB	e ⁻ Conf	N-Rule
318.0780	1	C ₁₆ H ₁₃ FNO ₅	100.00	318.0772	-0.8	-2.4	7.2	10.5	even	ok

(2E)-3-(3-fluorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2h)

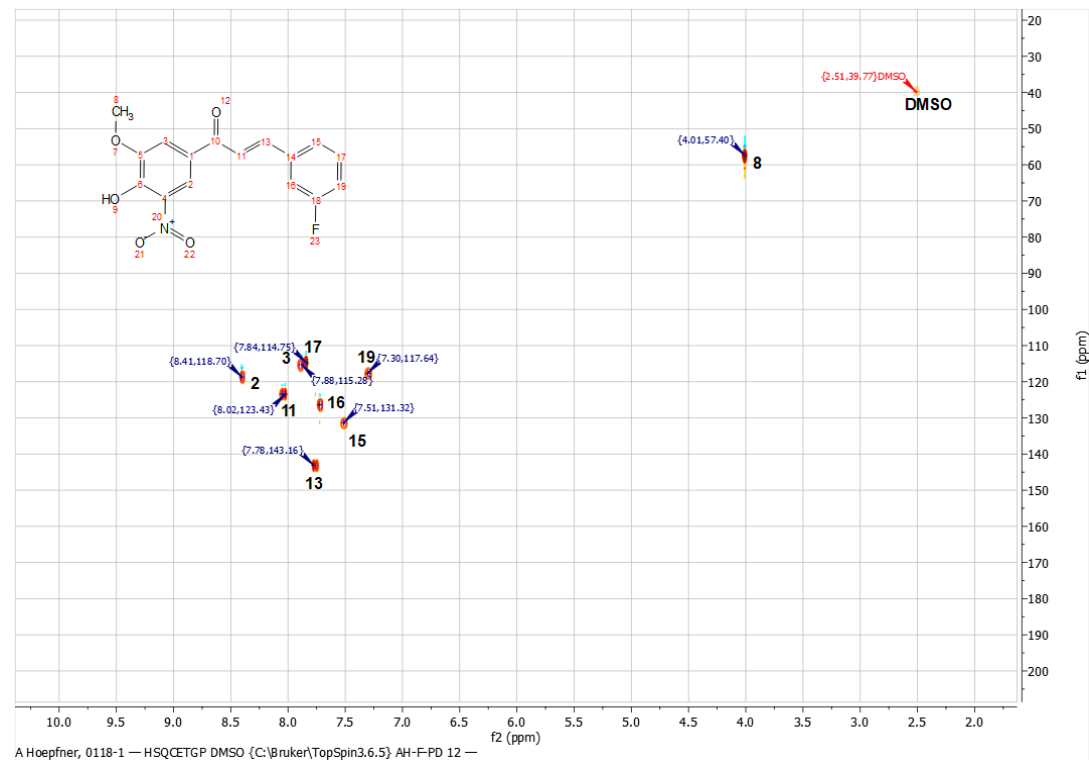
Proton (¹H) NMR spectrum



Carbon (¹³C) NMR spectrum

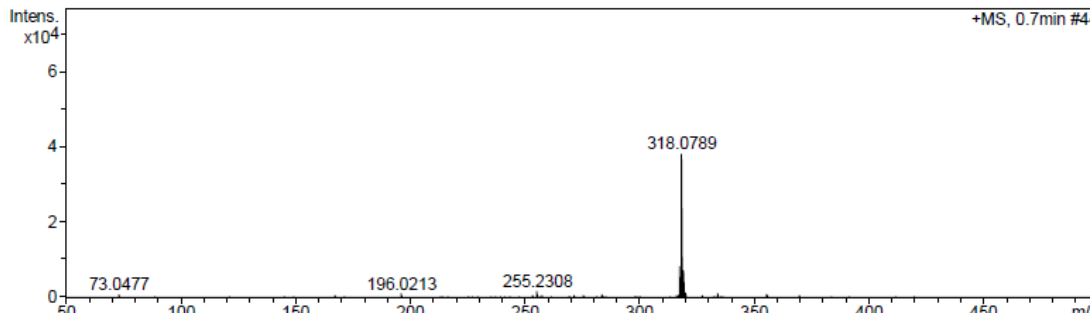


Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum



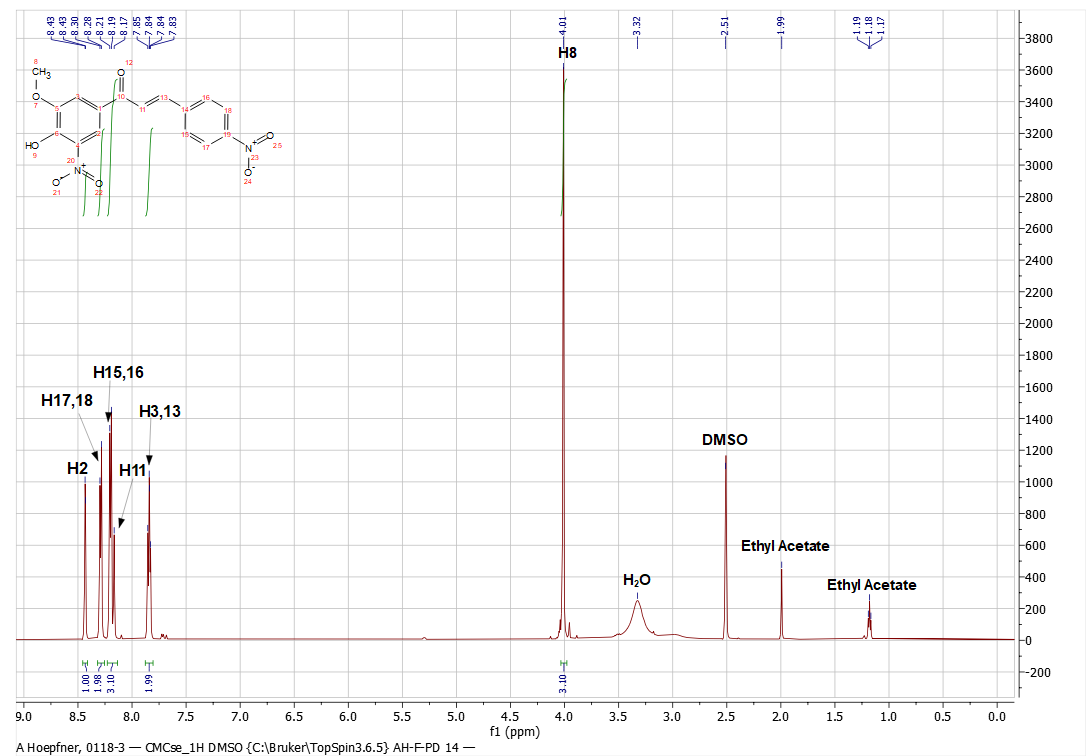
Mass spectrometry (MS) spectrum

Mass Spectrum SmartFormula Report

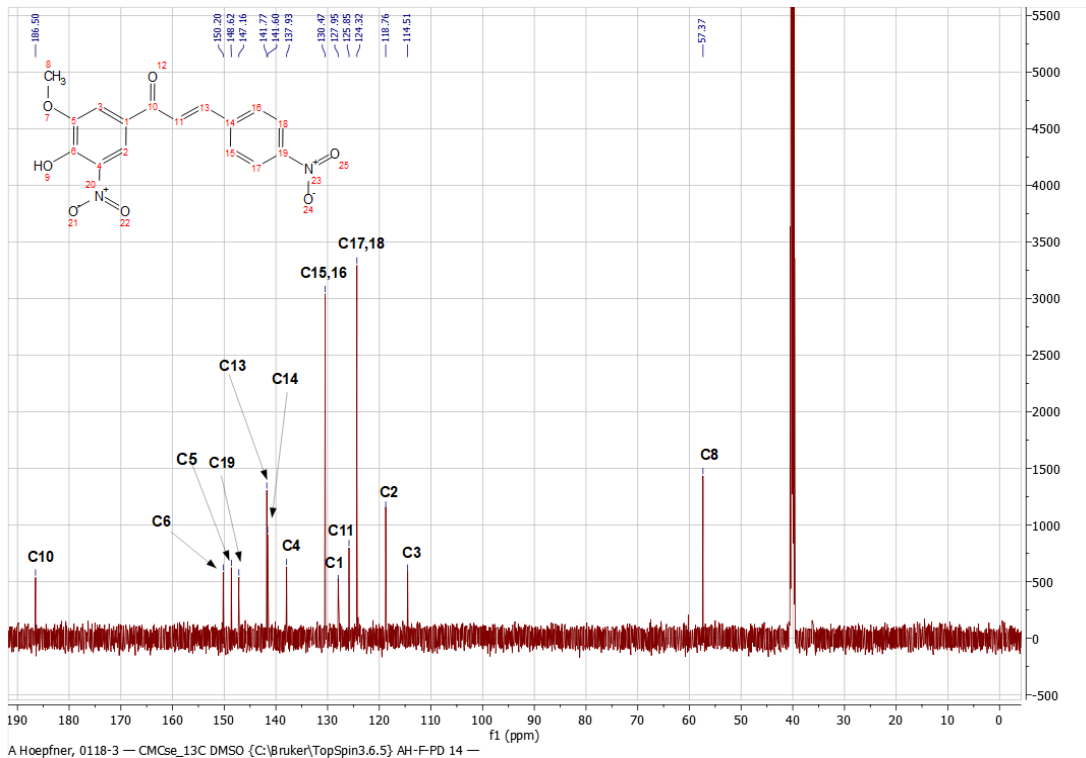
Analysis Info			Acquisition Date 2/16/2024 7:58:00 AM							
Analysis Name	D:\Data\16022024\LADMS000004.d		Operator	Dr JHL Jordaan						
Method	DEFAULT.m		Instrument / Ser#	micrOTOF-Q II 2010390						
Sample Name	20240118-1									
Comment										
Acquisition Parameter										
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar					
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C					
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min					
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste					
										
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e ⁻ Conf	N-Rule
318.0789	1	C 16 H 13 F N O 5	100.00	318.0772	-1.6	-5.1	5.6	10.5	even	ok

(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-(4-nitrophenyl)prop-2-en-1-one (2i)

