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Biocatalytic synthesis of novel oxidized aromatic compounds as potential anti-bacterial and anti-cancer agents

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Biocatalytic Synthesis of Novel Oxidized Aromatic Compounds as Potential Anti-Bacterial and Anti-Cancer Agents

A dissertation submitted by

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In fulfilment of the degree

Doctor of Philosophy

(Chemistry)

In the

Faculty of Science

School of Chemistry

University of the Witwatersrand

Supervisor: Dr Kevin Wellington

Co-supervisor: Professor Charles de Koning

Co-supervisor: Professor Dean Brady

DECLARATION

The work I am submitting is my own original work and all sources used in the present study have been acknowledged by means of full references. Furthermore, we have published some of this work as a manuscript and have referenced the publication as reference 155b.

NAME: TOZAMA OGUNLEYE

SIGNATURE: T. Ogunleye

DATE: 01 June 2015

PLACE: JOHANNESBURG

ABSTRACT

1.0 SYNTHESIS

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide and has accounted for 7.6 million deaths (13% of all deaths) in 2008. The number of effective drugs available has been reduced by chemo resistant malignant tumors. Similarly, bacterial infections are one of the world's most pressing public health problem. The major challenge in anti-bacterial treatment is due to the development of bacteria strains that are resistant to antibiotics. Each year more than 11 million people die from major infections such as MDR tuberculosis. In 2013, 9 million people fell ill with TB and 1.5 million died from the disease (WHO). Therefore there is a need for novel therapeutic alternatives such as the discovery of new anti-cancer and anti-bacterial agents. Benzofurans have attracted much attention due to their broad spectrum of pharmacological activities such as anti-cancer and anti-bacterial activities and one classical example is usnic acid. Most of the published synthesis of the benzofuran moiety involved the formation of annellated furan ring by intramolecular cyclisation of benzene, and these procedures involved a multi-step, rigorous reaction conditions and expensive catalyst. This research investigated the novel synthesis of benzofurans through the application of biocatalysis, where the reactions involved the use of the oxidative enzyme laccase to generate carbon-carbon bonds, carbon-oxygen and carbon-nitrogen bonds between aromatic compounds. The substrates used were *o*-diols from catechols **1**, *p*-quinone **2** from naphthoquinones and naphthohydroquinone **3**, which, when activated by the enzyme action, could be reacted with 1,3-diketones **4, 5** or coumarins **6** (Figure 1). The aim of synthesising different classes of compounds was to vary the functional groups and to increase the number of rings, so as to possibly increase the biological activities.

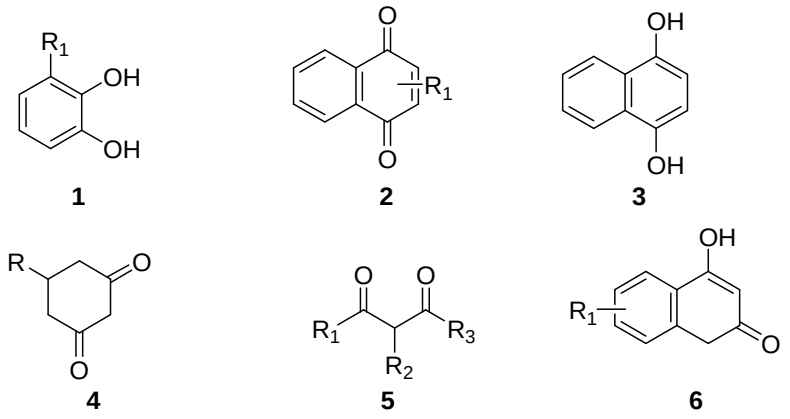


Figure 1: Substrates of laccase (**1, 2,3**) and co-reactants (**4,5,6**)

We exploited the application of a commercial laccase, Suberase® from Novozymes as the catalyst in these transformations. This oxidoreductase was successfully utilised in the synthesis of 5,6-dihydroxylated benzo[*b*]furans **7**, synthesis of C-C coupled substituted dicarbonyls to catechols **8**, benzofuro [3,2-*c*]chromen-6-one derivatives **9**, C-C coupled naphthoquinone **10** and C-C bis-coupled naphthoquinone derivatives **11** and **12** (Figure 2).

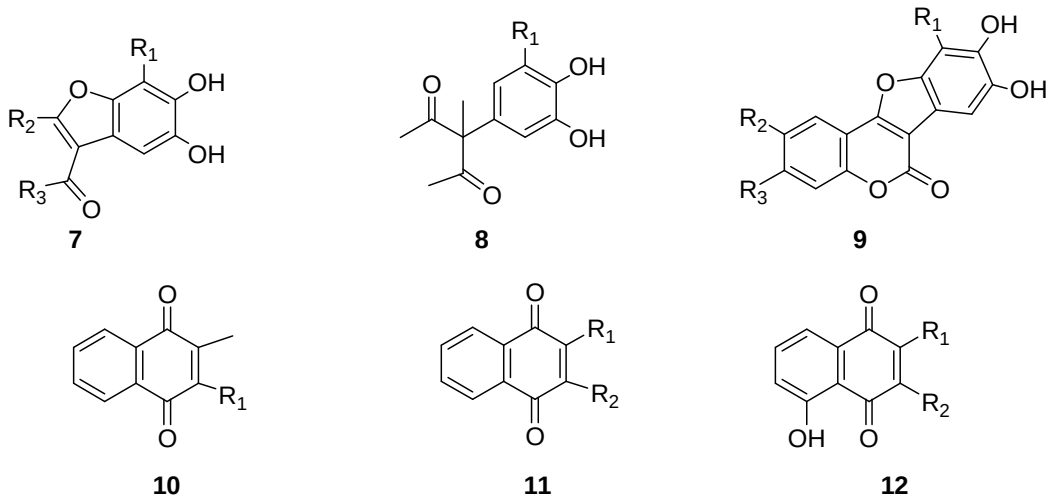
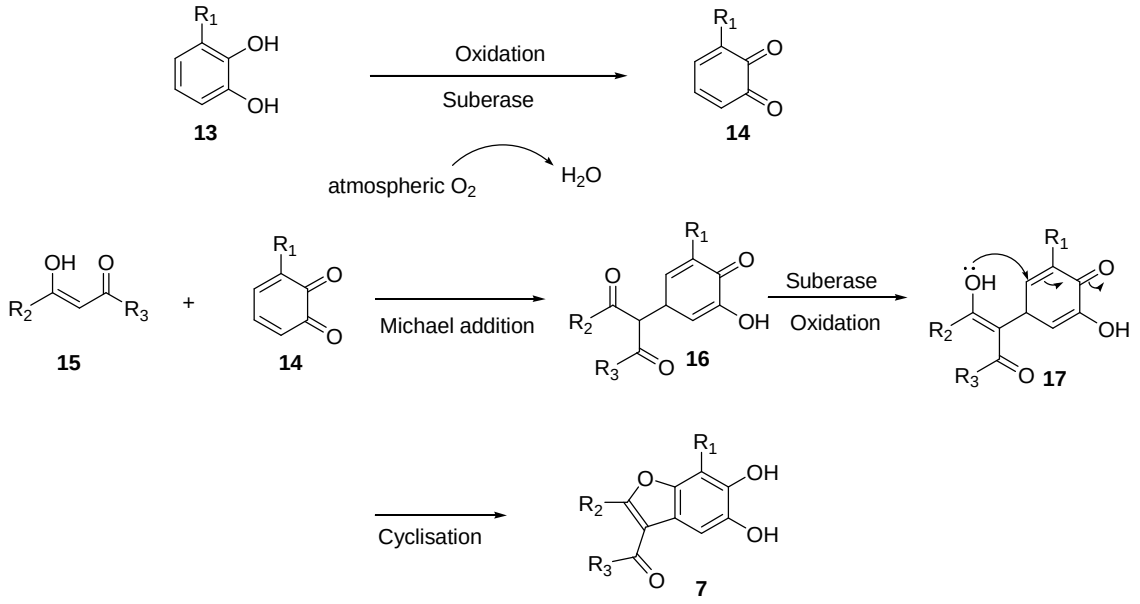


Figure 2: The classes of synthesised compounds synthesised in this study

As an example, the synthesis of benzofurans takes place by the *in situ* oxidation of catechol **13** facilitated by laccase to afford the ortho-quinone **14**. This allows for a Michael addition of the 1,3-dicarbonyl **15** with **14**. This is then followed by an oxa-Michael addition which allows for cyclisation as shown in Scheme 1 below to afford the benzofurans **7**.



Scheme 1: Synthesis of the 5, 6-dihydroxylated benzo[*b*]furans

Depending on the nature of the starting materials, a second reduction can only take place for un-substituted dicarbonyls at position 2. Interestingly, the substituted diketones formed a monocoupled product **8**. Such a mechanism has been reported when using traditional synthetic methods in the classical cross coupling mechanism using palladium (Pd), or Nickel (Ni) or iron (Fe). However, the 1,4-naphthoquinones unexpectedly gave a C-C bis coupled product **10** and **11** when reacted with 1,3-diketones in the presence of laccase. This suggests that the reaction has undergone two Michael addition reactions without cyclisation. This mechanism is also observed for the first time through the application of laccase.

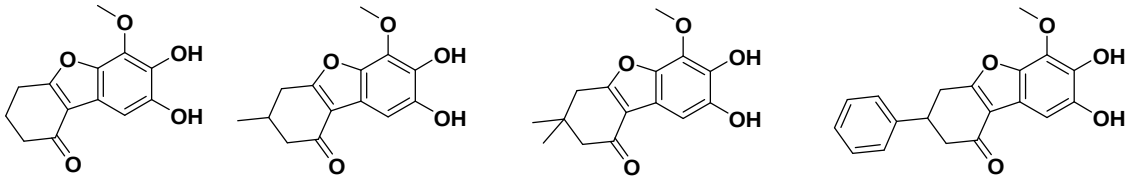
The method was broadly applicable but unsuccessful regarding fluorinated and acid catechols, which did not react with neither 1,3-dicarbonyls nor coumarins. Anthraquinones were also not susceptible to this reaction.