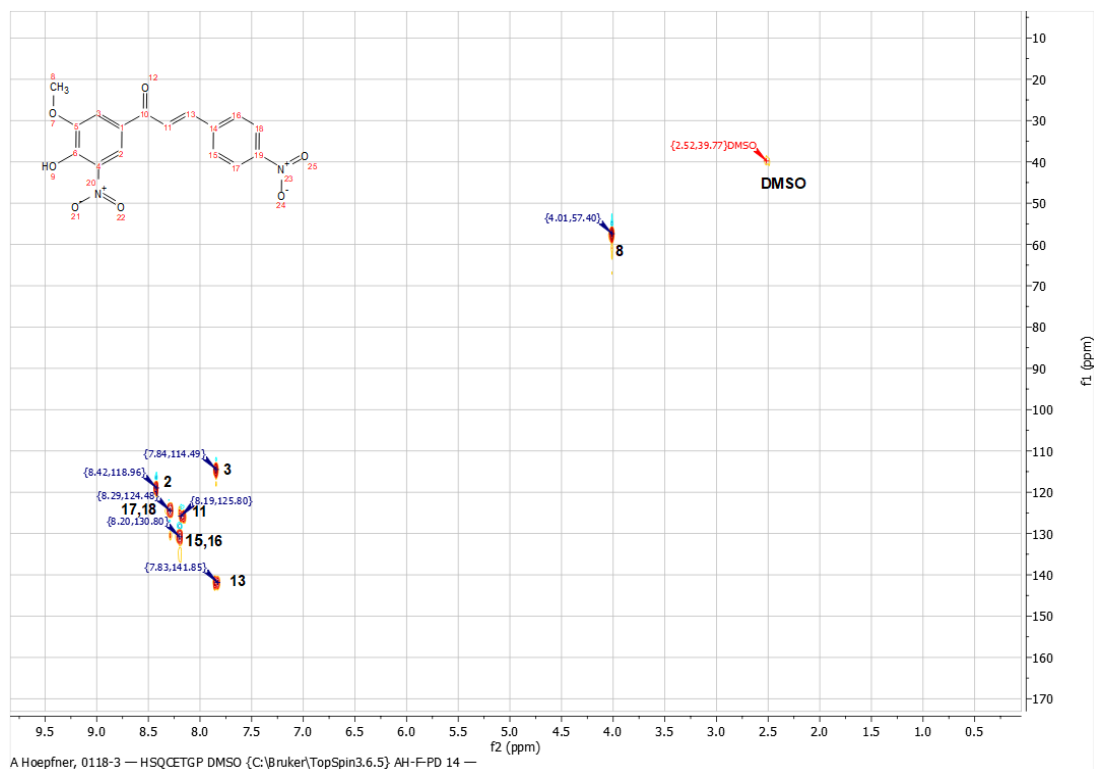
Proton (¹H) NMR spectrum



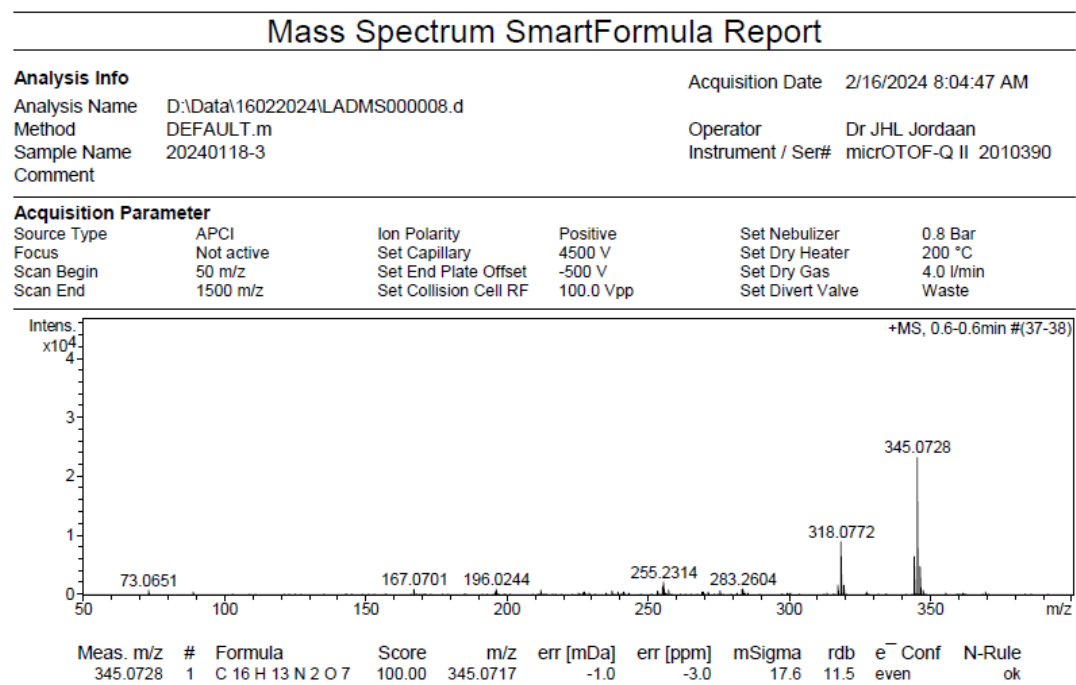
Carbon (¹³C) NMR spectrum



Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum



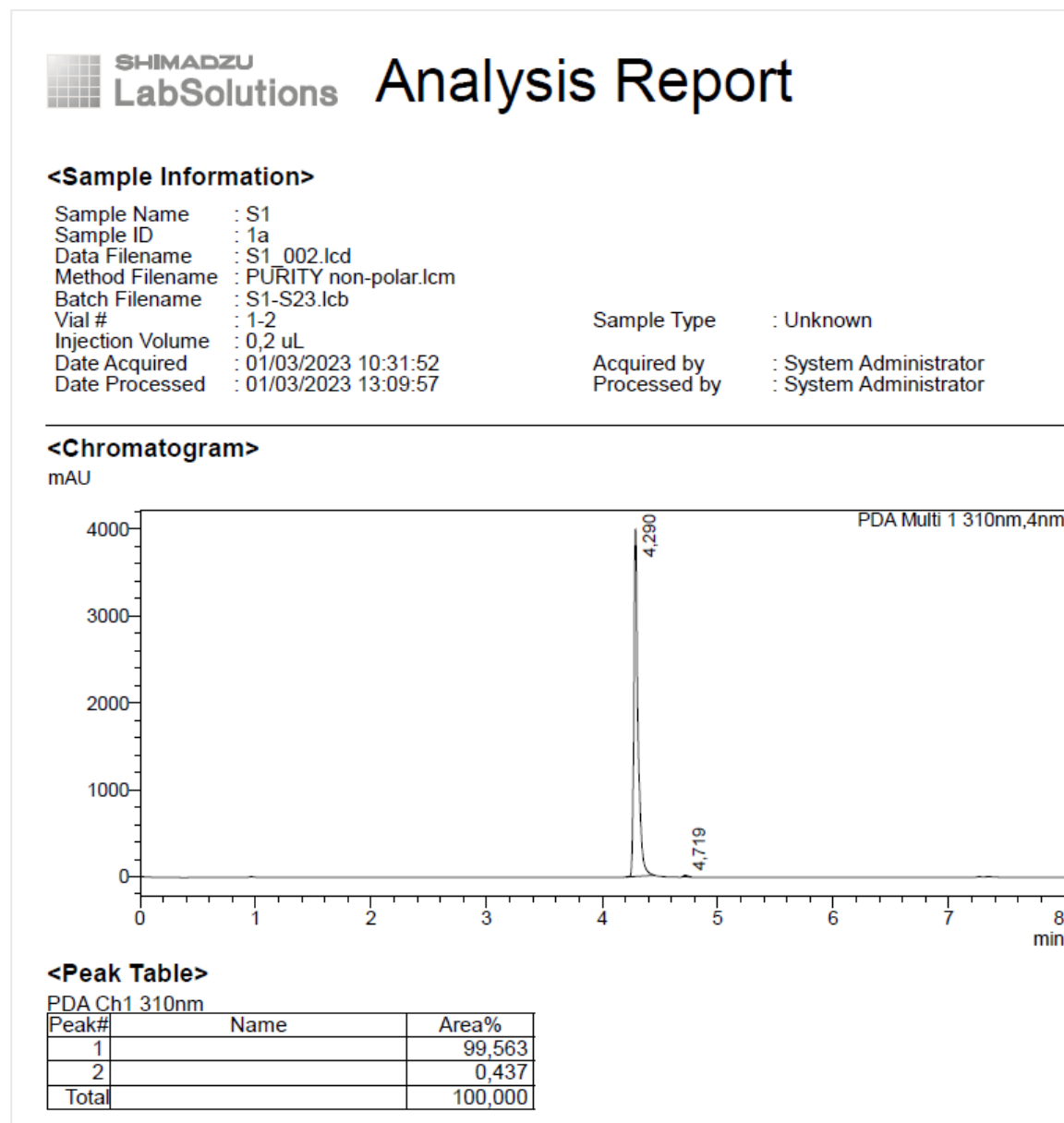
Mass spectrometry (MS) spectrum



ANNEXURE C

Article 1: High-performance liquid chromatography (HPLC) results of compounds

(2E)-3-(4-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3a)



(2E)-3-(2,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3b)



Analysis Report

<Sample Information>

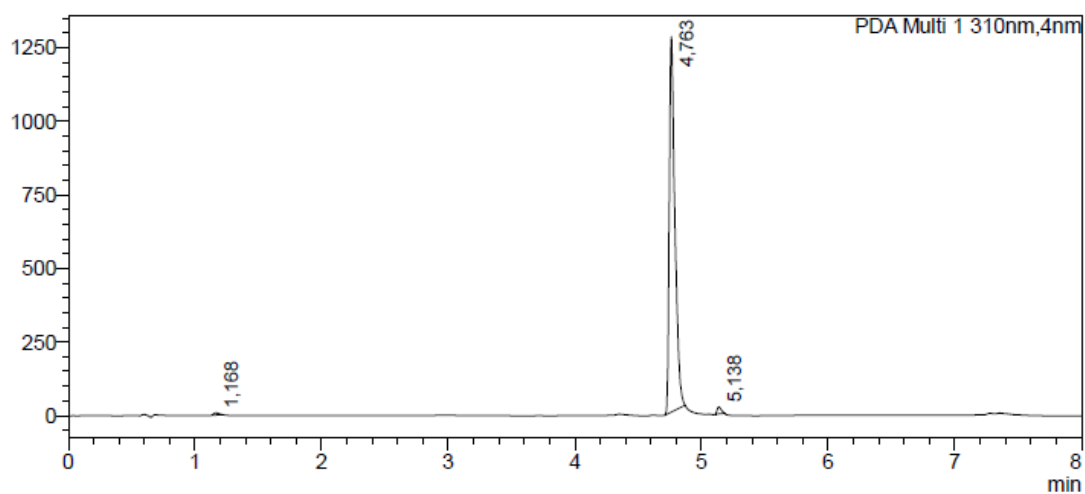
Sample Name : S2
Sample ID : 1b
Data Filename : S2_003.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-3
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 10:40:14
Date Processed : 01/03/2023 10:48:16

Sample Type : Unknown

Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		0,446
2		98,251
3		1,303
Total		100,000

(2E)-3-(3,4-dichlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3c)



Analysis Report

<Sample Information>

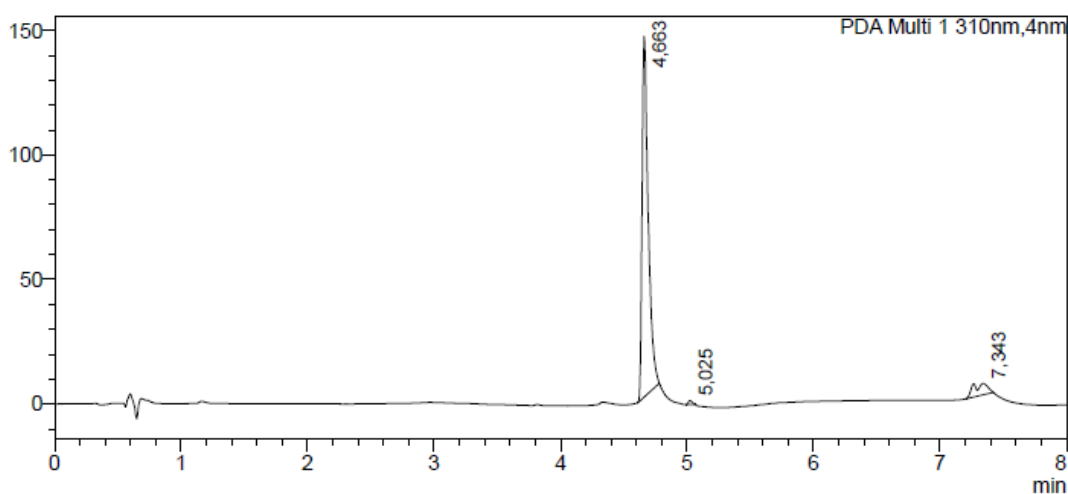
Sample Name : S3
Sample ID : 1e
Data Filename : S3_004.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-4
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 10:48:35
Date Processed : 01/03/2023 13:16:04

Sample Type : Unknown

Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		92,381
2		0,612
3		7,007
Total		100,000

(2E)-3-(3-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3d)



Analysis Report

<Sample Information>

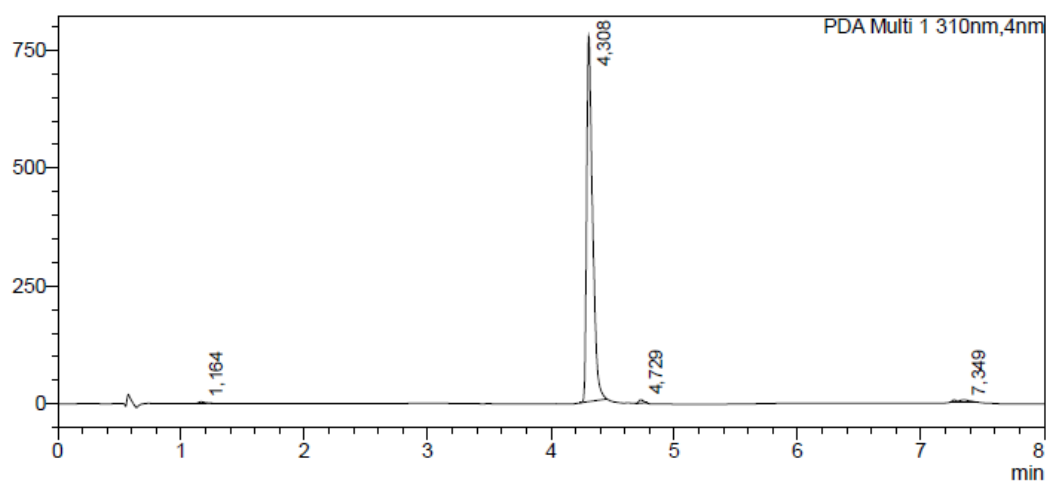
Sample Name : S22
Sample ID : 1f
Data Filename : S22_023.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-23
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 13:27:32
Date Processed : 01/03/2023 13:35:34

Sample Type : Unknown

Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		0,457
2		97,474
3		0,641
4		1,427
Total		100,000

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-ethoxyphenyl)prop-2-en-1-one (3e)



Analysis Report

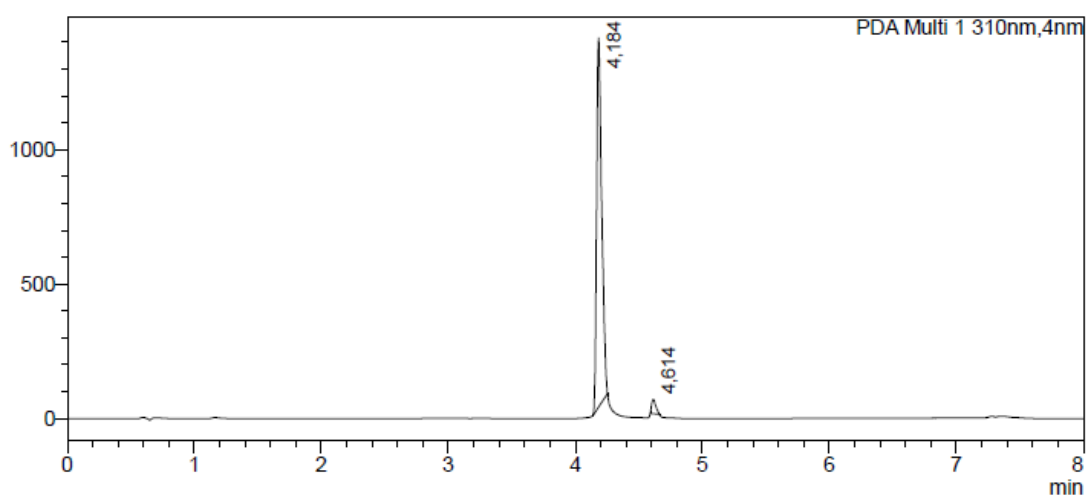
<Sample Information>

Sample Name : S4
Sample ID : 1h
Data Filename : S4_005.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-5
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 10:56:57
Date Processed : 01/03/2023 11:04:59

Sample Type : Unknown
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		96,940
2		3,060
Total		100,000

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-iodophenyl)prop-2-en-1-one (3f)



Analysis Report

<Sample Information>

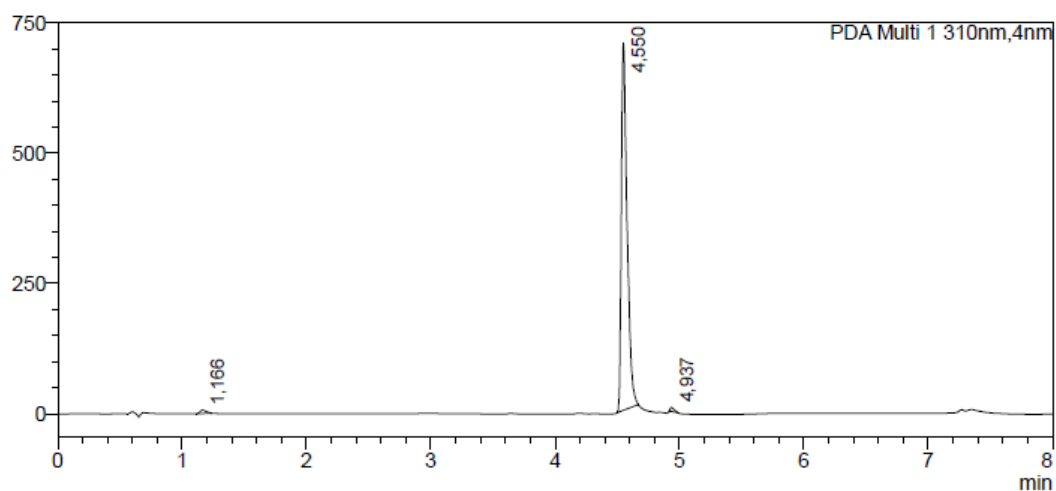
Sample Name : S5
Sample ID : 1i
Data Filename : S5_006.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-6
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 11:05:20
Date Processed : 01/03/2023 13:30:34

Sample Type : Unknown

Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		0,972
2		98,322
3		0,706
Total		100,000

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-[4-(methylsulfonyl)phenyl]prop-2-en-1-one
(3g)



SHIMADZU
LabSolutions

Analysis Report

<Sample Information>

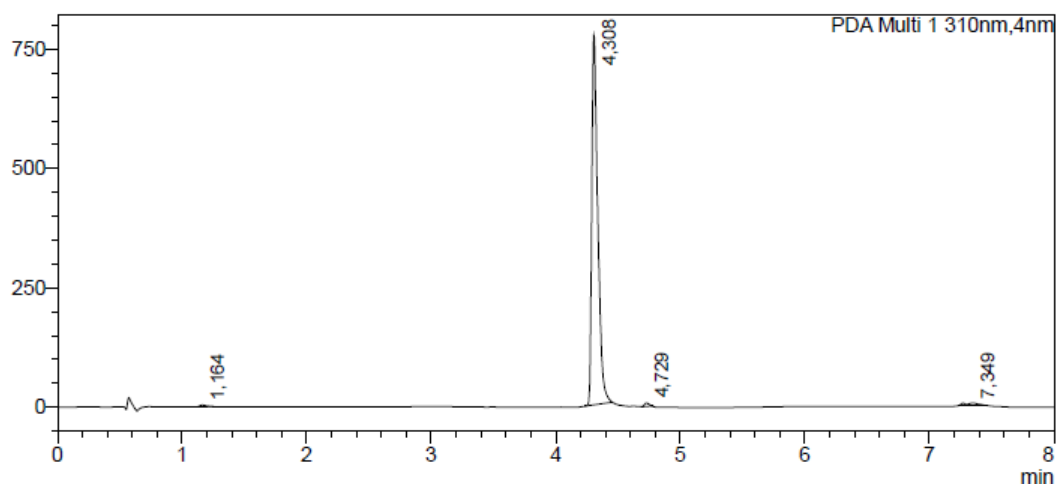
Sample Name : S22
Sample ID : 1f
Data Filename : S22_023.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-23
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 13:27:32
Date Processed : 01/03/2023 13:35:34

Sample Type : Unknown

Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		0,457
2		97,474
3		0,641
4		1,427
Total		100,000

(2E)-3-(4-bromo-2-fluorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (3h)



SHIMADZU
LabSolutions

Analysis Report

<Sample Information>

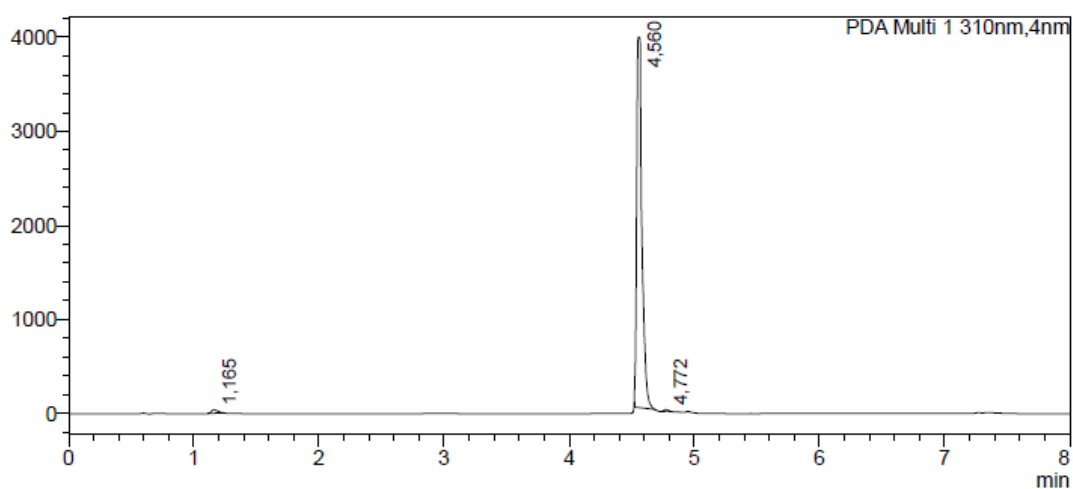
Sample Name : S7
Sample ID : 1k
Data Filename : S7_008.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-8
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 11:22:05
Date Processed : 01/03/2023 11:30:06

Sample Type : Unknown

Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		1,112
2		98,603
3		0,285
Total		100,000

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (3i)



SHIMADZU
LabSolutions

Analysis Report

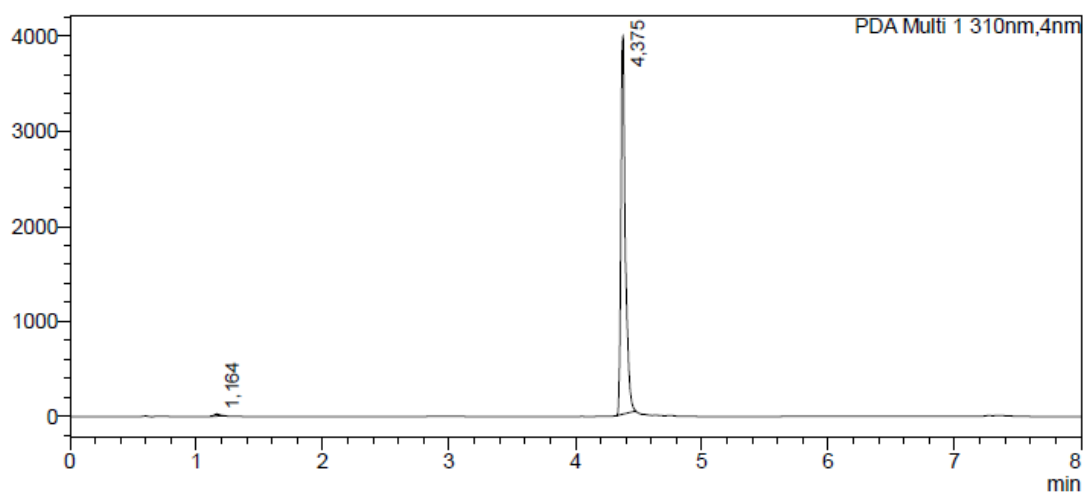
<Sample Information>

Sample Name : S8
Sample ID : 1I
Data Filename : S8_009.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-9
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 11:30:26
Date Processed : 01/03/2023 11:38:28

Sample Type : Unknown
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		0,765
2		99,235
Total		100,000

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(3-fluorophenyl)prop-2-en-1-one (3j)



SHIMADZU
LabSolutions

Analysis Report

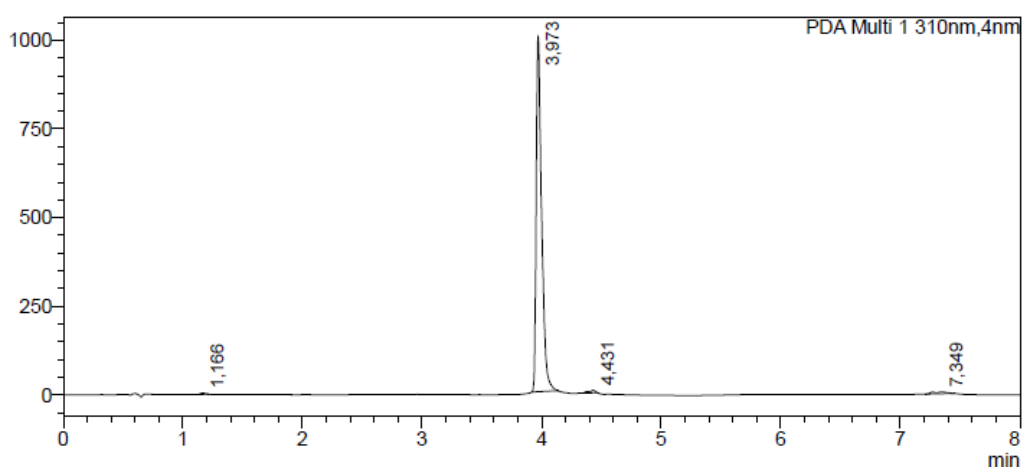
<Sample Information>

Sample Name : S9
Sample ID : 1m
Data Filename : S9_010.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-10
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 11:38:49
Date Processed : 01/03/2023 11:46:50

Sample Type : Unknown
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 310nm

Peak#	Name	Area%
1		0,352
2		97,506
3		0,816
4		1,326
Total		100,000

(2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(4-fluorophenyl)prop-2-en-1-one (3k)



Analysis Report

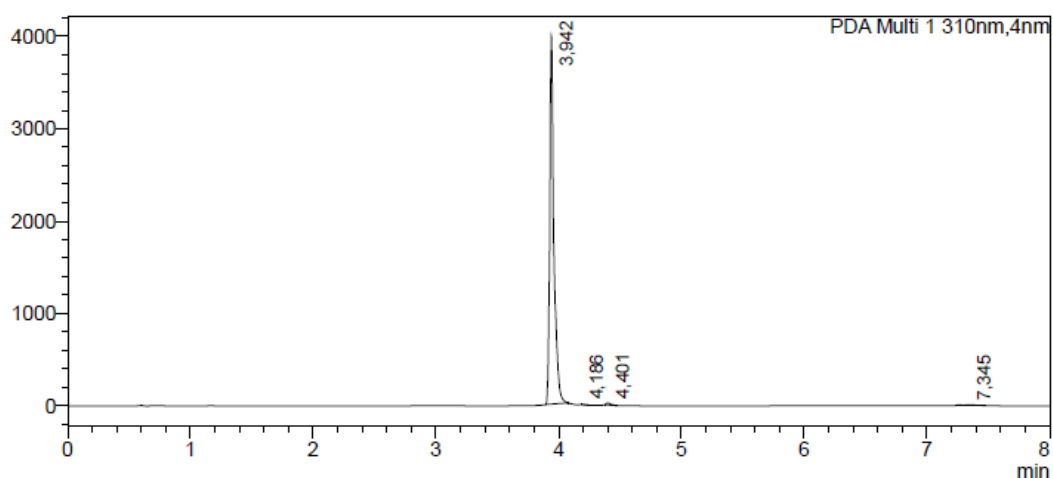
<Sample Information>

Sample Name : S10
Sample ID : 1n
Data Filename : S10_011.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-11
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 11:47:10
Date Processed : 01/03/2023 11:55:11

Sample Type : Unknown
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU

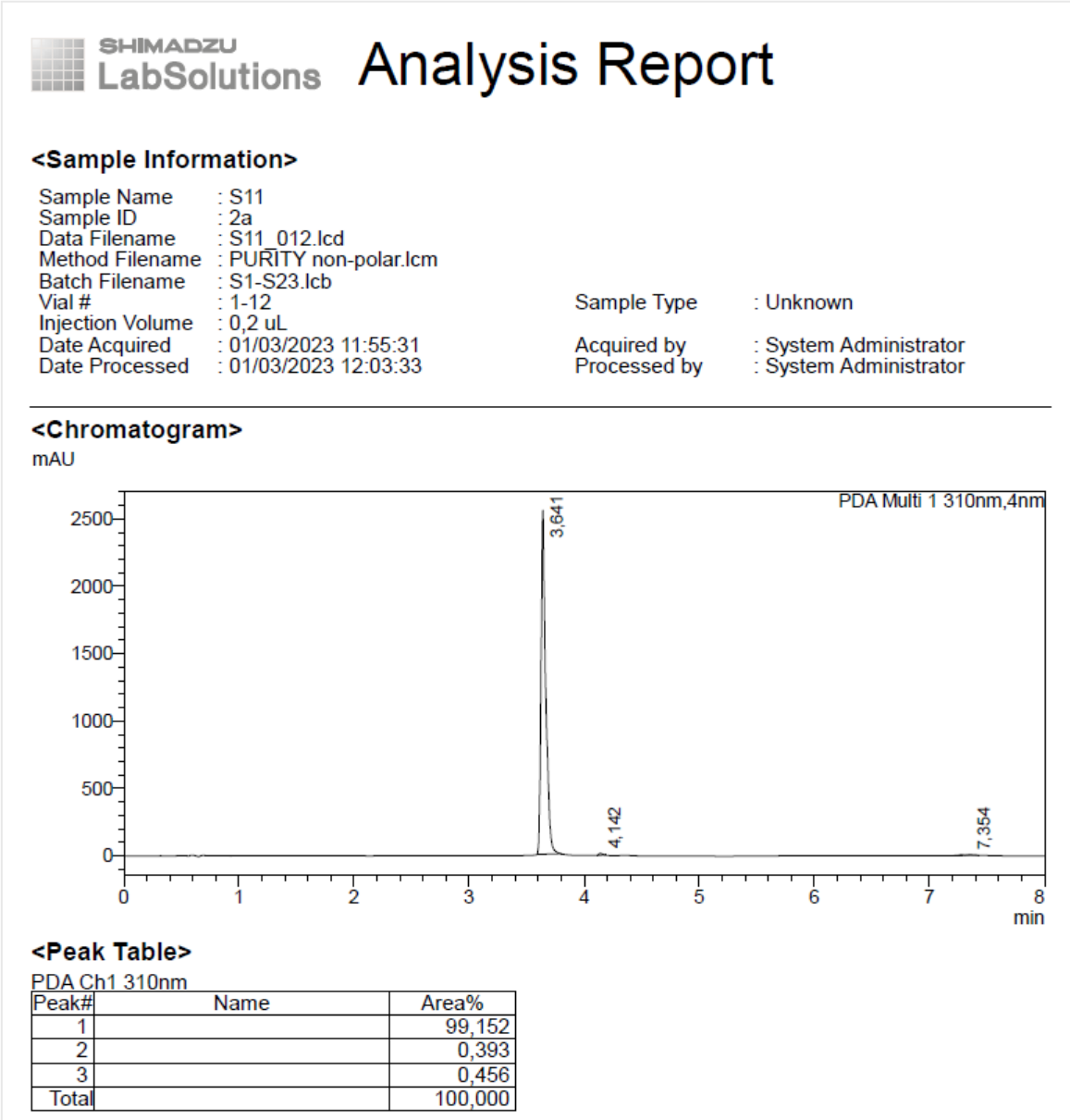


<Peak Table>

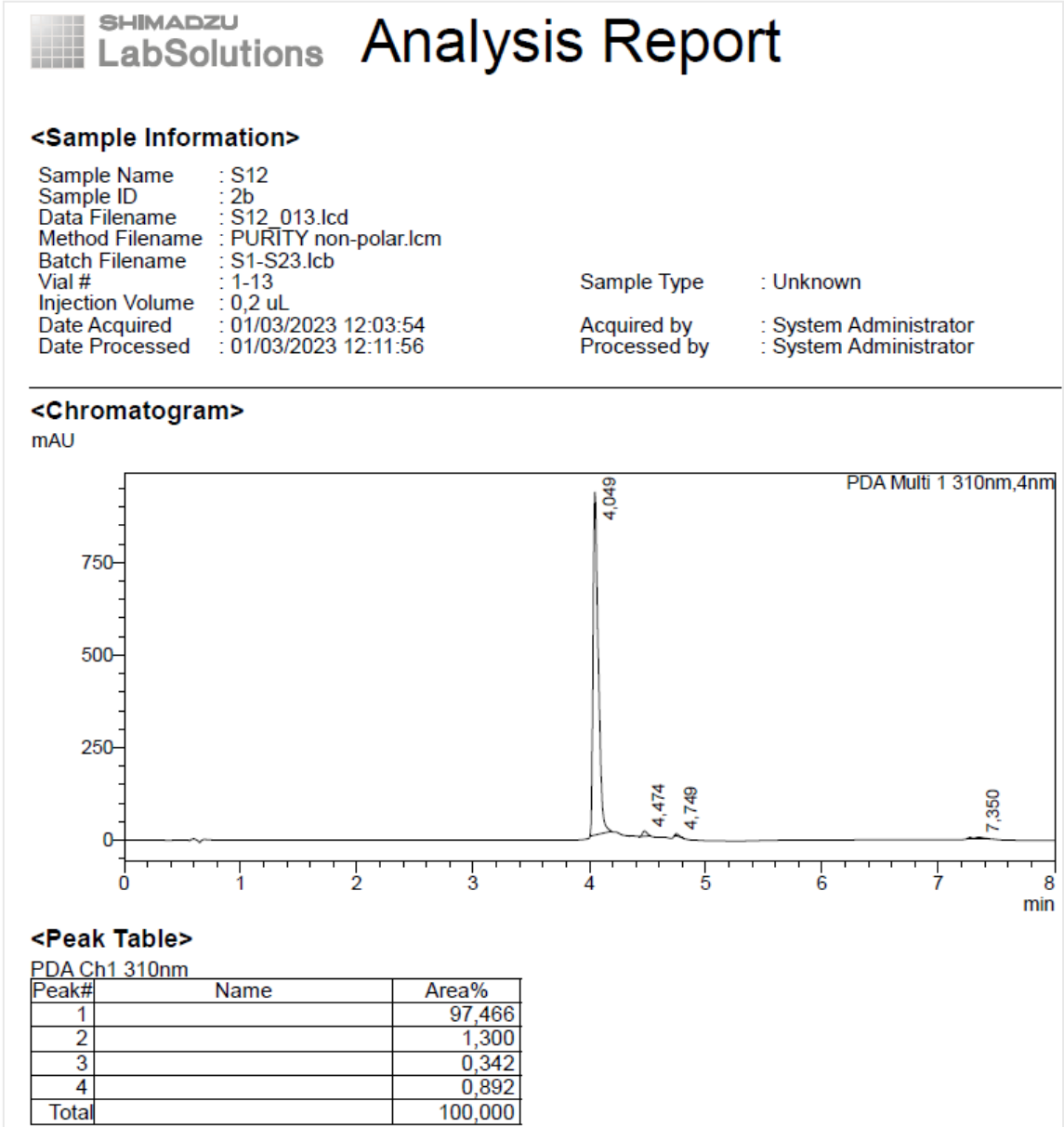
PDA Ch1 310nm

Peak#	Name	Area%
1		98,973
2		0,008
3		0,559
4		0,460
Total		100,000

1-[5-(4-chlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4a)



1-[5-(2,4-dichlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4b)



1-[5-(3,4-dichlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4c)



SHIMADZU
LabSolutions

Analysis Report

<Sample Information>

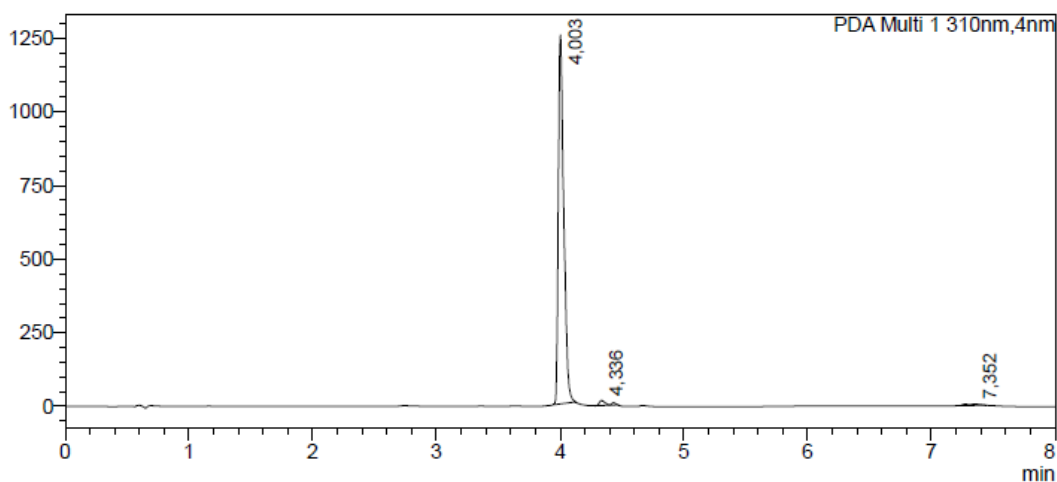
Sample Name : S13
Sample ID : 2e
Data Filename : S13_014.lcd
Method Filename : PURITY non-polar.lcm
Batch Filename : S1-S23.lcb
Vial # : 1-14
Injection Volume : 0,2 uL
Date Acquired : 01/03/2023 12:12:16
Date Processed : 01/03/2023 12:20:17