A selection of the benzofurans were derivatized with a well-known anti-TB drugs; isoniazid and thiosemicarbizide. The motif is that drugs that modulate several targets have a potential to improve balance of efficacy in addition to safety when compared to single target agents. A total of 69 compounds were generated in this study, of which 19 were known and 50 were novel.

2.0 BIOLOGICAL EVALUATION

Biological evaluation, both anti-cancer and anti-bacterial, of selected synthesised compounds was performed. Anticancer screening was against a number of cancer cell-lines, which included renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer cell lines.. The GI_{50}, TGI and LC_{50} data for many of these compounds were reported for the first time. Anticancer screening showed that the cytostatic effects of the 5,6-dihydroxylated benzo[*b*]furans were most effective against the melanoma (UACC62) cancer cell line with several compounds exhibiting potent growth inhibitory activities (GI_{50} = 0.77–9.76 μM), of which two compounds had better activity than the well-known anticancer agent etoposide (GI_{50} = 0.89 μM). One compound exhibited potent activity of (GI_{50} = 9.73 μM) against the renal (TK10) cancer cell line and two exhibited potent activity (GI_{50} = 8.79 and 9.30 μM) against the breast (MCF7) cancer cell line (Figure 2). The three numbers at the bottom in the figure above represent the GI_{50} of renal (TK10), melanoma (UACC62) and breast (MCF7) cancer respectively. Comparison of the structures relative to the biological activity showed

that the methoxy catechol was the most biologically active derivative, and the biological activity increased proportionally to the size of the 1,3-diketone.



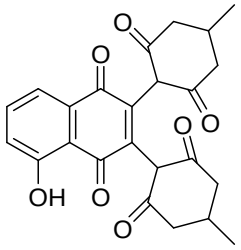
19 **20** **21** **22**

BIOLOGICAL ACTIVITY INCREASES

18	**19**	**20**	**21**
GI_{50}	**GI_{50}**	**GI_{50}**	**GI_{50}**
52.57, 6.04, 15.85	**51.65, 0.78, 8.79**	**18.69, 6.79, 28.44**	**9.73, 0.77, 9.33**

Figure 2: Structures of most biological active compounds on cancer cell-lines

Antibacterial evaluation involved screening selected compounds against*Mycobacterium smegmatis* MC^2155, *Staphylococcus aureus* 32710 (*MSSA*), *Bacillus subtilis* 168 and *Escherichia coli* DH10B. *Mycobacterium smegmatis* MC^2155 was used as a pre-screening model organism prior to screening compounds against *Mycobacterium tuberculosis (M.tb).* Only one compound, which belongs to the naphthoquinones (compound **22**, Figure 3), showed potency against *M. smegmatis* (6.25 µg/mL). This indicated that many benzofurans are not active against Gram negative and Gram positive bacteria, but have some application towards acid fast bacteria such as Mycobacteria.



22

Figure 3: Structure of the active 1,4-naphthoquinone derivative

Compound **22** was then screened against three drug susceptible *M.tb* strains (two with different mutations in the rpoB gene and one a drug susceptible strain called BE), and was active against all three strains at 10 µg/mL.

This study has demonstrated the applicability of oxidative biocatalysis to access novel furan compounds with activity towards cancer cell lines and acid fast bacteria.

DEDICATION

I would like to dedicate this thesis to the following people:

- My family: Mr Olalekan Samuel Ogunleye (husband), Anuoluwapo Ogunleye (son), Oluwanifemi Ogunleye (daughter) and Olaitan Oweseni (sister inlaw). Thank you for your love, support, patience and walking with me on this vibrant journey.
- My Parents: Mrs Patricia Bukelwa Qwebani and Mr Thandabantu Anorth Qwebani.

  Your love, support and encouragement has strengthened me greatly during the course of the PhD, thank you.

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If I stand tall it is because I stand on the shoulders of giants**.**

- My sincere gratitude to **Dr Kevin Wellington, Prof Dean Brady** and **Prof Charles de Koning**, for all your assistance, training, encouragement and time. You introduced me to science literature and allowed us to publish some of that literature before completing my doctorate. You became more than supervisors and equipped me with necessary skills and knowledge for the working world. Words can not fully express how grateful I am for being your student. I felt honoured being supervised by you for the qualities and values you give to your students, which I have embraced, and if the fruit is not yet visible it surely will be sooner or later and for that I will forever be grateful, thank-you.

- To **Dr Paul Steenkamp**, **Dr Natasha Kolesnikova**, **Dr Chris Van der Westhuyzen**, **Dr Dorothy Fallows, Dr Natalia Kurepina, Prof Gilla Kaplan and Mr Blas Peixoto** all your assistance and encouragement to the success of this thesis is highly appreciated. When the puzzle was still in pieces you assisted me to complete it, I will forever be **grateful**, **THANK-YOU**. I would also like to extend my gratitude to both **Dr Kevin Wellington, Prof Charles de Koning and Prof Gilla Kaplan's laboratories,** it has been great working with all the **team members.**

- I would like to thank all my sponsors for thr financial support: The **Council of Scientific Industrial Research (CSIR), Department of Science Technology (DST) Biocatalysis Initiative**, **Aurum Institute** and **TATA Africa**. This research would have not been possible without your assistance, **THANK YOU**.

- I will be a pretender to the throne if I don't mention the names of some of my support structure, without their encouragement and help I might not have

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PUBLICATIONS AND CONFERENCES

Publications from this thesis are as follows:

1. A One-Pot Laccase-Catalysed Synthesis of 5, 6-Dihydroxylated Benzo[b]furans and Catechol Derivatives and their Anticancer Activity. Wellington, K.; Qwebani-Ogunleye, T.; Kolesnikova, N. I.; Brady, D.; de Koning, C. *Archiv Der Pharmazie*, **2013**, *346*, 266.
2. Biocatalytic synthesis of coumestans and their anticancer activity. Wellington, K.; Ogunleye, T.; Kolesnikova, N. I.; Brady, D.; de Koning, C. (To be submitted to the *Journal of medicinal chemistry*).
3. Anticancer and antibacterial activity of 5,6-dihydroxylated benzo[b]furan analogues. Ogunleye, T.; Wellington, K.; Kolesnikova, N. I.; Brady, D.; de Koning, C. (To be submitted to the *Journal of medicinal chemistry*).
4. A laccase-catalysed in-situ C-C coupling of 1,3 diketones to 1,4- dihydroxynaphthalene and 5-hydroxy-1,4-naphthoquinone. Ogunleye, T.; Wellington, K.; Kolesnikova, N. I.; Brady, D.; de Koning, C. (To be submitted to *Applied Microbiology and Biotechnology Journal).*
5. Naphthalene-1,4-dione analogue as potential bacteriostatic agent for *Mycobacterium smegmatis* and *Mycobacterium tuberculosis.* Ogunleye, T.; Wellington, K.; Kolesnikova, N. I.; Brady, D.; de Koning, C. (To be submitted to *Applied Microbiology and Biotechnology Journal).*

Parts of this thesis have been presented in three conferences, as a poster as well as for oral presentation:

(Presenting author underlined)

1. Oral Presentation: BIT's 3rd Symposium on enzymes and Biocatalysis, China, 2012.

Title: Biocatalytic Synthesis of Benzo[b]furan and Catechol Derivatives and Their Biological Activity

Author: Tozama Ogunleye Kevin Wellington Dean Brady & Charles de Koning

2. Poster: SACI Conference, University of the Witwatersrand, South Africa, 2011.

Title: Synthesis of oxidized aromatic compounds via Laccase oxidation of hydroxyl groups.

Authors: Tozama Ogunleye Kevin Wellington Dean Brady & Charles de Koning

3. Poster: ASSAf-DST-NRF Second annual South African young scientists' conference, 2011.

Title : Studies on the synthesis and antimicrobial evaluation of benzofuran analogues.

Authors: Tozama Ogunleye Kevin Wellington Dean Brady & Charles de Koning

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ABBREVIATIONS

ABTS	:	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)
Ach	:	Acetylcholine
AChE	:	Acetylcholinesterase
ACS	:	American Chemical Society
ACVS	:	δ-(L-α-Aminoadipyl)-L-cysteine-D-valine synthetase
Bcl2	*:*	*B-cell CLL/lymphoma 2*
BuChE	:	Butyrylcholinesterase
CDK	:	Cyclins
CFU	:	Colony Forming Unit
C NMR	:	Carbon nuclear magnetic resonance
CSIR	:	Council for Scientific and Industrial Research
DARQs	:	Diarylquinolines
DMF	:	Dimethylformamide
DNA	:	Deoxyribonucleic acid
DSM	:	Dihydrostreptomycin
E.coli	*:*	*Escherichia coli*
EPA	:	Environmental Protection Agency
ETH	:	Ethambutol
EtOAC	:	Ethyl acetate
FCTC	:	Framework Convention on Tobacco Control
FDA	:	Food and Drug Administration
5-FU	:	Fluorouracil
FRCA	:	Fluconazole-resistant *Candida albicans*
GCI	:	Green Chemical Institute
GI_{50}	:	50% Cell growth inhibition
GPCRs	:	G-protein-coupled receptors
HBV	:	Hepatitis B virus

HCl	:	Hydrochloric acid
HCV	:	Hepatitis C virus
HIV	:	Human Immunodeficiency Virus
HIV-1	:	Human Immunodeficiency Virus-1
H NMR	:	Proton nuclear magnetic resonance
HPV	:	Human papillomavirus
HSCTs	:	Hematopoietic stem cell transplants
Hz	:	Hertz
IC_{50}	:	The drug concentration causing 50% inhibition of the desired activity
INH	:	Isonicotinyl hydrazine
IPNS	:	Isopenicillin N synthase
LC_{50}	:	Lethal concentration (that kills 50% of the cells)
MIC	:	Minimum inhibitory concentration
MDR	:	Multi drug-resistance
MIDA	:	Methyliminodiacetic acid
MRSA	:	Methicillin resistant *Staphylococcus aureus*
mRNA	:	Messenger ribonucleic acid
M. smegatis	:	*Mycobacterim smegmatis*
MSSA	:	Methicillin susceptible *Staphylococcus aureus*
Mtb	:	*Mycobacterim tuberculosis*
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Nicotinamide adenine dinucleotide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NCI	:	National Cancer Institute
NNRTIs	:	Nucleoside reverse transcriptase inhibitors
NRPS	:	Nonribosomal peptide synthetase
PA824	:	Nitroimidazopyran
PDA	:	Photodiode array