Sample Type : Unknown

Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU

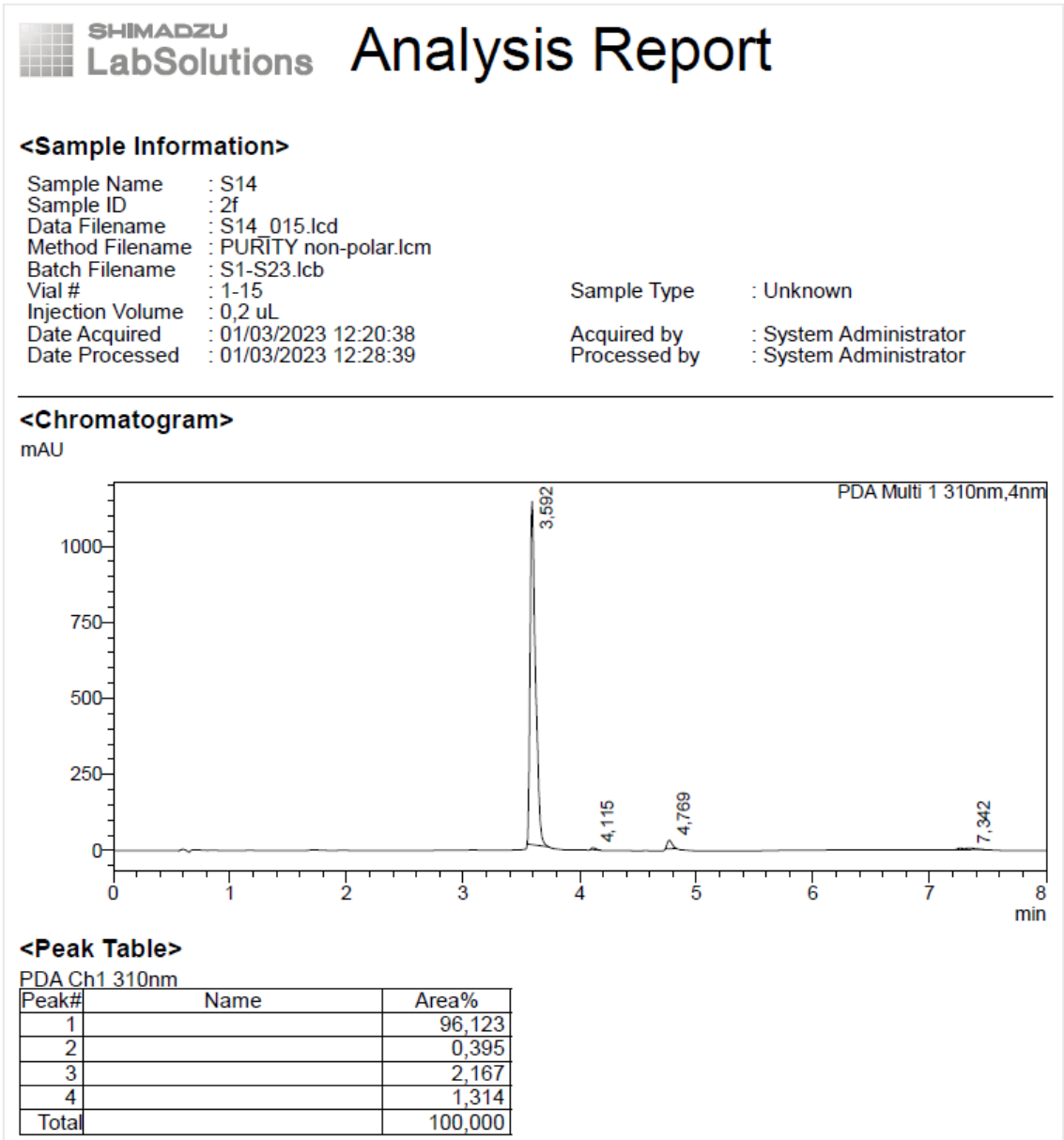


<Peak Table>

PDA Ch1 310nm

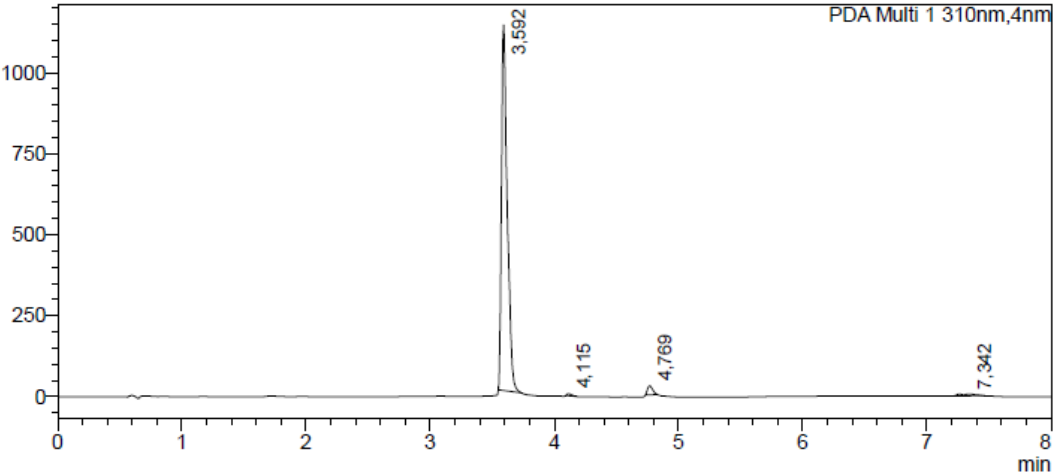
Peak#	Name	Area%
1		97,110
2		1,896
3		0,993
Total		100,000

1-[5-(3-chlorophenyl)-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4d)



<Chromatogram>

mAU



PDA Multi 1 310nm,4nm

3.592

4.115

4.769

7.342

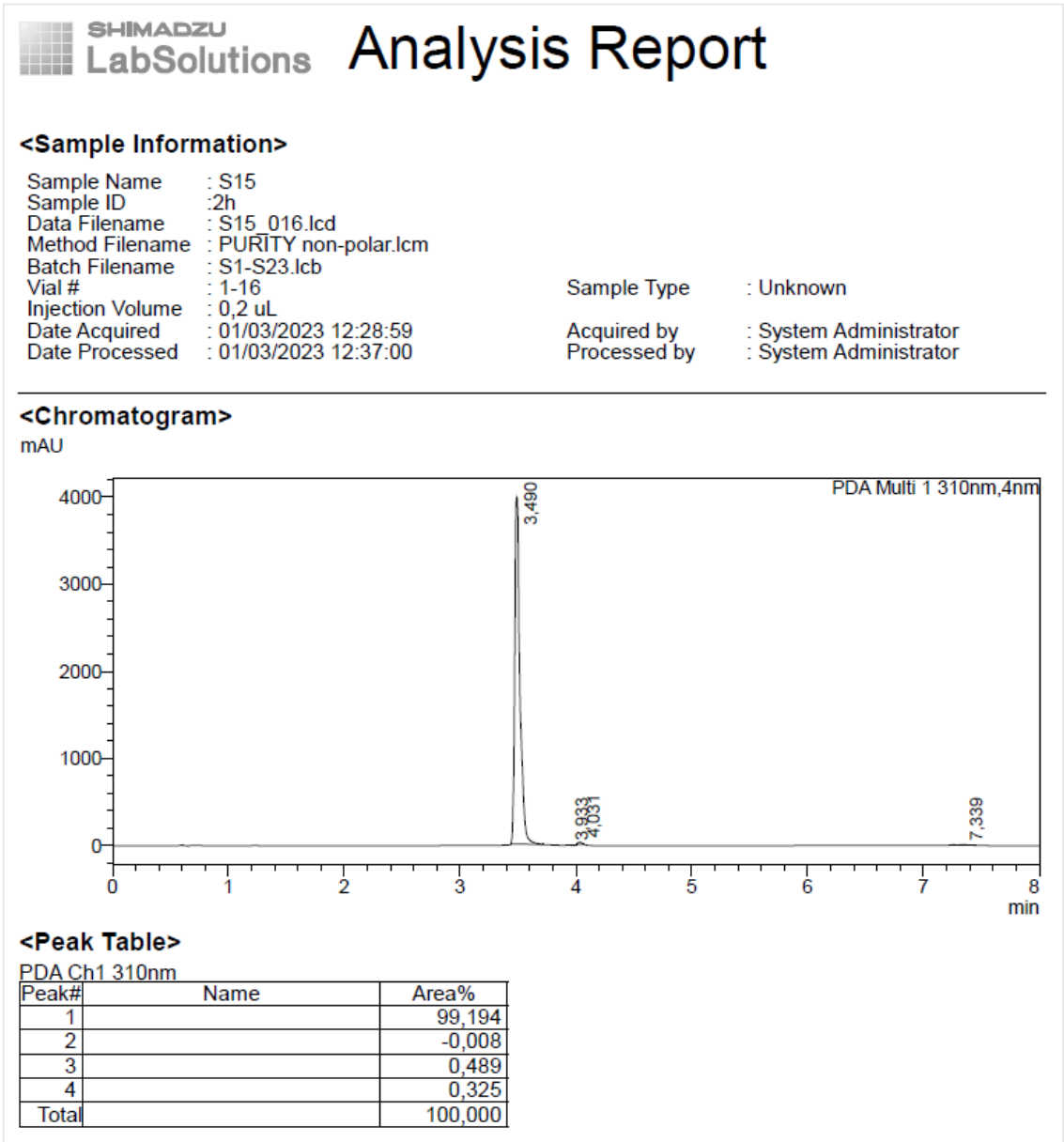
min

<Peak Table>

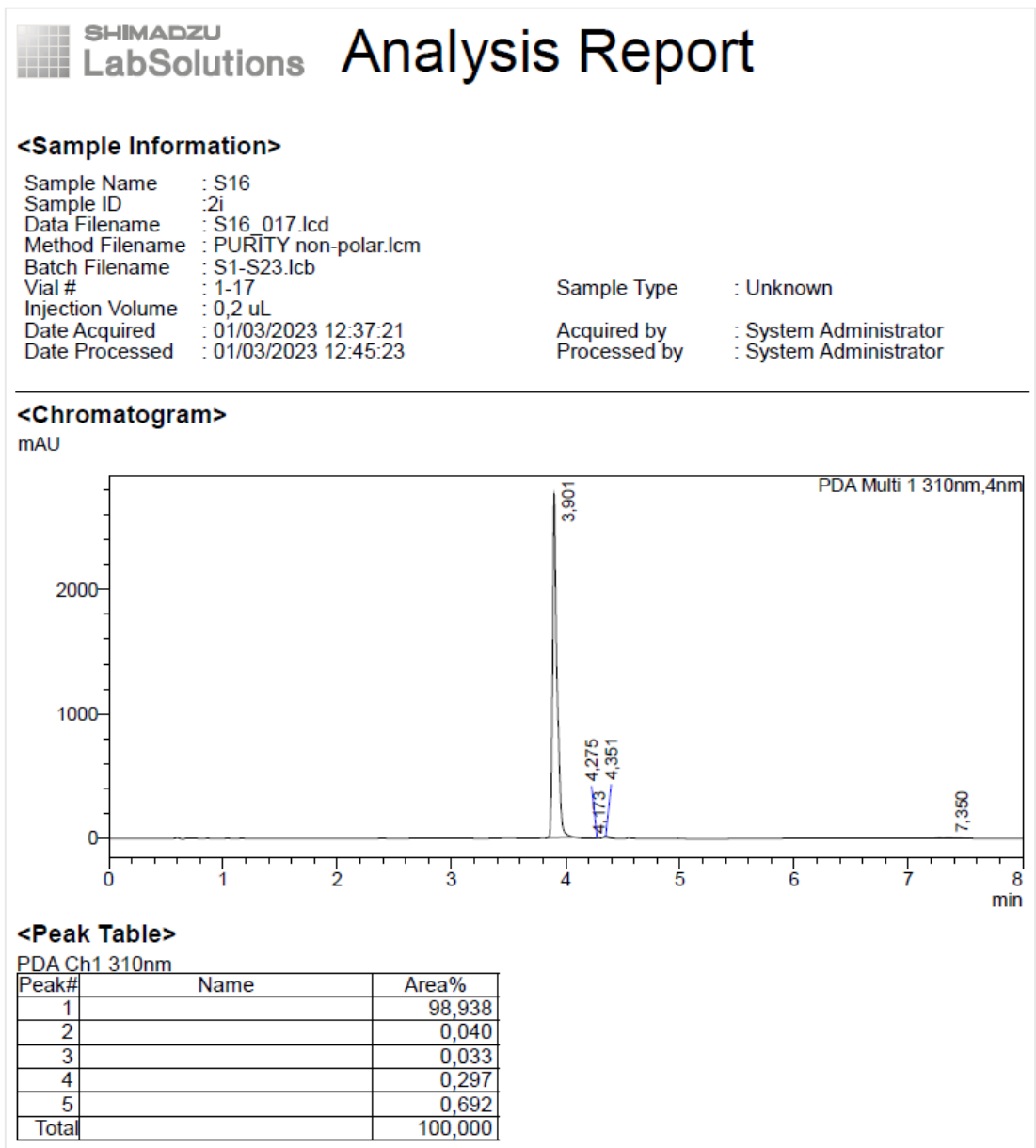
PDA Ch1 310nm

Peak#	Name	Area%
1		96,123
2		0,395
3		2,167
4		1,314
Total		100,000

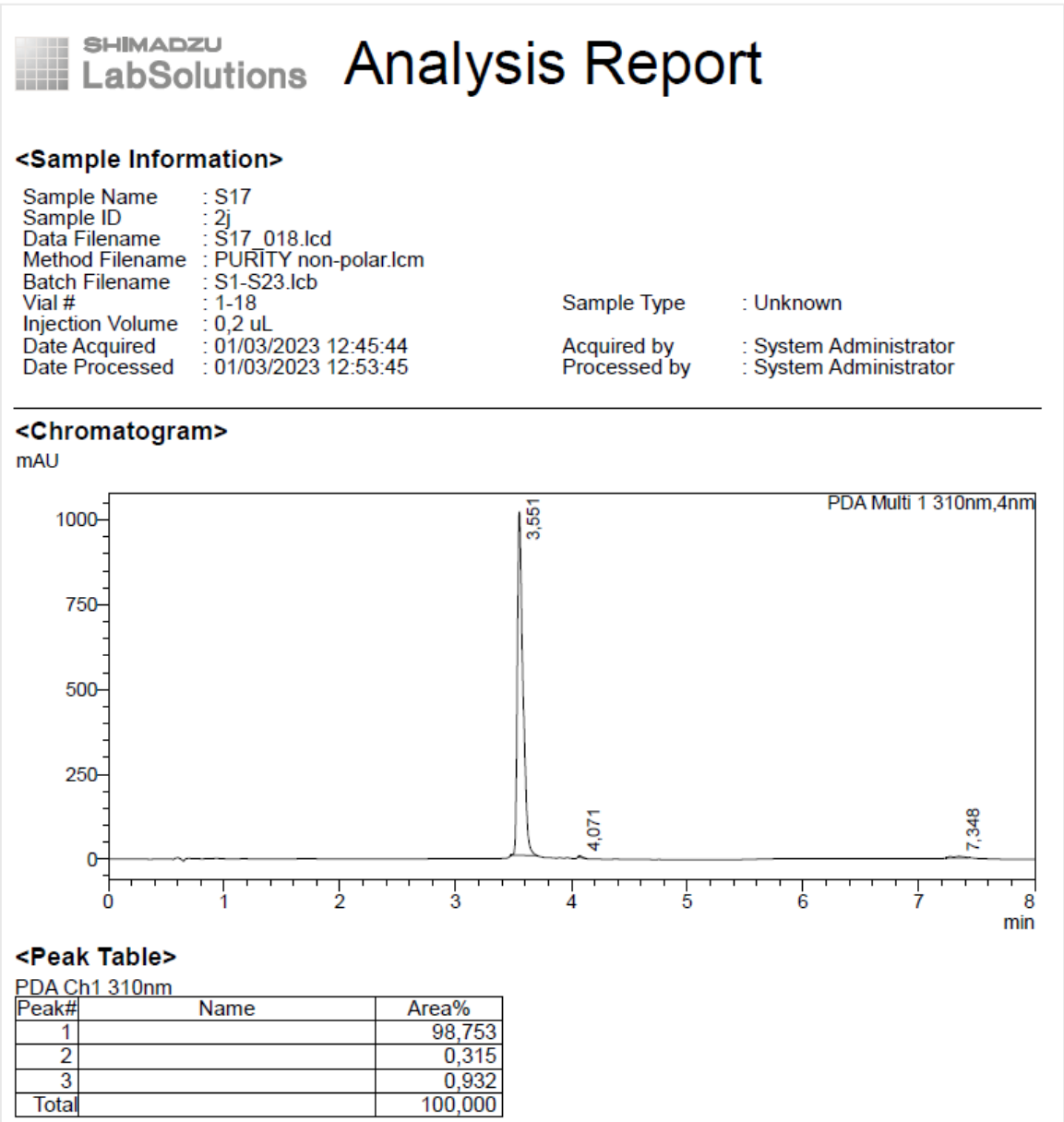
1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-ethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4e)