PDT	:	Photodynamic therapy
ppm	:	Parts per million
pRb	:	Retinoblastoma protein
PYR	:	Pyrazinamide
PZA	:	Pyrazinoic acid
QSAR	:	Quantitative structure-activity relationship
RIF	:	Rifampicin
RLZ	:	Rifalazil
RMP	:	Rifampicin
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal ribonucleic acid
ROS	:	Reactive oxygen species
rt	:	Room temperature
S315T	:	Amino acid residue 315
SBDD	:	Structure-based drug discovery
SCF	:	Skp1 – Cullin – F-box protein
SMCC	:	Suzuki-Miyaura cross coupling
SRB	:	Sulforhodamine
STR	:	Stremptomycin
TB	:	Tuberculosis
TDR	:	Total drug-resistance
TGI	:	Total cell growth inhibition
TK10	:	Renal TK10
TLC	:	Thin Layer Chromatography
TMEDA	:	Tetramethylethylenediamine
TNF	:	Tumour necrosis factor
TRAIL	:	Tumor necrosis factor-related apoptosis-inducing ligand
TRI	:	Toxic Release Inventory

tRNA	:	Transfer ribonucleic acid
UACC62	:	A melanoma
UPLC	:	Ultra-high Performance Liquid Chromatography
VEGF	:	Vascular endothelial growth factor
VP16	:	Etoposide

BACTERIA AND CANCER; AN OVERVIEW

1.0 BACKGROUND

An overwhelming evidence has shown association between certain bacteria and cancer.[1a] The number of drugs available for treating malignant tumours and bacterial infections has been reduced by the development of chemoresistant and antibiotic resistance. Polyhydroxylated compounds have attracted much attention due to their broad spectrum of pharmacological activities such as anti-cancer and anti-bacterial activities and one classical example is usnic acid as previously mentioned. The structural motif of a great number of these compounds contain fused aromatic rings, quinones and hydroxyl moieties. These moieties have the ability to undergo redox cycling, this process is when compounds catalytically cycle and generate ROS such as hydrogen peroxide and superoxide which damages the cell. ROS also inhibit active efflux which is responsible for moving antibiotics out of the cell. The scope of this overview is broad therefore a wide range of reports is presented. Synthesis and mode of action of selected antibiotics, search for new drug candidates, cell circle in cancer cells, cancer in Africa and cancer treatment will be discussed.

According to the World Health Organization (WHO) bacterial infection is one of the world's most pressing public health problems.[1b] Bacteria are a diverse group of organism that are classified as Gram negative, Gram positive and acid fast bacteria according to the crystal violet staining technique used for preliminary identification based on cell wall composition.[2] Gram positive bacteria retain the dye stain and appear violet, Gram negative bacteria loose the stain and appear red, while acid-fast bacteria do not stain (Figure 1).[2] The acid fast bacteria have a similar cell wall structure to the Gram negative bacteria, but in addition to the peptidoglycan it also has mycolic acids.[2]

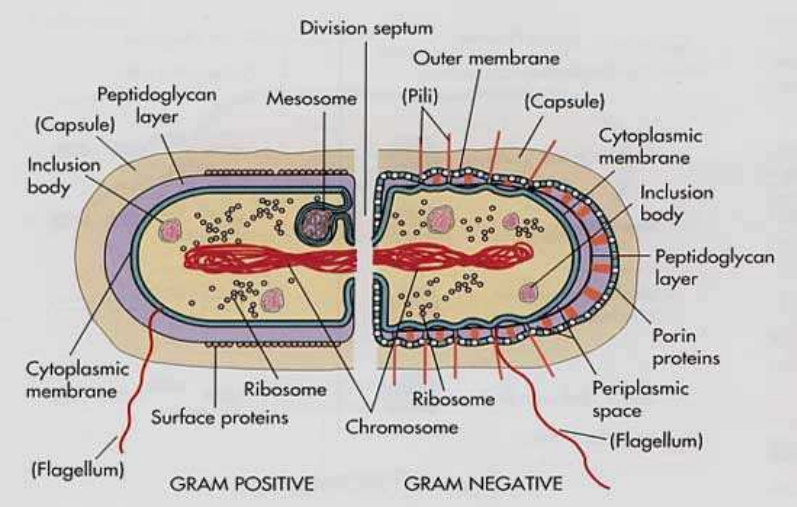


Figure 1: Structure of a gram positive and gram negative bacteria[3]

Almost every type of bacterium is becoming less responsive to antibiotic treatment, resulting in antibiotic resistance.[1] Antibiotic resistance is the ability of microorganism to survive the exposure to one or more antibiotics. These antibiotic-resistant bacteria can quickly spread to family members, schoolmates, and co-workers thus threatening the community with a new strain of infectious disease that is difficult to cure and more expensive to treat.[4] Therefore there is a need for new potent antibiotic agents that are affordable. Figure 2 depicts drug susceptible, non-resistant bacteria as well as drug resistant bacteria.

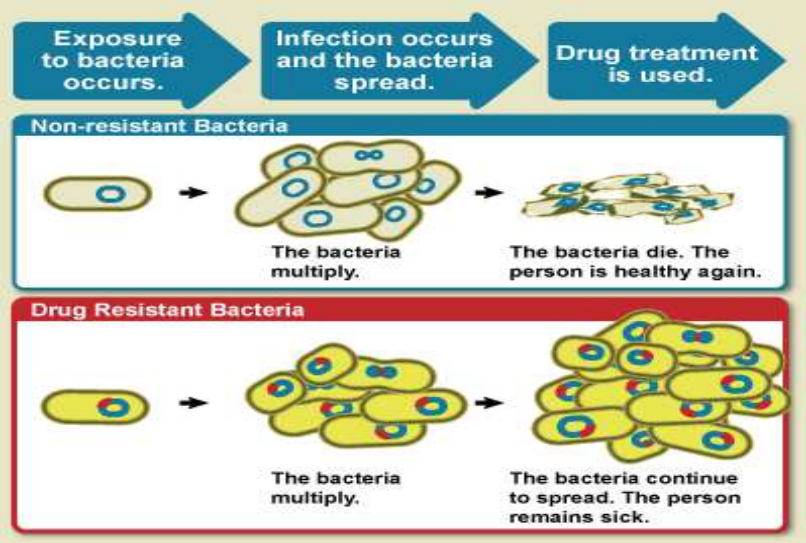


Figure 2: Drug susceptible, non-resistant and drug resistant bacteria[4]

Astonishingly, there has been a decline in the approvals of new antibiotics in an era where drug resistant and extreme drug resistant bacteria are posing a great threat to human health.[5-7] This is due to the common failure of new antibiotic drug candidates in the pipeline of pharmaceutical companies. The molecular framework of the first-line antibiotics contain fused aromatic rings, quinones and hydroxyl moieties. This framework has the ability to undergo redox cycling, this process is when compounds catalytically cycle and generate reactive oxygen species (ROS) such as hydrogen peroxide and superoxide which damages the cell. ROS also inhibit active efflux which is responsible for moving antibiotics out of the cell.

Historically antibiotics such as penicillin G **1**, tetracycline **2**, erythromycin **3** and cephalosporin C **4** gave rise to a large fraction of the approved antibiotics (Figure 3) and the process of semi-synthesis continues to be the predominant avenue to successful new ones [8]

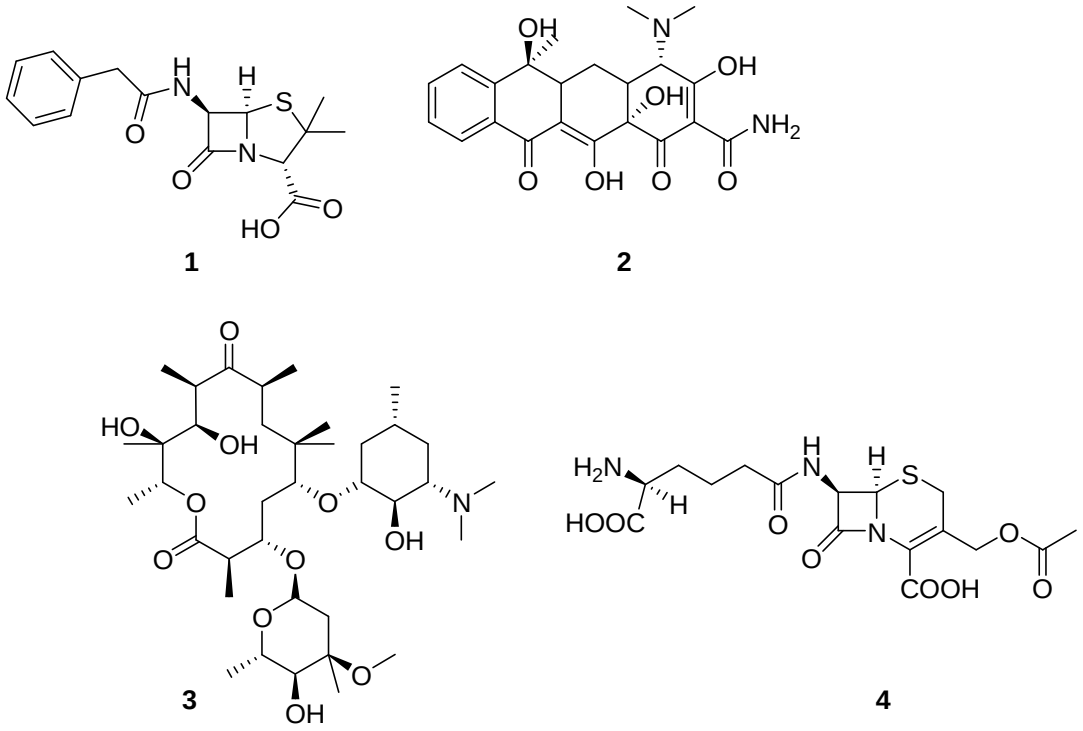


Figure 3: The first antibiotics to be discovered

Penicillins were the first antibiotics to be discovered in **1928,** by Sir Alexander Fleming, a professor of bacteriology.[9] He noticed zones of inhibition where a contaminating mould was growing in a petri-dish culture of the Gram positive bacterium *Staphylococcus aureus*. He later named the mould *Penicillium rubrum*.[9] The inhibition of bacterial growth was found to be caused by a compound that was named penicillin, one of a family of compounds characterized by a fused beta lactam, a carboxylic acid and one or more amino acid side chains. In **1930** Cecil George Piane, a pathologist at the royal infirmary in Sheffield, discovered that penicillin can be used in the treatment of a gonococcal eye infection in infants (a cause of ophthalmia neonatorum).[10-12] In **1936** the Australian scientist Howard Florey and a team of researchers from the University of Oxford proved the *in vivo* bactericidal action of the drug. [13] John Bumstead and Ovan Hess saved a dying patient's life using penicillin in **1942**. Merck and company also used

penicillin to treat the burn victims from the Coconut Grove fire in Boston in **1942**, and was found to be crucial in combating *Staphylococcus* bacteria which infect skin grafts.[14] The narrow spectrum of activity however led to derivitization to antibiotics such as ampicillin **5**, flucloxacillin **6**, dicloxacillin **7** and methicillin **8** as shown in Figure 4.

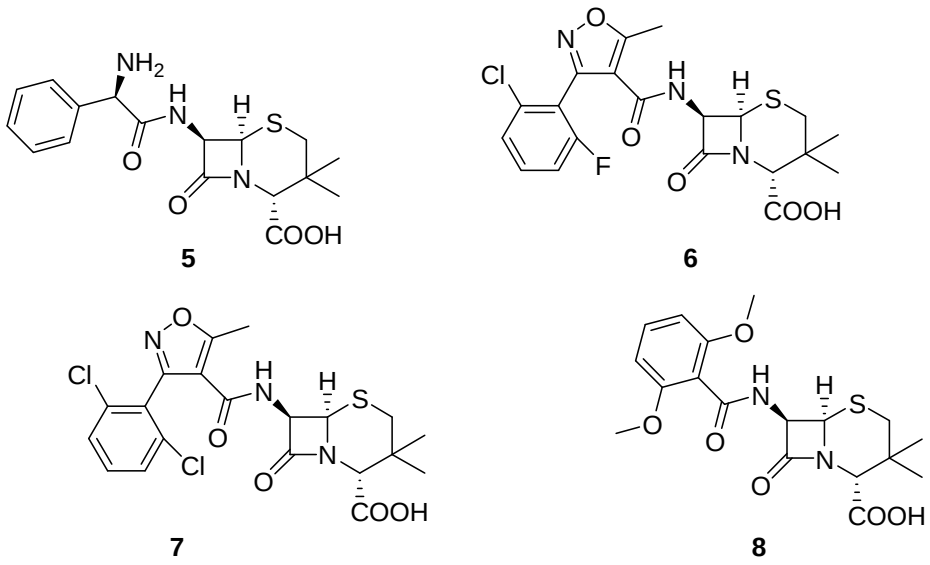


Figure 4: Semisynthetic analogues of penicillin[15]

All the derivatives shown in the Figure 4 above are very effective against both Gram positive and Gram negative with the exception of methicillin-resistant *Staphylococcus aureus* (MRSA) strain which has subsequently emerged.[15] In **1945** tetracyclines **2** were discovered by Benjamin Minge. They are produced by the *Streptomyces* genus of actinobacteria.[16] Tetracyclines are characterized by their exceptional chemotherapeutic efficacy against a wide range of both Gram positive and Gram negative bacteria.[16] The main indications for the use of tetracyclines are infections due to *Escherichia coli* and *Haemophilus influenza*, infections of the bile duct, bacterial respiratory disorders including bronchitis prophylaxis, mixed infections arising from the mouth, pharynx, or intestinal tract, brucellosis, tularaemia, plague and other pasteurelloses, leptospirosis, lymphogranuloma inguinale, cholera, and rickettsiosis.[17] Because of the development of strains

of microorganisms resistant to the tetracyclines, these antibiotics have lost some of their usefulness. They are no longer the drugs of first choice for treatment of staphylococcal, streptococcal, or pneumococcal infections.

In **1952** a broad spectrum antibiotic called erythromycin **3** was discovered by Mc Guire and co-workers.[18] It was isolated from the actinomycete *Saccharopolyspora erythrae*. Erythromycin, commonly administered as the ester prodrug erythromycin ethylsuccinate (EES). It is a macrocyclic 14 membered lactone ring, with 10 asymmetric centres and 2 sugar moieties (L-cladinose and D-desosamine).[19] It is on the WHO's list of essential medicine as the most important medication needed in a basic health system.[19] It inhibits the growth of *Staphylococcus aureus*, *Haemophilus influenzae*. A second major group of beta-lactams is the cephalosporins **4** that were discovered in **1956**. These are broad type of antibiotics from *Cephalosporium acremonium*, a sewer fungus.[19] They differ from penicillin by the presence of a 6 instead of a 5 membered ring as well as the different substituent groups. They are used for treatment of *Salmonella typhi*, the bacterium that causes typhoid fever. It is most effective against Gram positive bacteria; however its derivatives are effective on Gram negative bacteria as well.

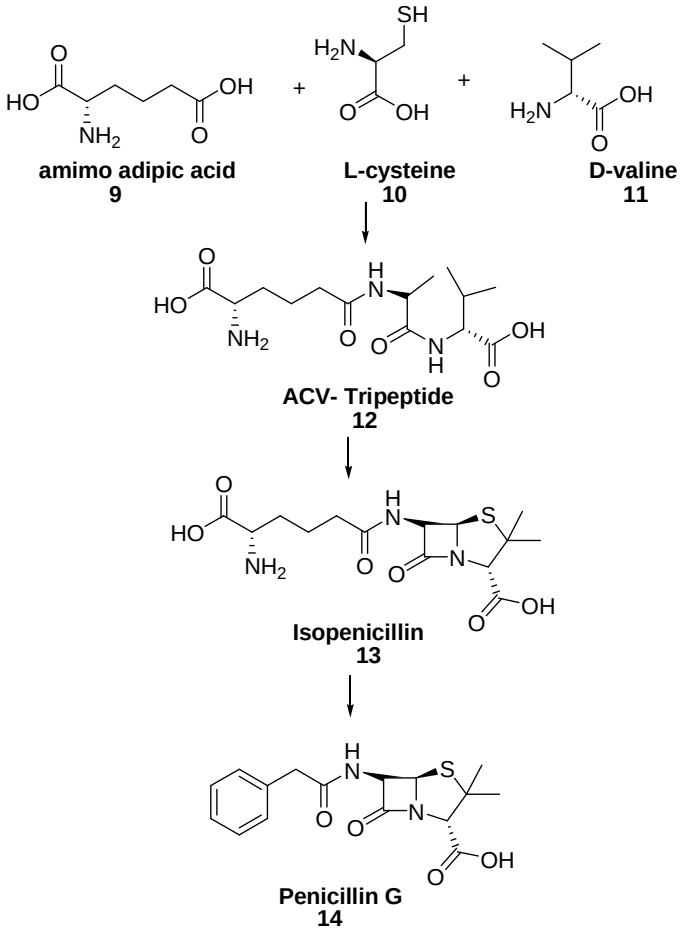
1.1 BIOSYNTHESIS, SYNTHESIS AND MODE OF ACTION OF SELECTED ANTIBIOTICS

Understanding the mode of action of anti-bacterial drugs not only guides the design and synthesis of novel antibiotics, it also helps to implement measures to prevent the development of resistance.[20] In this section we will discuss the mode of action of the first four discovered antibiotics.

1.1.1 PENICILLIN (1928)

Bio-Synthesis

Initially we will look at the biosynthesis of penicillin G (benzyl penicillin) as an example. This involves what 3 sequential biosynthetic steps. The first step involves the condensation of three amino acids; L-α-aminoadipic acid **9**, L-cysteine **10**, and L-valine **11** to form a tripeptide named δ-(l-α-aminoadipyl)-L-cysteine-D-valine (ACV) **12** as shown in Scheme 1.[21, 22, 23]



Scheme 1: Bio-synthesis of penicillin G.[21]

Prior to tripeptide condensation, the amino acid L-valine undergoes epimerization to become D-valine.[24, 25] The enzyme responsible for this first step is called δ-(L-α-aminoadipyl)-L-cysteine-D-

valine synthetase (ACVS), a nonribosomal peptide synthetase (NRPS). The second step is the oxidative conversion of linear ACV into the bicyclic intermediate isopenicillin N **13** by isopenicillin N synthase (IPNS), which is encoded by the gene *pcbC*.[21, 22] Isopenicillin N is a very weak antibiotic.[21] The final step is a transamination by isopenicillin N N-acyltransferase (encoded by the gene *penDE*), in which the α-aminoadipyl side-chain of isopenicillin N is removed and exchanged for a phenylacetyl side-chain to form penicillin G **14**. [21]

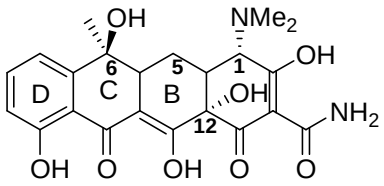
Mode of Action

β-lactam antibiotics act as bactericides, wherein they disrupt the synthesis of the peptidoglycan layer of the bacterial cell walls.[26] This is achieved by binding of the β-lactam ring to the enzyme called DD-transpeptidase, an enzyme responsible for catalysing the peptidoglycan cross-linkages. This then causes an imbalance between cell wall production and degradation, which eventually leads to cell death through osmolysis.