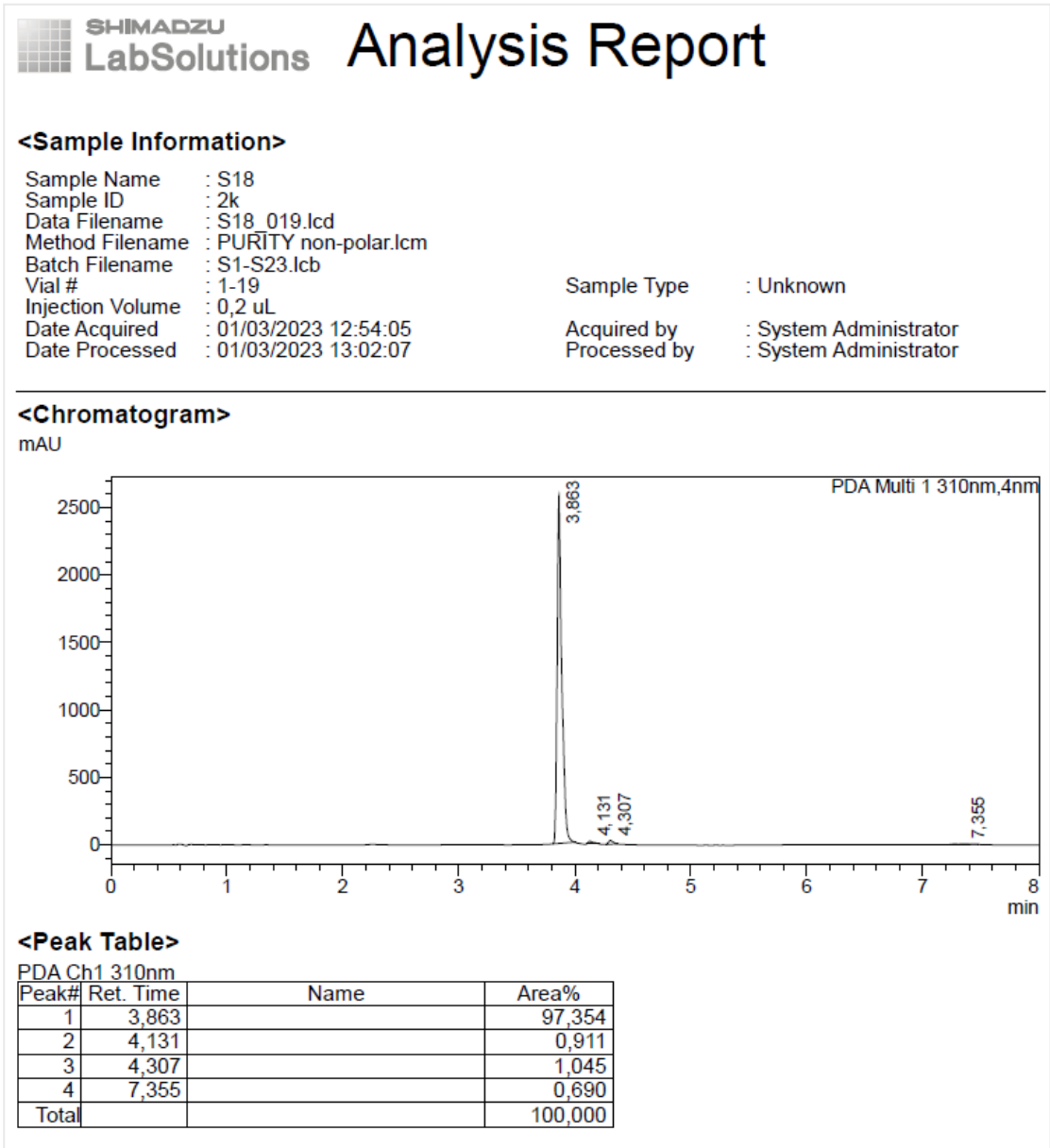
1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-iodophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4f)



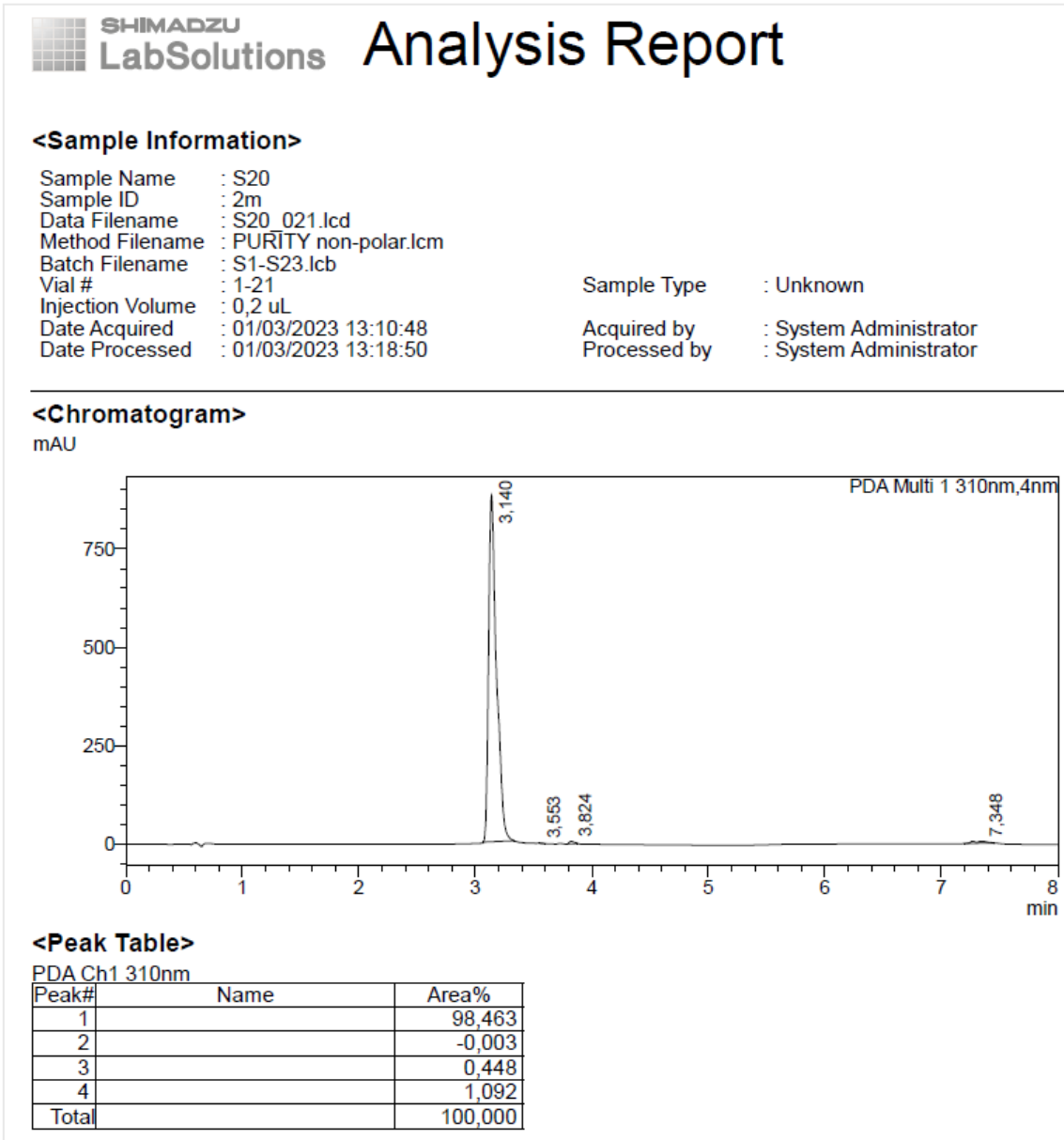
1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-[4-(methylsulfanyl)phenyl]-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4g)



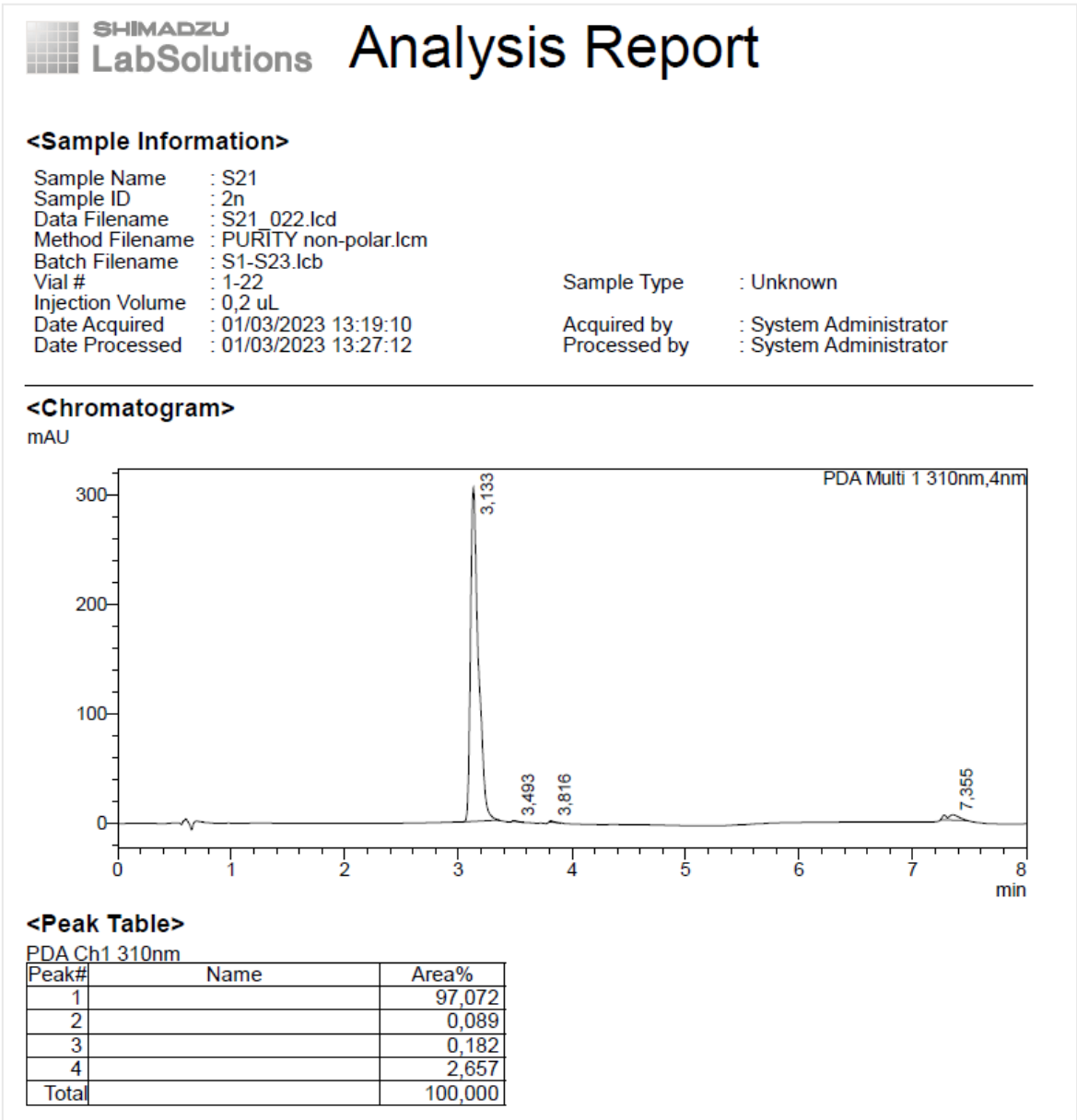
1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-bromo-2-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4h)



1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(3-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4i)

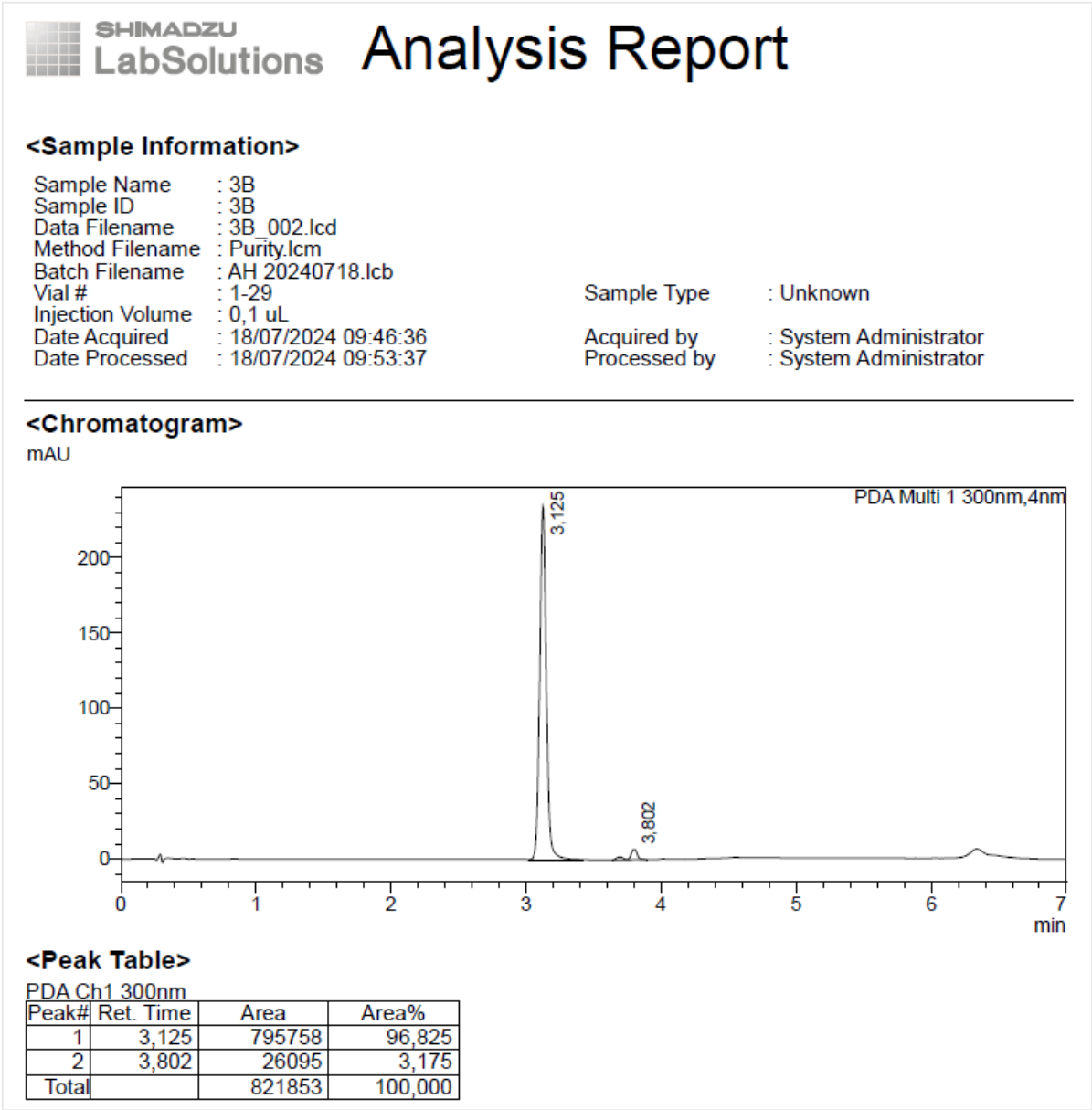


1-[3-(3,4-dihydroxy-5-nitrophenyl)-5-(4-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethan-1-one (4j)



Article 2: High-performance liquid chromatography (HPLC) results of compounds

(2E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1a)



(2E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1b)