1.1.2 TETRACYCLINES (1945)

Synthesis

Tetracycline has four linearly annulated six-membered rings with five asymmetric centres at C4, C4a, C5a, C6 and C12 as shown in Figure 5 below.[27]

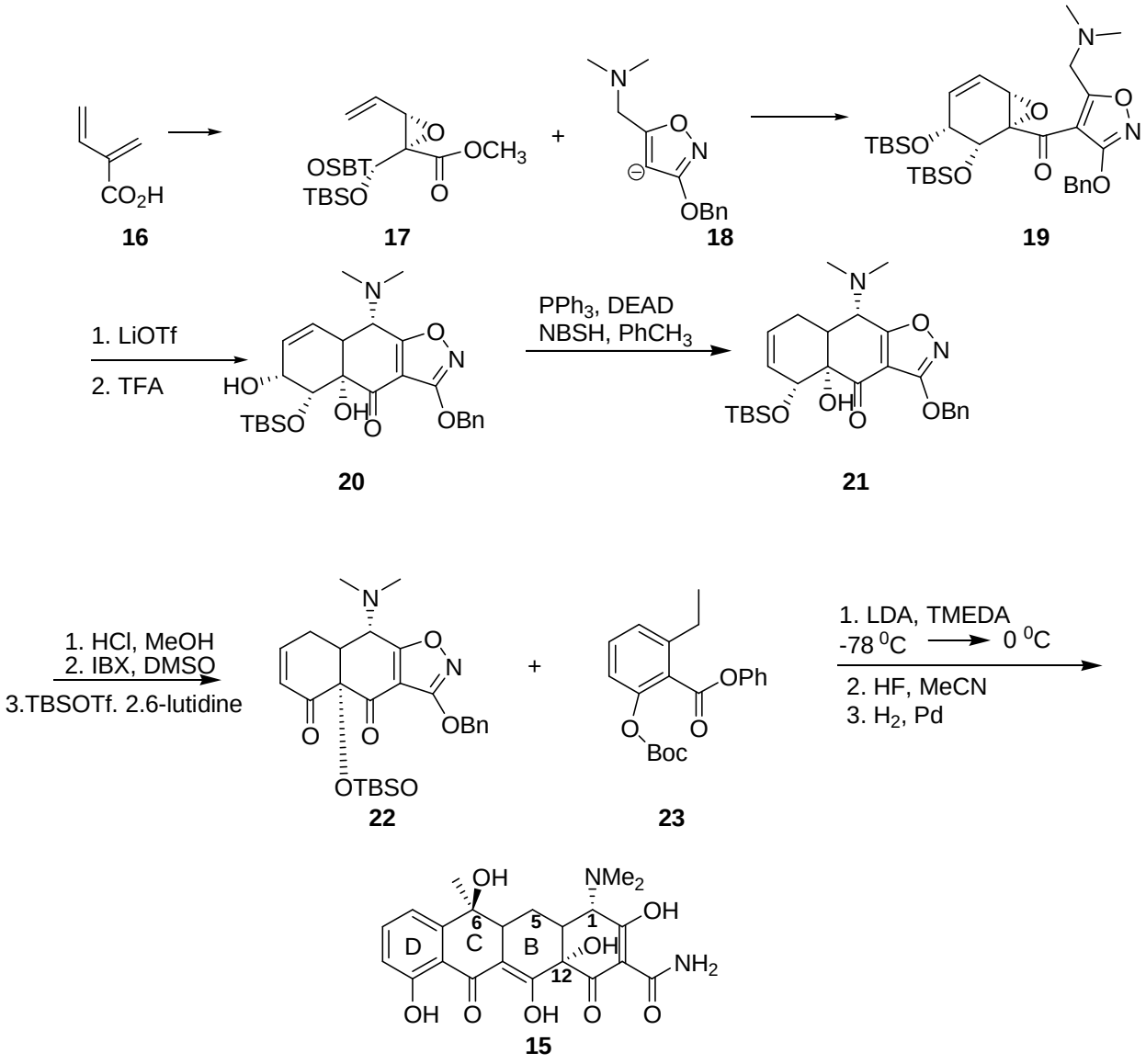


15

Figure 5: Structure of tetracycline

They can be prepared from cultures of several species of *Streptomyces*.[27] The first synthesis to be reported was by Woodward and a group from Pfizer.[27] A number of alternative synthetic

approaches were later developed by Shemyakin, Stork and more recently Meyers.[28,29] However, we have elected to discuss the improved synthetic pathway developed by Stork as shown in Scheme 2 below.[29] The most formidable challenge posed by tetracycline structure is the densely functionalized fused ring structure. The first step is the synthesis of the enantiomerically-pure ester **17** as shown in Scheme 2. This is prepared through fermentation of benzoic acid **16** and 1,2-dihydrodiol with Alcaligenes eutrophus B9. This is followed by epoxidation, rearrangement and silylation. Acylation of the epoxide with **18** resulted in ketone **19**.

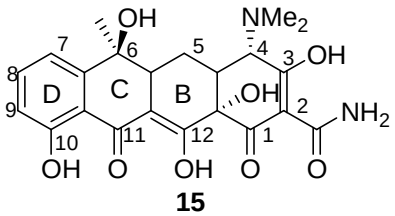


Scheme 2: Stork's synthetic pathway of tetracyclines[28]

The ketone undergoes a C-C bond formation after selective desilylation with TFA in the presence of LiOTf. Construction of AB ring system was accomplished through the treatment of a ketone with lithium triflate at 60 °C and subsequent TBS deprotection to give tricycle **20.** Double bond migration and reduction were carried out with triphenylphosphine, diethyl azodicarboxylate and o-nitrobenzylsulfonylhydrazine in 74% yield, giving allylsilyl ether **21**. This is then followed by deprotection of TBS group with IBX and protection of tertiary alcohol with TBSOTf to afford α,β-unsaturated enone 22. The final step is the Michel-Diekmann reaction of **22** with **23** to form **15**.[29]

Mode of action

Tetracyclines act as bacteriostatics for both Gram positive and Gram negative bacteria, with the exceptions such as *Pseudomonas aeruginosa*. The B and C diketone system of the C11 and C12 is the active centre, C4 and C5a are crucial for the antimicrobial activity (Figure 5).[30,31] This pharmacophore is composed of the linear fused tetracycle, naturally occurring (α) stereochemical configuration at the 4a, 12a (A-B ring junction) and 4 (dimethyl amino group) positions and the presence of the keto-enol system at positions 11, 12 and 12a in the proximity of the D ring.



15

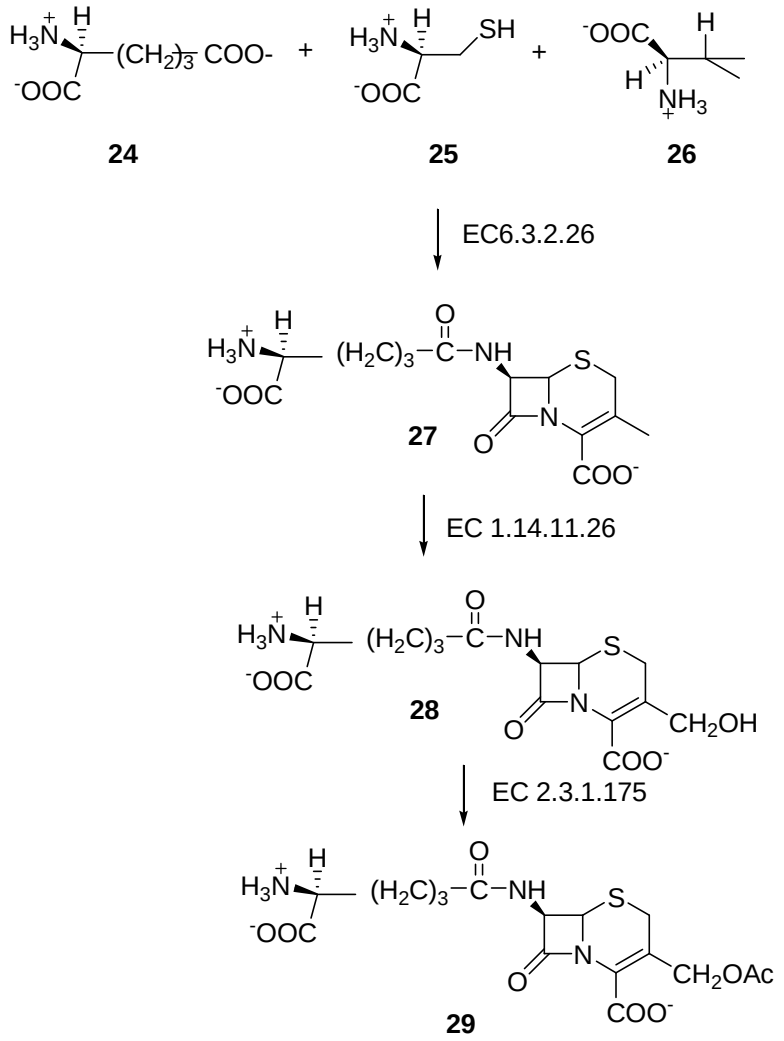
Figure 6: Molecular frame-work of a tetracyclines

Tetracyclines are strong chelating agents and their antibacterial activity is influenced by the chelation of metal ions.[32] The chelation site is comprised of the β-diketone system (11 and 12 positions), the enol (1 and 2 positions) and the carboxamide (position 2) groups of the A system. They bind to the 30S ribosome of the bacteria and thereby preventing attachment of amino acyl tRNA to the RNA ribosome complex.[30,31]

1.1.3 CEPHALOSPORINS (1956)

Synthesis

First generation cephalosporins are active predominantly against Gram positive bacteria and successive generations are also active against Gram negative bacteria. For this class of compound we will discuss the biosynthesis of cephalosporin C as an example. The first step in the biosynthesis is the deacetoxyacephalosporin A biosynthesis. This comprises the reaction of the aminoadipate **24**, L-cysteine **25** and L-valine **26** in the presence of N-(5-amino-L-carboxypentanoyl)-L-cysteinyl-D-valine synthase. This enzyme contains 4-phosphopantatethiene which may be involved in the reaction.[33]



Scheme 3: Bio-synthesis of cephalosporin C[33]

The resulting product is the deacetoxycephelasporin C **26** is oxidised to deacetylcephalosporin C **27** by deacetylcephalosporin C hydroxylase. Deacetylcephasporin C acetyltransferase catalyses the final step in the biosynthesis of cephalosporin C **29**.

Mode of action

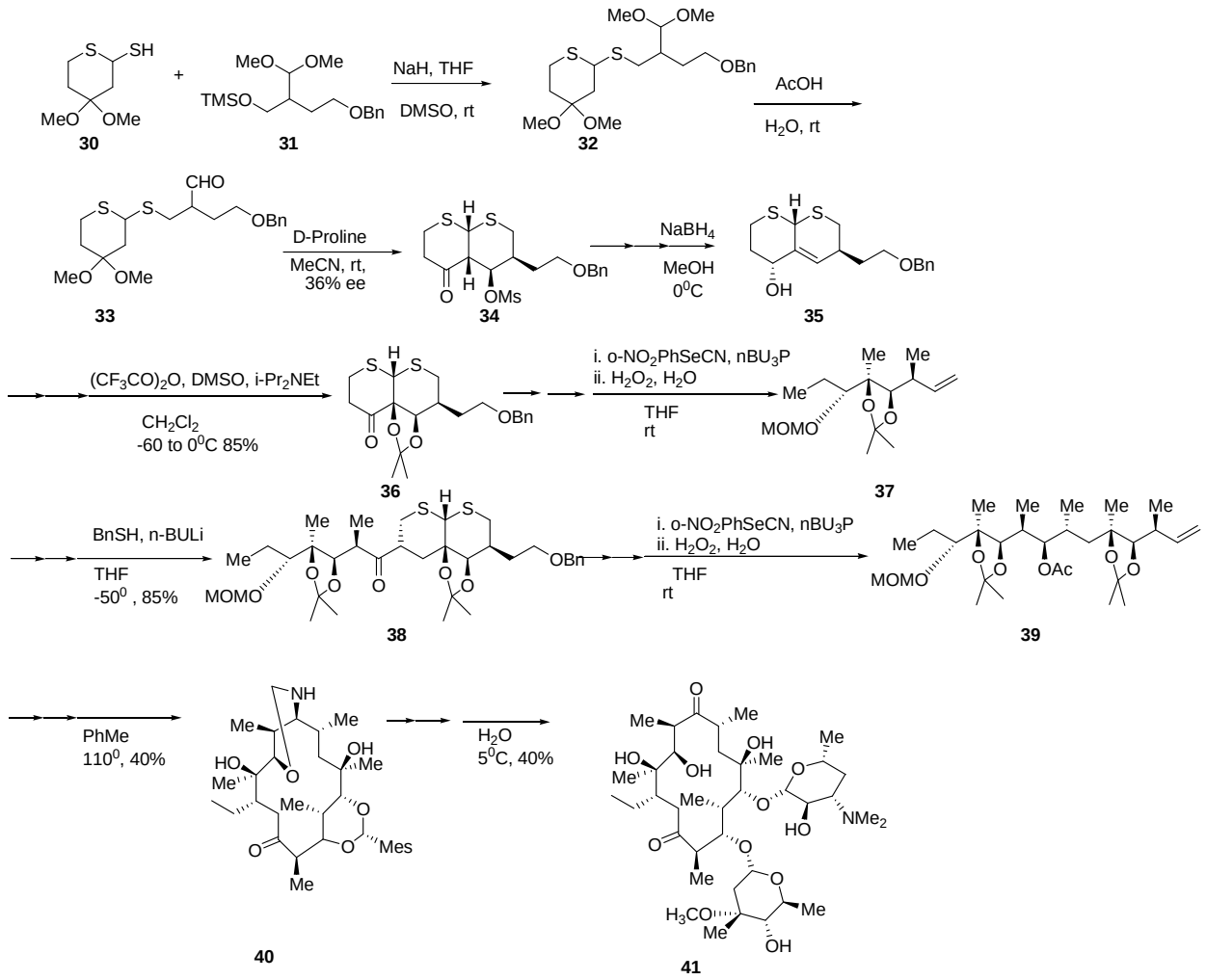
Cephalosporins act like penicillin in the inhibition of formation of peptidoglycan cross-links in the bacterial cell wall, but are less susceptible to β-lactamases.[34] Biological activity is also achieved by binding the β-lactam ring of the cephalosporin to the enzyme called DD-

transpeptidase, which is responsible for catalysing the formation of cross-links. This mechanism irreversibly inhibits cross-linkage of the peptidoglycan by mimicking the DD site.

1.1.4 ERYTHROMYCIN (1952)

Synthesis

The 10 stereogenic centres and a number of substituents make erythromycin challenging to synthesize. However, Robert Woodward was the first person to successfully synthesize the antibiotic, using hydrolysis and stereospecific aldolization (Scheme 4).[35] The key steps in the reaction sequence are such that compound **30** and **31** are coupled together to form compound **32** (scheme 4). Compound **32** undergoes hydrolysis to form aldehyde **33**, followed by proline-catalysed stereoselective intramolecular aldol reaction to form enone **34**. This is then followed by induced elimination of the mesylate to form compound **35**. After this comes a Grieco dehydration to give compound **37**. Through a series of reduction and oxidations compound **37** was reacted with compound **36** to form a dithiadecalin **38**. The final step involves hydration to give the desired erythromycin product **41**.



Scheme 4: The Woodward synthesis of Erythromycin[35]

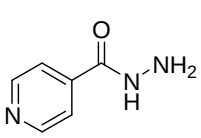
Mode of action

The erythromycins act as bacteriostatics.[36] They bind to 50S subunit of the bacterial 70S rRNA complex. This then inhibits protein synthesis, a processes critical for cell life and replication. It interferes with aminoacyl translocation preventing the transfer of the tRNA bound at the A site of the rRNA complex.[36] Without this translocation, the A site remains occupied thus the addition of tRNA and thereby inhibits transfer of its attached amino acid to polypeptide. The cell then stops the production of functional proteins, which ultimately results in cell death.

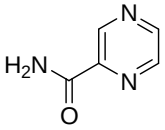
1.2 A SEARCH FOR NEW DRUG CANDIDATES

The government and non-governmental organizations globally, have started to invest in antibiotic drug research and development. For this project we attempted the search for new drug entities that could be used for the treatment of tuberculosis. This is an infectious disease caused by an acid fast bacterium called *Mycobacterim tuberculosis.* We selected this disease as a target as South Africa has the third highest number of TB infections globally.[1b]

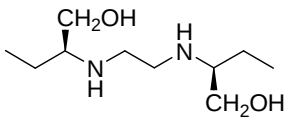
The first-line anti-TB drugs that can be used for drug susceptible TB strain are; isoniazid (INH) **42**, pyrazinamide (PYR) **43**, ethambutol (ETH) **44**, stremptomycin (STR) **45** and rifampicin (RIF) **46** (Figure 7).[37]



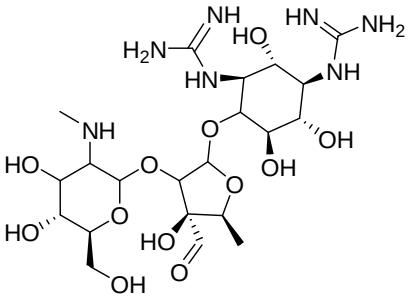
Isoniazid 42



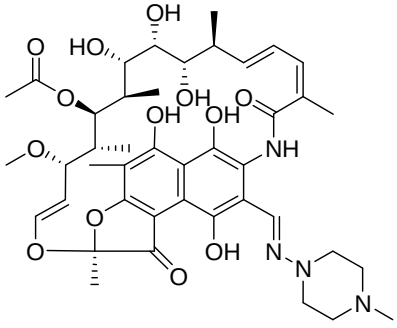
Pyrazinamide 43



Ethambutol 44



Streptomycin 45



Rifampicin 46

Figure 7: First-line TB drugs.[37]

Soon after the first anti-tuberculosis agents were introduced to humans the emergence of drug-resistant isolates of *M.tb* was already observed.[38-40] Since the early 1990s, there has been a great concern within public health institutions over the emergence of multiple drug resistant

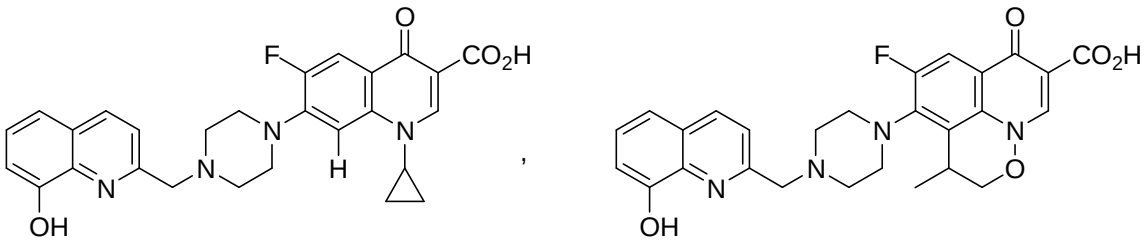
tuberculosis (MDR-TB). This is caused by *M.tb* strains resistant to at least two first-line anti-TB drugs, isoniazid (INH) **42** and rifampicin (RIF) **46**. MDR-TB develops during treatment of drug sensitive TB when the course of antibiotics regimen is interrupted and the levels of drug in the body are insufficient to completely eliminate the bacteria.[41] *In vitro* studies showed that spontaneous mutations in *M.tb* can be associated with drug resistance, while selective (antibiotic) pressure can lead to enhanced accumulation of these drug resistant mutants.[42,43] The efficient selection of drug resistance in the presence of a single antibiotic led investigators to recommend combination therapy, using more than one antibiotic to reduce the emergence of drug resistance during treatment.[44-46] TB control has indeed been effective where combination treatment is properly managed.[47]

In this study we adopted the use of the *Mycobacterium smegmatis* as the model for compounds to be further evaluated for anti-TB activity. This was primarily because it takes 3 months to grow *M. tuberculosis,* whereas it takes only about 4 days to grow *M. smegatis*. These two types of bacterial species belong to the same family and have many physiological similarities. The *M. smegmatis* model involves pre-screening a library of compounds against *M. smegmatis* and selecting the reduced number of compounds that proved positive to screen against *M. tuberculosis* (*M.tb).*

1.2.1 ANOTHER CLASS OF COMPOUNDS

1.2.1.1 FLUOROQUINOLONES

The fluoroquinolones are a promising class of drugs for the treatment of TB.[48] When they are used for treatment they are well distributed throughout the body, which explains their efficacy against intracellular mycobacteria. Fluoroquinolones are registered as second-line anti-TB drugs.[48-51] Two examples are moxifloxacin **47** and gatifloxacin **48** and these are candidates for shortening TB treatment (Figure 8) as they have exhibited the lowest minimum inhibitory concentrations (MICs).[52--58] They have also exhibited the greatest bactericidal activity as expressed in the rate of fall in Colony Forming Units (CFU).[55,59-61] CFU is the estimate of the number of viable bacterial cells in the sample. The lesser the CFU the more active is the drug as this means that the bacteria's ability to multiply via binary fission has been hindered.