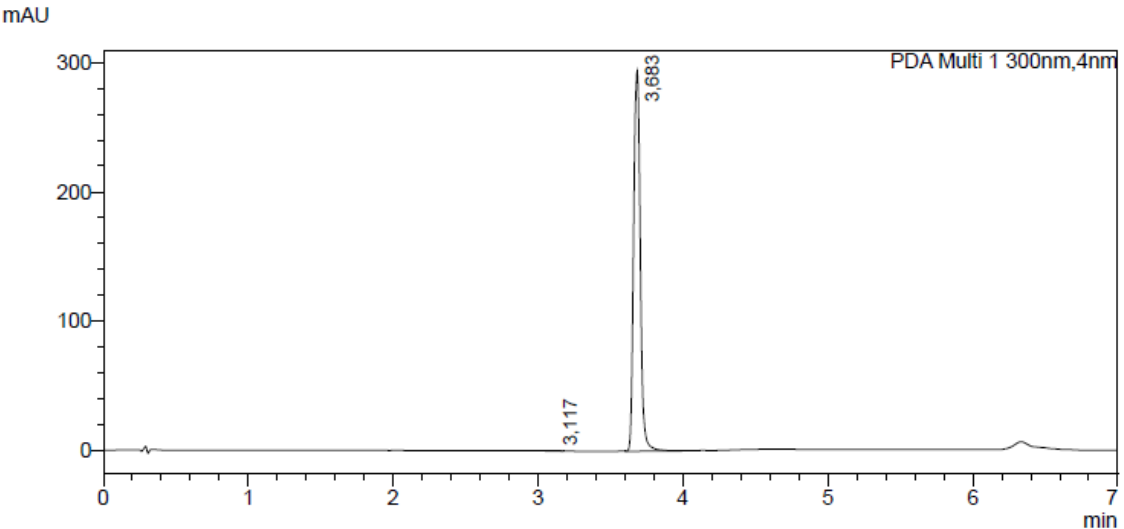
SHIMADZU
LabSolutions

Analysis Report

<Sample Information>

Sample Name	: 3C	Sample Type	: Unknown
Sample ID	: 3C	Acquired by	: System Administrator
Data Filename	: 3C_003.lcd	Processed by	: System Administrator
Method Filename	: Purity.lcm		
Batch Filename	: AH 20240718.lcb		
Vial #	: 1-30		
Injection Volume	: 0,1 uL		
Date Acquired	: 18/07/2024 09:53:57		
Date Processed	: 18/07/2024 10:00:59		

<Chromatogram>



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	3,117	913	0,097
2	3,683	939078	99,903
Total		939991	100,000

(2E)-3-(3,4-dichlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1c)

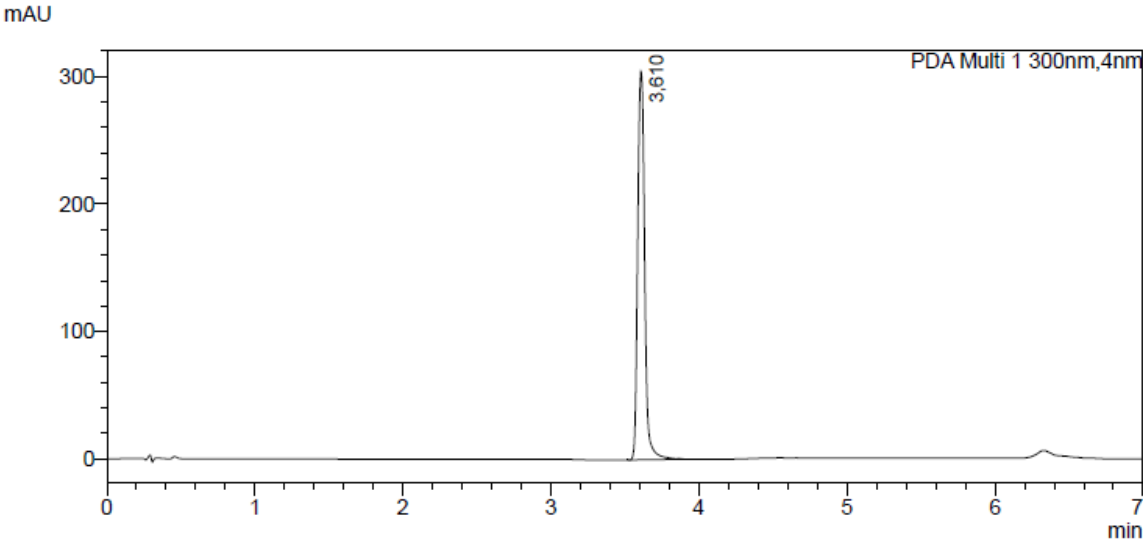


Analysis Report

<Sample Information>

Sample Name	: 3D	Sample Type	: Unknown
Sample ID	: 3D		
Data Filename	: 3D_004.lcd		
Method Filename	: Purity.lcm		
Batch Filename	: AH 20240718.lcb		
Vial #	: 1-31		
Injection Volume	: 0,1 uL		
Date Acquired	: 18/07/2024 10:01:18	Acquired by	: System Administrator
Date Processed	: 18/07/2024 12:28:34	Processed by	: System Administrator

<Chromatogram>



<Peak Table>

PDA Ch1 300nm			
Peak#	Ret. Time	Area	Area%
1	3,610	998011	100,000
Total		998011	100,000

(2E)-3-(3-chlorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1d)



Analysis Report

<Sample Information>

Sample Name : 3A

Sample ID : 3A

Data Filename : 3A_002.lcd

Method Filename : Purity.lcm

Batch Filename : AH 20240718.lcb

Vial # : 1-28

Injection Volume : 0,1 uL

Date Acquired : 18/07/2024 12:18:18

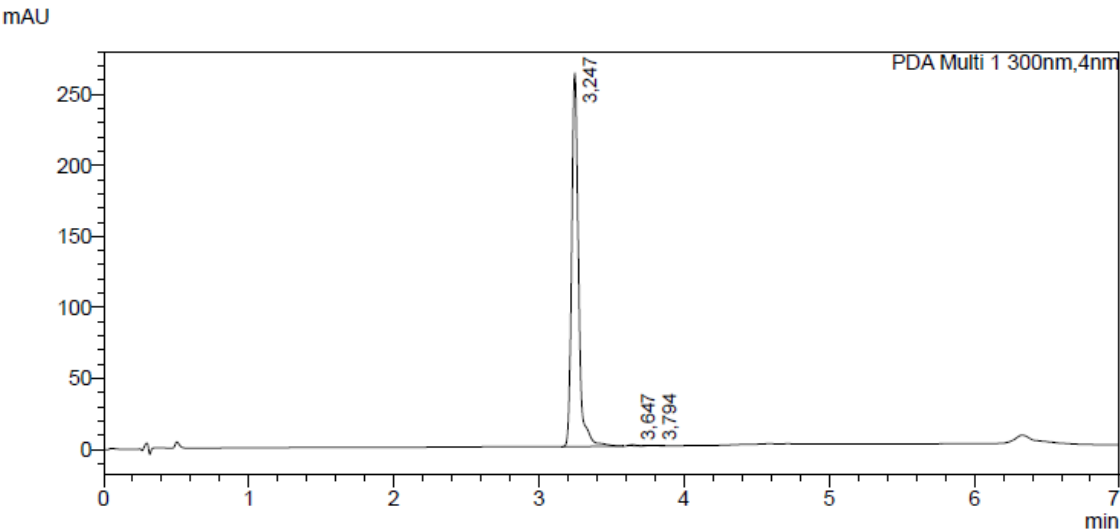
Date Processed : 18/07/2024 12:25:20

Sample Type : Unknown

Acquired by : System Administrator

Processed by : System Administrator

<Chromatogram>

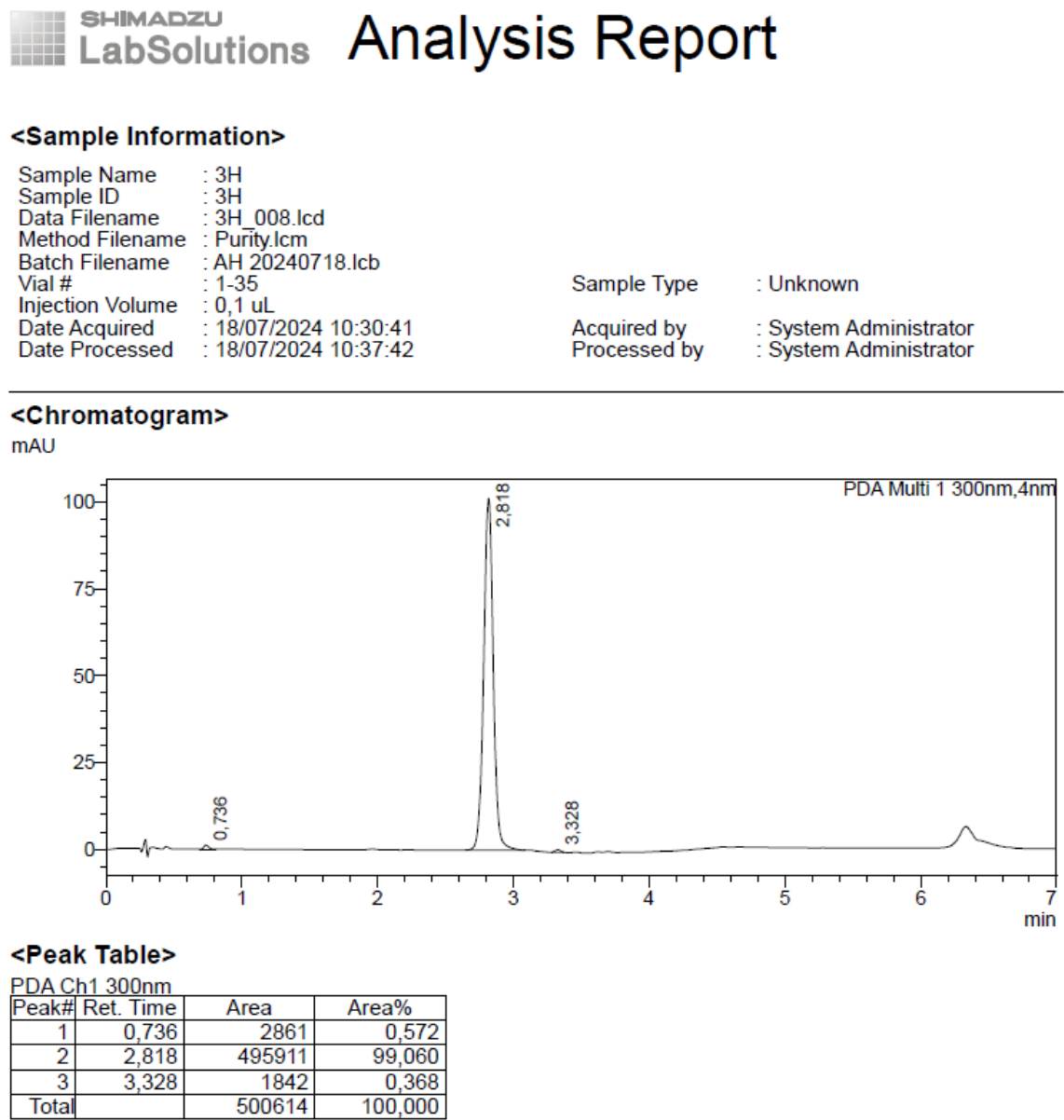


<Peak Table>

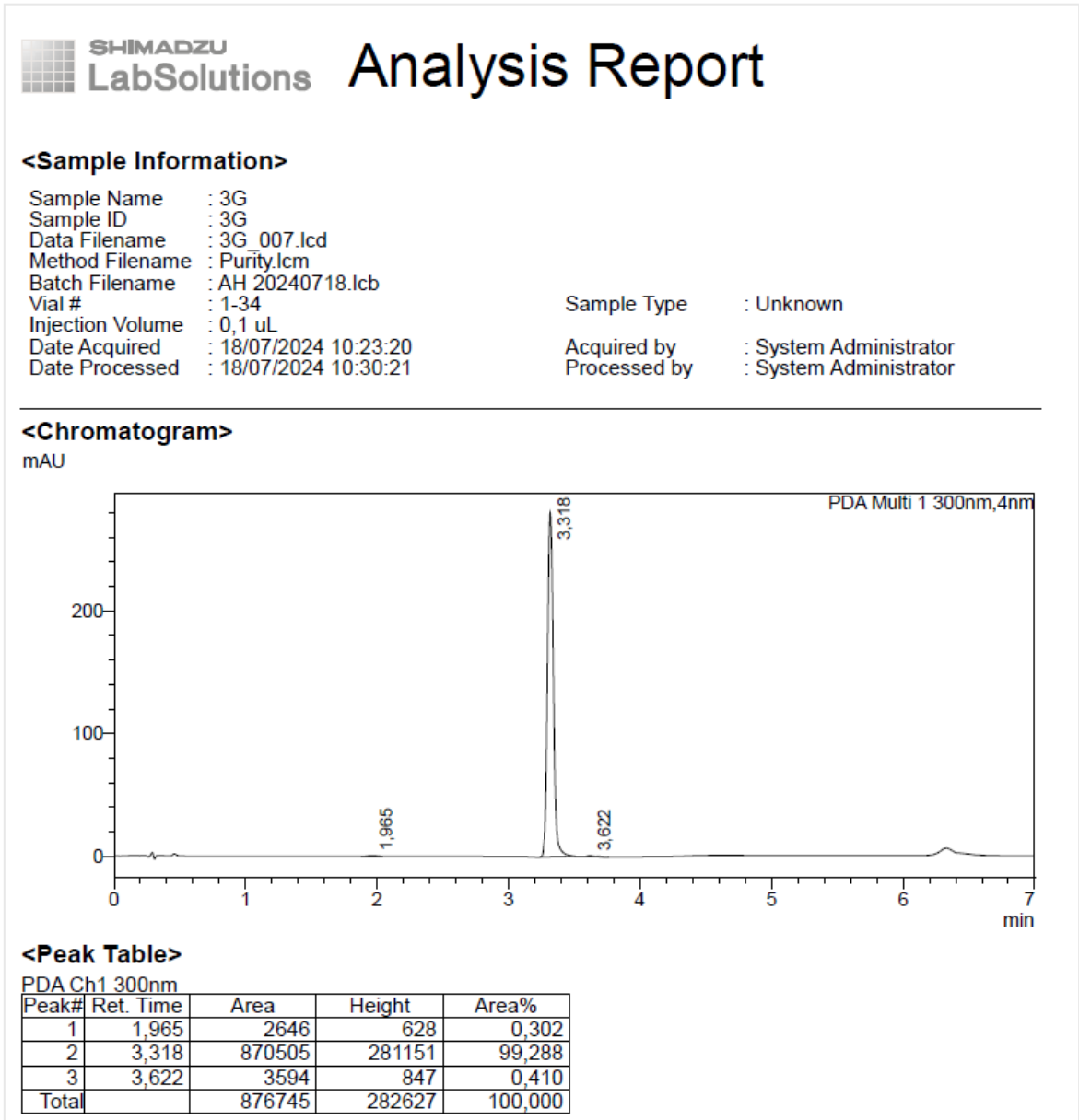
PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	3,247	871227	99,520
2	3,647	2011	0,230
3	3,794	2192	0,250
Total		875431	100,000

(2E)-1-(4-hydroxy-3-methoxyphenyl)-3-[4-(methylsulfonyl)phenyl]prop-2-en-1-one
(1e)



(2E)-1-(4-hydroxy-3-methoxyphenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one
(1f)



(2E)-3-(4-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1g)