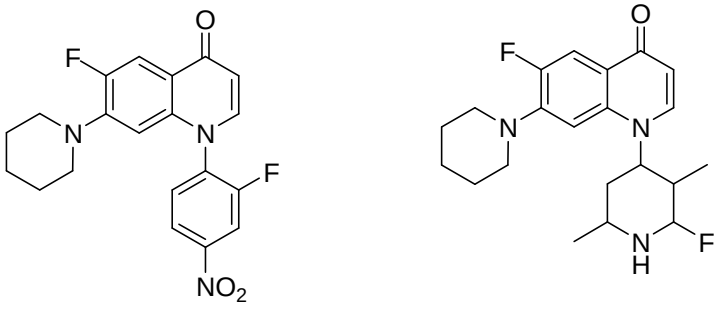


47
Moxifloxacin

48
Gatifloxacin

Figure 8: Fluoroquinolone derivatives

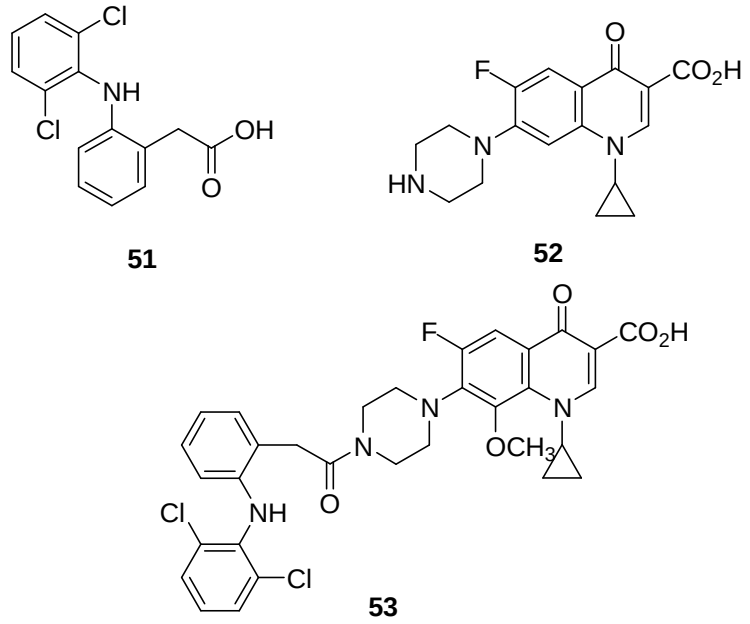
Bifunctional fluoroquinolone–hydroxyquinolines were also evaluated for antimycobacterial activity.[62] The compounds that exhibited the best activity are the 7-piperidinyl-1-(2-fluoro-4-nitrophenyl)quinolone **49** and 7-piperidinyl -(3,5-dimethylpiperazinyl)quinolone **50** derivatives, which exhibited 97% and 98% bacterial inhibition, respectively (Figure 9).



49 **50**

Figure 9: Fluoroquinolone derivatives

Among the synthesized compounds **51** (Figure 10) was found to be the most active compound *in vitro* with a MIC of 0.0383 µM and was more potent than the first-line anti-tubercular drug isoniazid (MIC = 0.1822 µM).[63]



51 **52** **53**

Figure 10: Diclofenac **51** and fluoroquinolone derivatives

This compound was suited for further modification to obtain a more efficacious and potent anti-tuberculosis drug. Various diclofenac acid hydrazones and amides have also been synthesized

and evaluated for *in vitro* and *in vivo* anti-mycobacterial activities against *M.tb*.[63] Preliminary results indicated that most of the compounds demonstrated better *in vitro* antimycobacterial activity (MIC = 0.0383–7.53 µM) than diclofenac **51** (MIC = 21.10 µM) and ciprofloxacin **52** (MIC = 9.41 µM).

Mechanism of Action of Fluoroquinolones

Moxifloxacin **47** is a broad-spectrum antibacterial agent with activity against both Gram-positive and Gram-negative bacteria.[63] It inhibits bacterial DNA gyrase, an enzyme that is essential for the maintenance of DNA supercoils, that are required for chromosomal replications.[63]

1.2.1.2 RIFAMYCIN DERIVATIVES

Rifampin is considered to be the cornerstone in the current treatment of TB.[65] Rifamycin derivatives, such as rifapentine **53**, rifabutin **54** and rifalazil **55** in Figure 12, have been synthesized from rifampicin to improve antimycobacterial activity and prolong their half-life. Rifapentine was approved by the FDA in 1998 for the treatment of TB. Rifapentine appears to be safe and well-tolerated at once-weekly dosing and is currently being evaluated in Phase III efficacy trials for treatment of latent tuberculosis.[62]

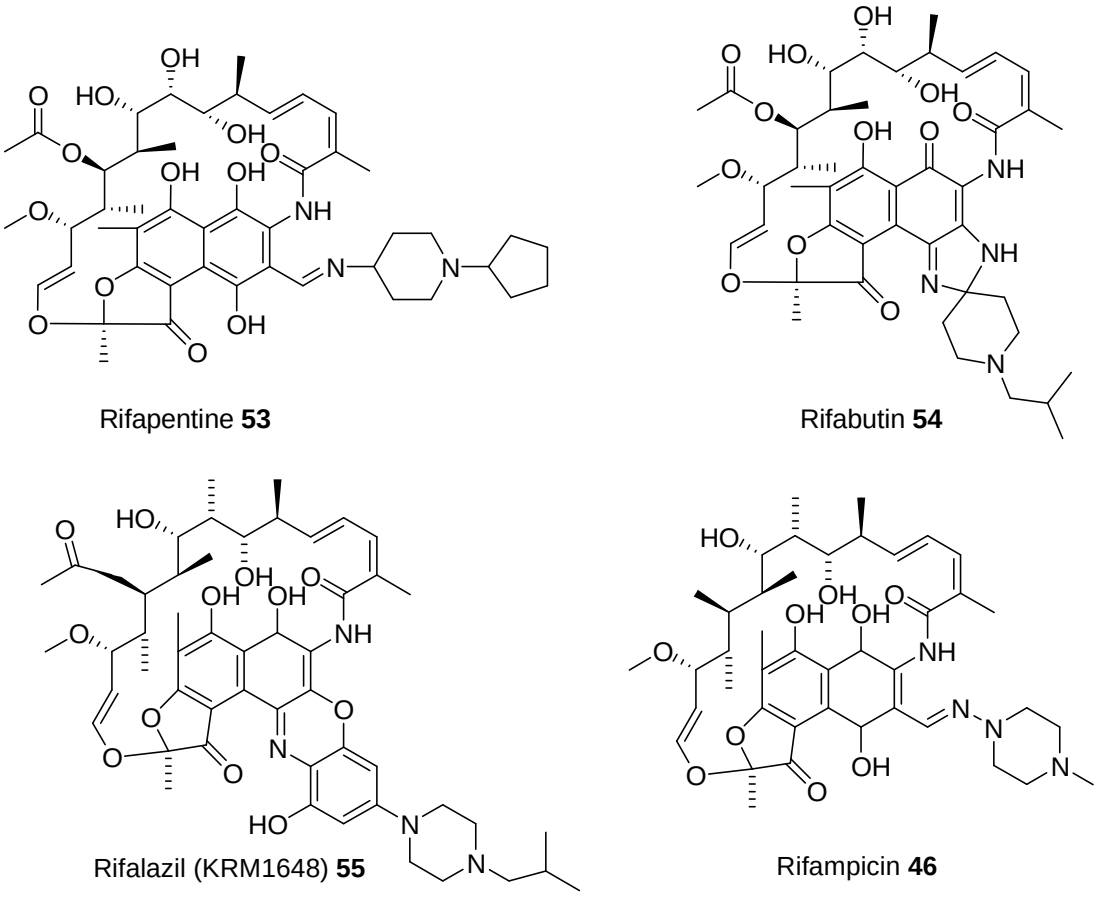


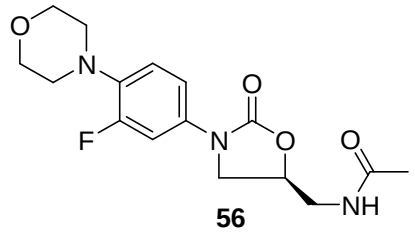
Figure 11: Structures of Rifamycin derivatives.