Analysis Report

<Sample Information>

Sample Name : 3F

Sample ID : 3F

Data Filename : 3F_006.lcd

Method Filename : Purity.lcm

Batch Filename : AH 20240718.lcb

Vial # : 1-33

Injection Volume : 0,1 uL

Date Acquired : 18/07/2024 10:16:00

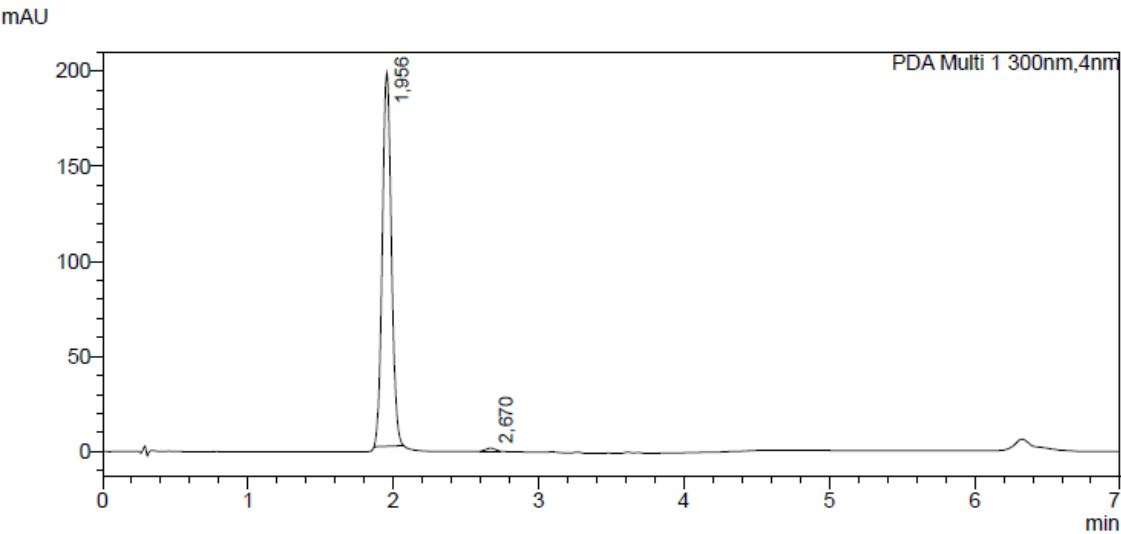
Date Processed : 18/07/2024 12:32:01

Sample Type : Unknown

Acquired by : System Administrator

Processed by : System Administrator

<Chromatogram>



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	1,956	853435	99,226
2	2,670	6656	0,774
Total		860091	100,000

(2E)-3-(3-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1h)



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LabSolutions

Analysis Report

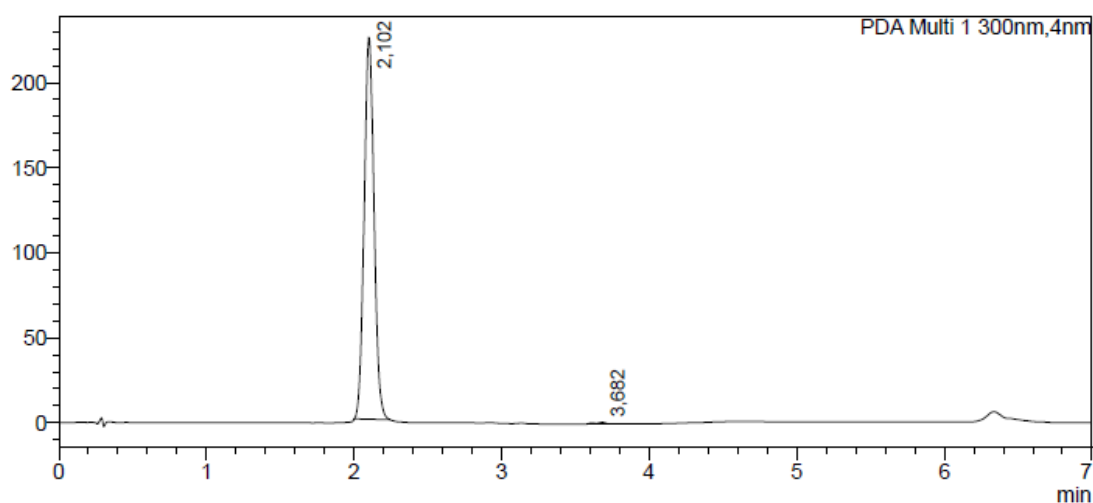
<Sample Information>

Sample Name : 3E
Sample ID : 3E
Data Filename : 3E_005.lcd
Method Filename : Purity.lcm
Batch Filename : AH 20240718.lcb
Vial # : 1-32
Injection Volume : 0,1 uL
Date Acquired : 18/07/2024 10:08:39
Date Processed : 18/07/2024 10:15:40

Sample Type : Unknown
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	2,102	1049339	99,701
2	3,682	3144	0,299
Total		1052483	100,000

(2E)-3-(4-bromo-2-fluorophenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1i)

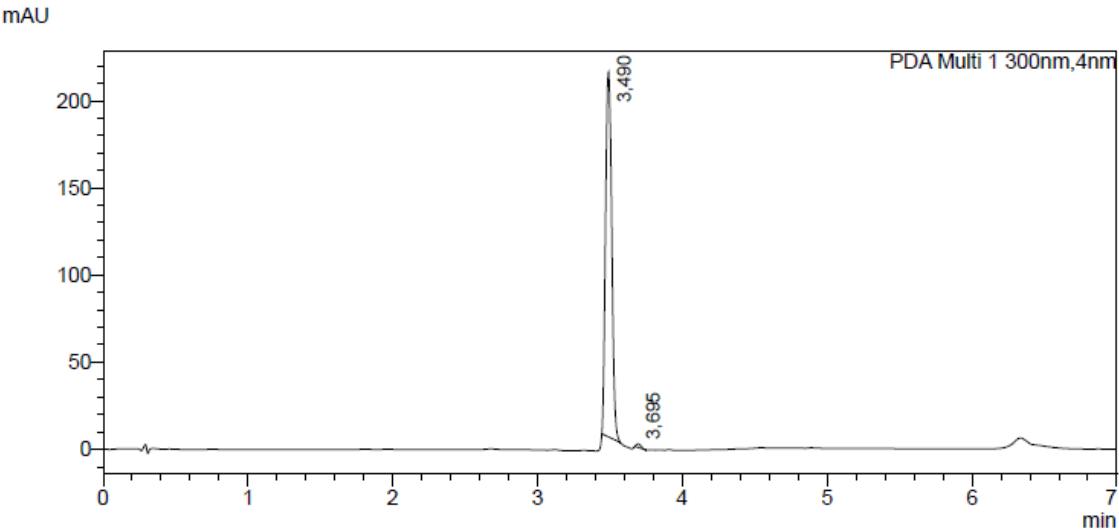


Analysis Report

<Sample Information>

Sample Name	: 3K	Sample Type	: Unknown
Sample ID	: 3K	Acquired by	: System Administrator
Data Filename	: 3K_011.lcd	Processed by	: System Administrator
Method Filename	: Purity.lcm		
Batch Filename	: AH 20240718.lcb		
Vial #	: 1-38		
Injection Volume	: 0,1 uL		
Date Acquired	: 18/07/2024 10:52:44		
Date Processed	: 18/07/2024 10:59:46		

<Chromatogram>



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	3,490	622561	99,328
2	3,695	4215	0,672
Total		626776	100,000

(2E)-3-(4-ethoxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (1j)



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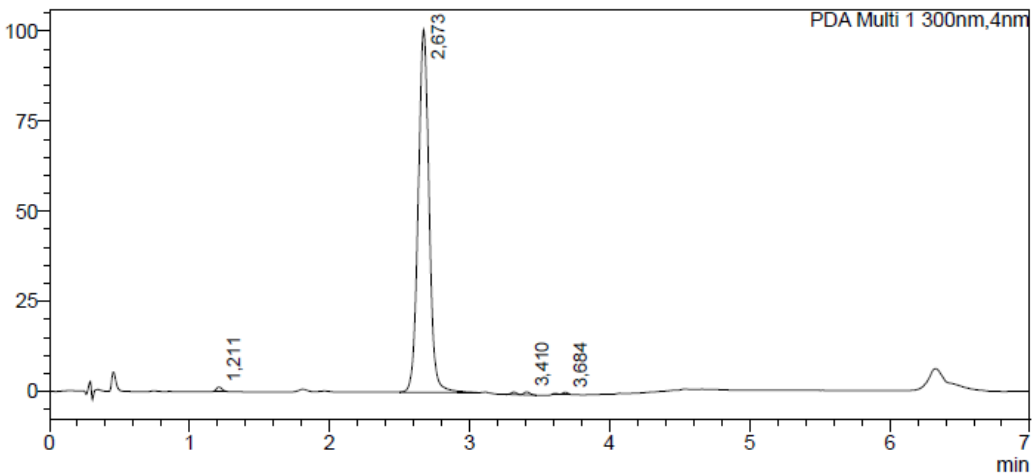
Analysis Report

<Sample Information>

Sample Name	: 3J	Sample Type	: Unknown
Sample ID	: 3J		
Data Filename	: 3J_010.lcd		
Method Filename	: Purity.lcm		
Batch Filename	: AH 20240718.lcb		
Vial #	: 1-37		
Injection Volume	: 0,1 uL		
Date Acquired	: 18/07/2024 10:45:23	Acquired by	: System Administrator
Date Processed	: 18/07/2024 10:52:24	Processed by	: System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	1,211	2752	0,498
2	2,673	544050	98,527
3	3,410	3763	0,681
4	3,684	1620	0,293
Total		552185	100,000

(2E)-3-(4-chlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2a)



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LabSolutions

Analysis Report

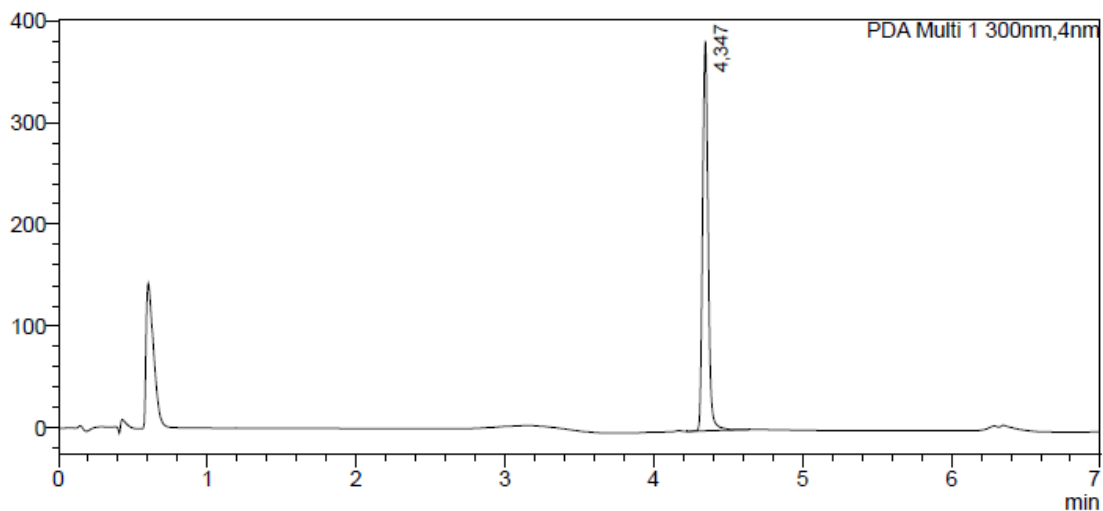
<Sample Information>

Sample Name : 4A002
Sample ID : 4A002
Data Filename : 4A002_014.lcd
Method Filename : Purity Polar.lcm
Batch Filename : AH 20240718.lcb
Vial # : 1-40
Injection Volume : 0,1 uL
Date Acquired : 18/07/2024 11:14:46
Date Processed : 18/07/2024 13:11:24

Sample Type : Unknown
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	4.347	993971	100,000
Total		993971	100,000

(2E)-3-(2,4-dichlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one
(2b)



Analysis Report

<Sample Information>

Sample Name : 4A003

Sample ID : 4A003

Data Filename : 4A003_015.lcd

Method Filename : Purity Polar.lcm

Batch Filename : AH 20240718.lcb

Vial # : 1-41

Injection Volume : 0,1 uL

Date Acquired : 18/07/2024 11:22:07

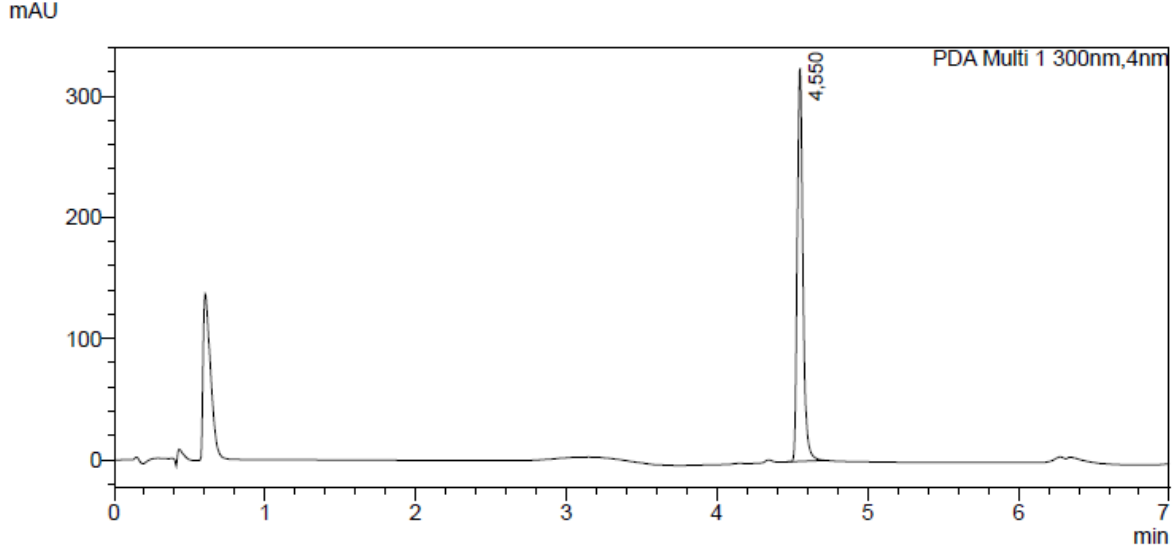
Date Processed : 18/07/2024 13:14:22

Sample Type : Unknown

Acquired by : System Administrator

Processed by : System Administrator

<Chromatogram>



<Peak Table>

PDA Ch1 300nm			
Peak#	Ret. Time	Area	Area%
1	4,550	849593	100,000
Total		849593	100,000

(2E)-3-(3,4-dichlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one
(2c)



Analysis Report

<Sample Information>

Sample Name : 4A004

Sample ID : 4A004

Data Filename : 4A004_016.lcd

Method Filename : Purity Polar.lcm

Batch Filename : AH_20240718.lcb

Vial # : 1-42

Injection Volume : 0.1 uL

Date Acquired : 18/07/2024 11:29:29

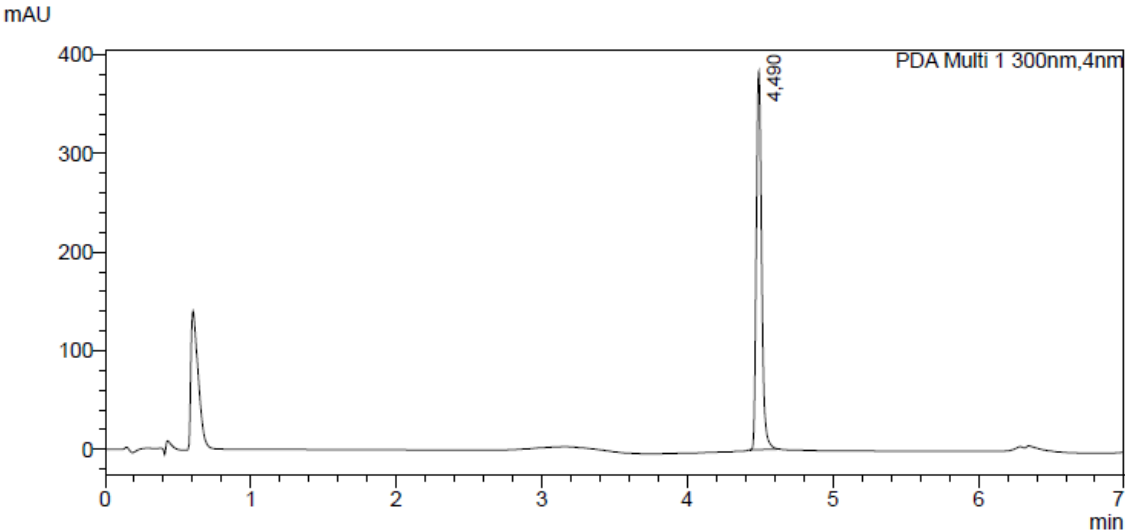
Date Processed : 18/07/2024 13:16:47

Sample Type : Unknown

Acquired by : System Administrator

Processed by : System Administrator

<Chromatogram>



<Peak Table>

PDA Ch1 300nm			
Peak#	Ret. Time	Area	Area%
1	4.490	982913	100.000
Total		982913	100.000

(2E)-3-(3-chlorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2d)



Analysis Report

<Sample Information>

Sample Name : 4A001

Sample ID : 4A001

Data Filename : 4A001_013.lcd

Method Filename : Purity Polar.lcm

Batch Filename : AH 20240718.lcb

Vial # : 1-39

Injection Volume : 0,1 uL

Date Acquired : 18/07/2024 11:07:25

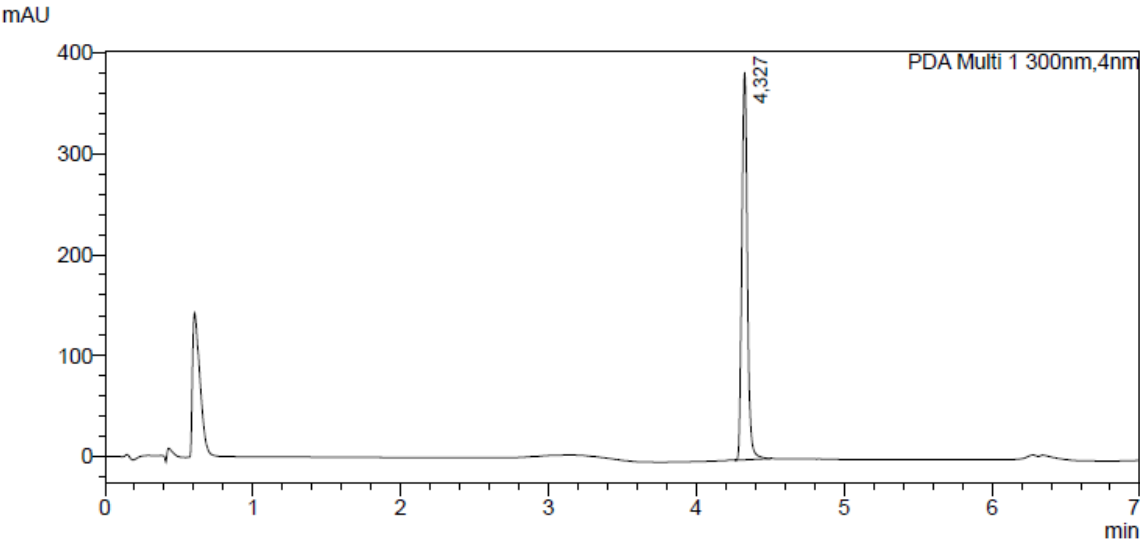
Date Processed : 18/07/2024 13:08:59

Sample Type : Unknown

Acquired by : System Administrator

Processed by : System Administrator

<Chromatogram>

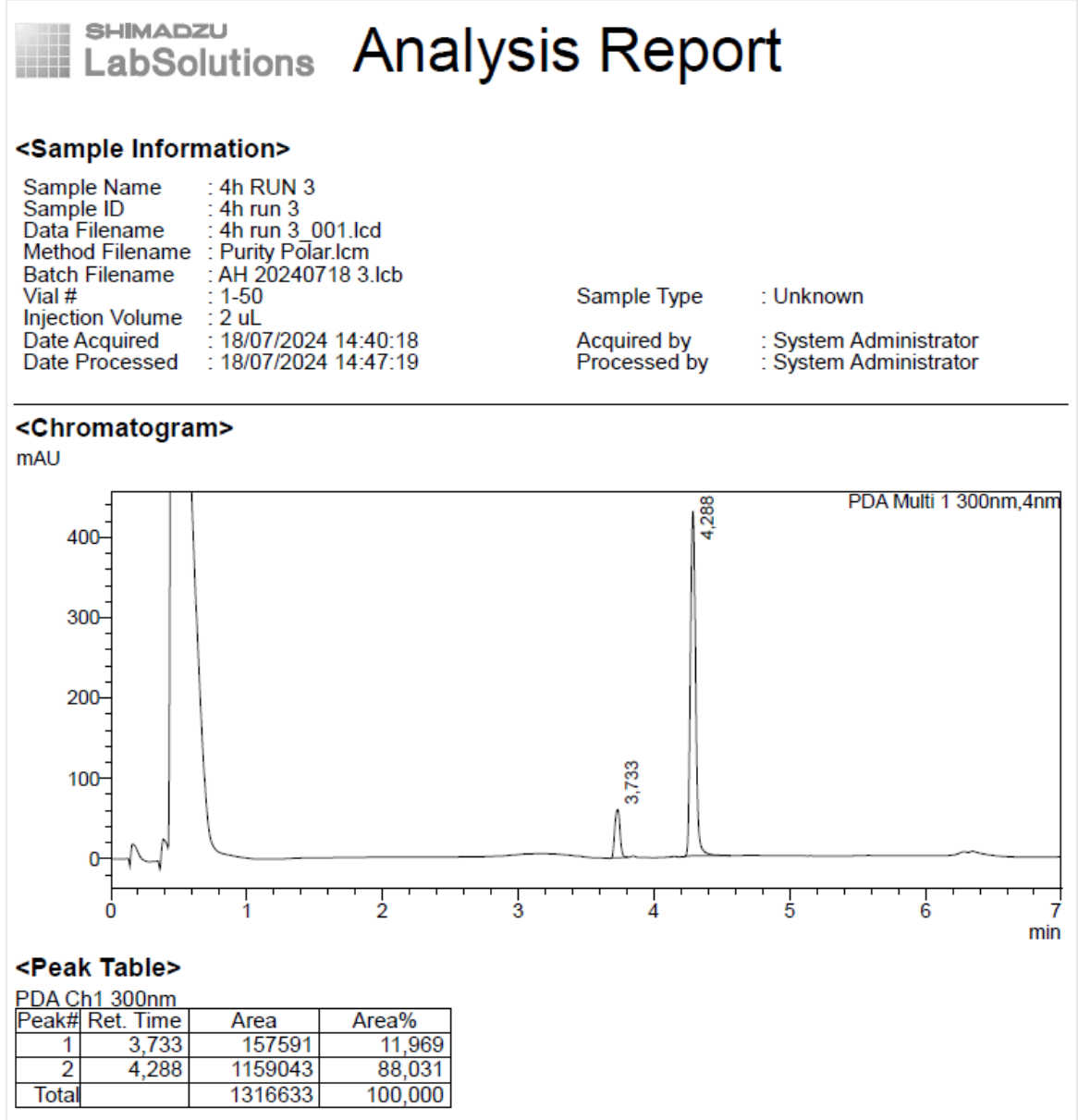


<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	4,327	1011484	100,000
Total		1011484	100,000

(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-[4-(methylsulfonyl)phenyl]prop-2-en-1-one (2e).



(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (2f)



Analysis Report

<Sample Information>

Sample Name : 4A007

Sample ID : 4A007

Data Filename : 4A007_019.lcd

Method Filename : Purity Polar.lcm

Batch Filename : AH_20240718.lcb

Vial # : 1-45

Injection Volume : 0,1 uL

Date Acquired : 18/07/2024 11:51:32

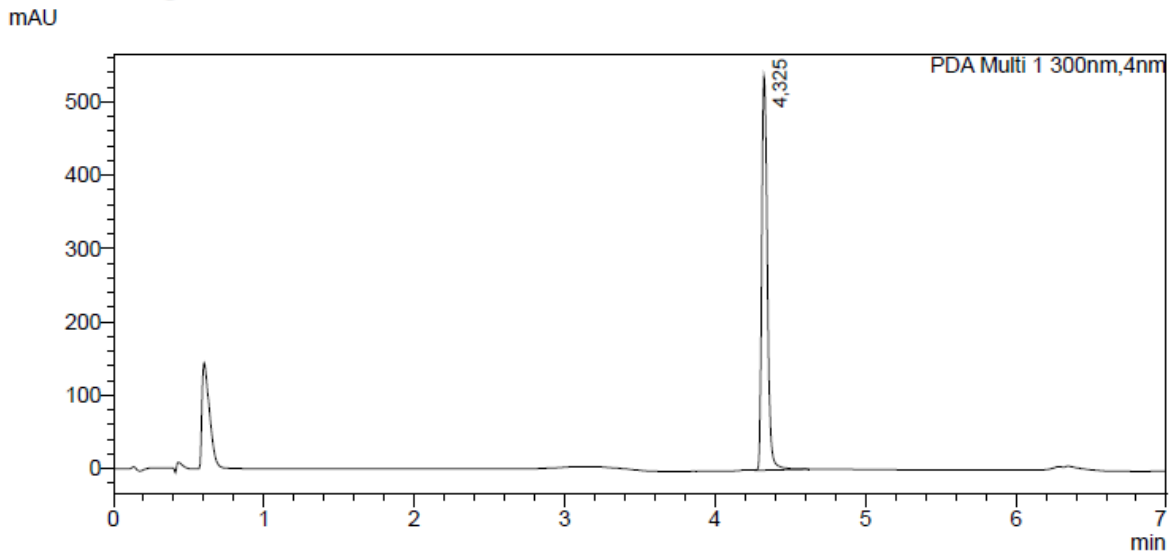
Date Processed : 18/07/2024 11:58:34

Sample Type : Unknown

Acquired by : System Administrator

Processed by : System Administrator

<Chromatogram>



<Peak Table>

PDA Ch1 300nm			
Peak#	Ret. Time	Area	Area%
1	4,325	1424897	100,000
Total		1424897	100,000

(2E)-3-(4-fluorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2g)



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Analysis Report

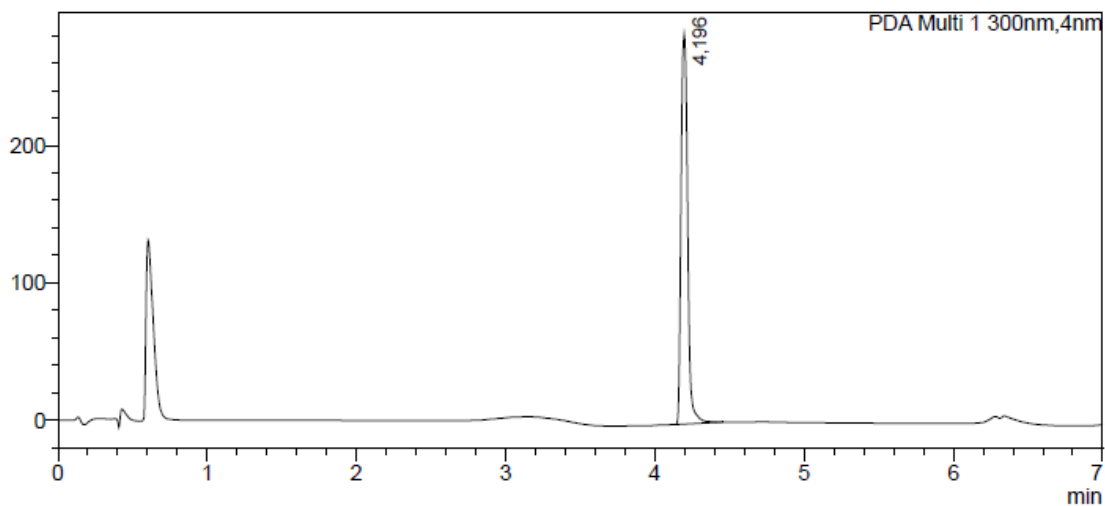
<Sample Information>

Sample Name : 4A006
Sample ID : 4A006
Data Filename : 4A006_018.lcd
Method Filename : Purity Polar.lcm
Batch Filename : AH 20240718.lcb
Vial # : 1-44
Injection Volume : 0,1 uL
Date Acquired : 18/07/2024 11:44:11
Date Processed : 18/07/2024 11:51:13

Sample Type : Unknown
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	4.196	867682	100,000
Total		867682	100,000

(2E)-3-(3-fluorophenyl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (2h)



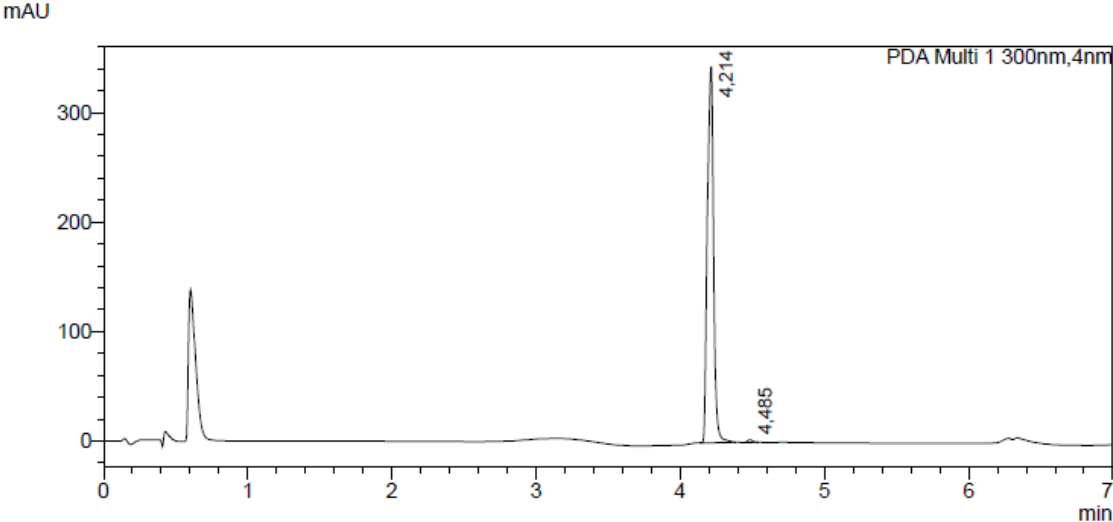
SHIMADZU
LabSolutions

Analysis Report

<Sample Information>

Sample Name	: 4A005	Sample Type	: Unknown
Sample ID	: 4A005	Acquired by	: System Administrator
Data Filename	: 4A005_017.lcd	Processed by	: System Administrator
Method Filename	: Purity Polar.lcm		
Batch Filename	: AH 20240718.lcb		
Vial #	: 1-43		
Injection Volume	: 0,1 uL		
Date Acquired	: 18/07/2024 11:36:49		
Date Processed	: 18/07/2024 11:43:51		

<Chromatogram>



<Peak Table>

PDA Ch1 300nm			
Peak#	Ret. Time	Area	Area%
1	4,214	1029061	99,462
2	4,485	5568	0,538
Total		1034629	100,000

(2E)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)-3-(4-nitrophenyl)prop-2-en-1-one (2i)



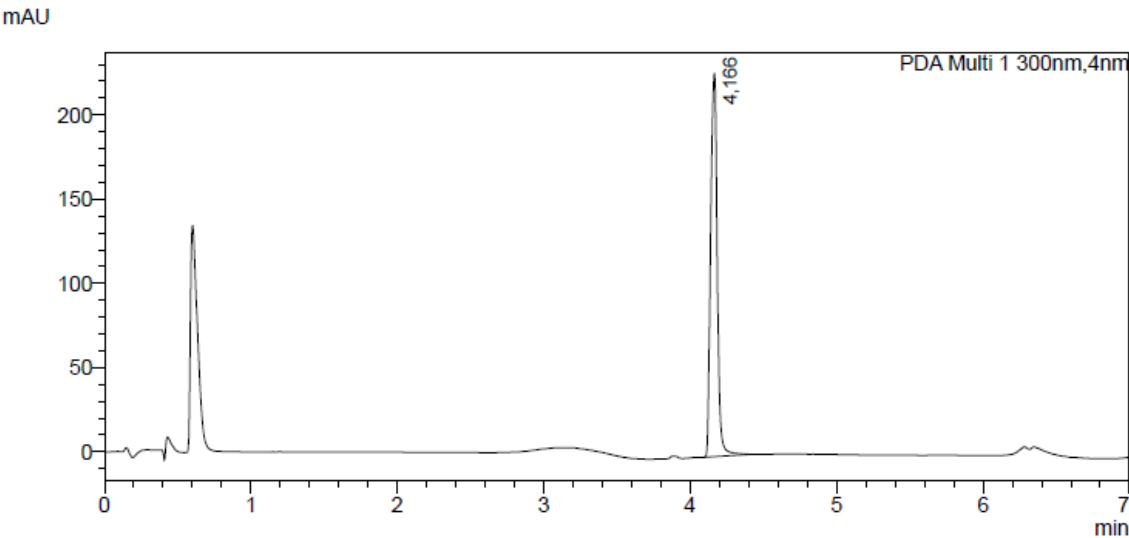
SHIMADZU
LabSolutions

Analysis Report

<Sample Information>

Sample Name	: 4A009	Sample Type	: Unknown
Sample ID	: 4A009	Acquired by	: System Administrator
Data Filename	: 4A009_021.lcd	Processed by	: System Administrator
Method Filename	: Purity Polar.lcm		
Batch Filename	: AH 20240718.lcb		
Vial #	: 1-47		
Injection Volume	: 0,1 uL		
Date Acquired	: 18/07/2024 12:06:14		
Date Processed	: 18/07/2024 12:13:15		

<Chromatogram>



<Peak Table>

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	4,166	722180	100,000
Total		722180	100,000