Rifalazil (RLZ) **55** is a new semisynthetic rifamycin derivative with a long pharmacokinetic half-life. It is highly active against a range of bacteria strains including *M.tb, Mycobacterium avium*, *Chlamydia trachomatis*, *Chlamydia pneumoniae,* and *Helicobacter pylori*.[66] RLZ is more active than RIF or rifabutin **53** against *M. tuberculosis* in mice both *in vitro* and *in vivo*.[67]

1.2.1.3 OXAZOLIDINONES

Oxazolidinones were discovered at DuPont in the 1970s and later developed by Pharmacia & Upjohn. They are a new class of compounds that are active against a variety of Gram-positive

bacteria, including *M. tb*. Linezolid **56** is the first oxazolidinone to be developed and approved by the FDA to treat single- or multiple-resistant Gram-positive bacterial infections (Figure 12).[68]

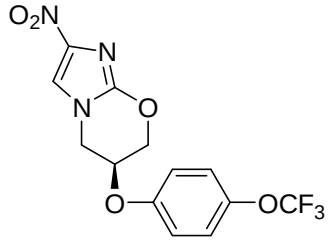


56

Figure 12: Structure of Linezolid.

1.2.1.4 NITROIMIDAZOPYRAN

A particularly promising candidate for TB treatment is nitroimidazopyran (PA824) **57**, derived from 5-nitroimidazoles (Figure 13).[69]



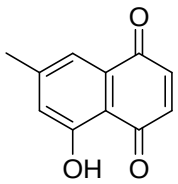
57

Figure 13: Nitroimidazopyran, PA824.

PA824 is highly active, with a *MIC* as low as 0.015–0.250 µg/ml against *M.tb* and MDR-TB. PA824 is a prodrug that requires activation by a bacterial F420-dependent glucose-6-phosphate dehydrogenase and nitroreductase to activate components that then inhibit bacterial mycolic acid and protein synthesis.[70]

1.2.1.5 NEW ANTIMICROBIALS

In most parts of Southern Africa, plants are used for medicinal purposes, including as antibacterial agents. The structure of biologically active compounds in plants varies; some compounds belong to classes of compounds such as alkaloids, terpenoids, coumarins/chromones, peptides and phenols. Naphthoquinones are among the compounds being investigated and they exhibit a range of pharmacological properties such as antibacterial,[71] antiviral,[72] trypanocidal,[73] anticancer,[74] antimalarial[75] and antifungal[76] activity. A detailed study of a series of synthetic and plant-derived naphthoquinone derivatives of the 7-methyljuglone **58** (Figure 14) scaffold on antibacterial activity against *M.tb* was reported by Mahapatra and co-workers.[77]



58

Figure 14: 7-Methyljuglone.

The aim of their study was to examine the synthesis, antibacterial activity and cytotoxicity of a series of naphthoquinones.[78] The naphthoquinone, 7-methyljuglone **58**, has previously been isolated and identified as an active component of root extracts of *Euclea natalensis* which displays anti-tubercular activity. Mehapatra and co-workers showed that 7-methyljuglone exhibited the most potent and most selective anti-tubercular activity of all the compounds tested.

It is well known that HIV increases a person's susceptibility to TB infection due to the decline of CD4+ lymphocytes in number and function, resulting in a weakened immune system incapable

of preventing the growth and local spread of *M.tb*.[79] A challenge arises in the interaction of HIV and TB drugs in HIV and TB patients. For example, rifampin has been recommended not to be used concurrently with almost all the anti-HIV nucleoside reverse transcriptase inhibitors NNRTIs and protease inhibitors due to drug-drug interactions.[80] Therefore, there is an urgent need for new compounds that can target TB but do not interfere with drugs that are used to treat HIV. Recent research for example that done by Xu has done an evaluation of anti-HIV active pyranocoumarin for their activity against *M.tb*.[80] Focused libraries were synthesized **59-67** and evaluated for their anti-TB activity in primary screening assays (Figure 15). The structural framework of library is similar to one of our libraries called benzo[3,2]chromen-6-one and they are discussed in Chapter 4.

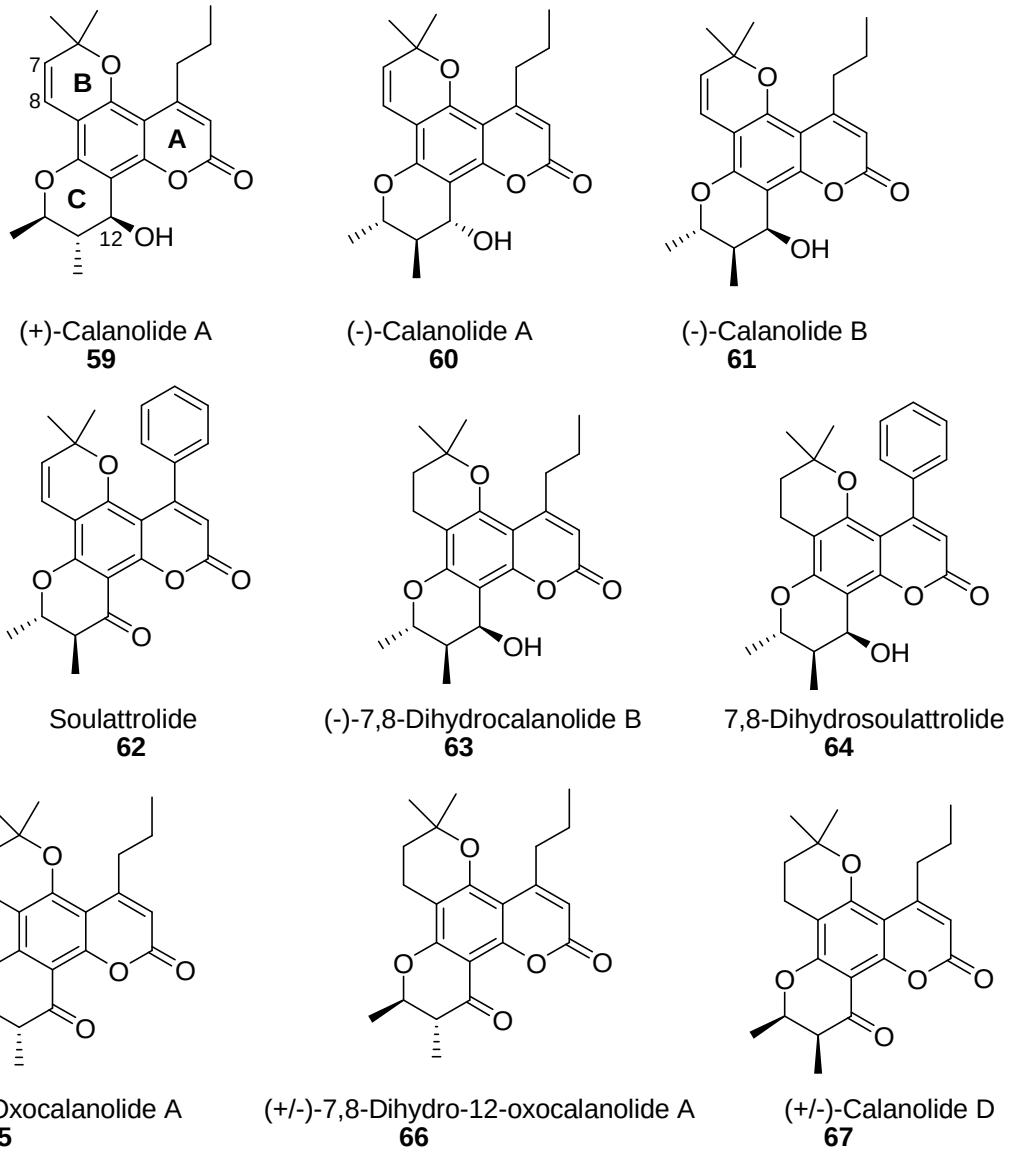
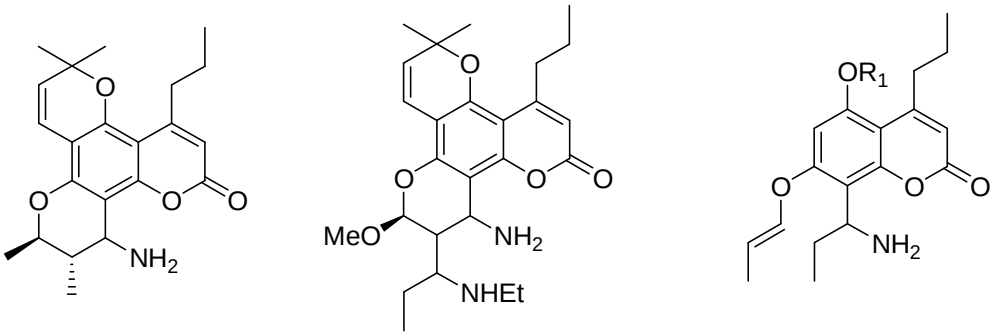


Figure 15: Pyranocoumarins evaluated for anti-TB activity.

Although this library was biologically active none were potent and the compounds shown to be active were further assessed to determine their MIC values. The amino derivatives (**68**, **69** and **70**) exhibited MIC values of 16 µg/mL each (Figure 16).

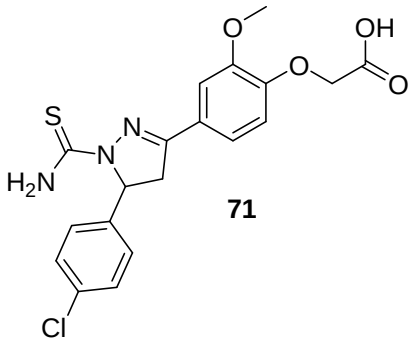


68 **69** **70**

Figure 16: The amino pyranocoumarin derivatives **68-70** most active against TB.[80]

1.2.1.6 PHENOXYACETIC ACID DERIVATIVES

A series of novel phenoxyacetic acid derivatives have also been synthesized and their *in vitro* activity was evaluated against *M.tb* H37Rv and an INH-resistant *M.tb* strain.[81] Among the synthesized compounds, compound **71** was found to be the most active against the *M.tb* H37Rv and INH-resistant strains exhibiting a minimum inhibitory concentration (MIC) of 0.06 µg/ml (Figure 17).



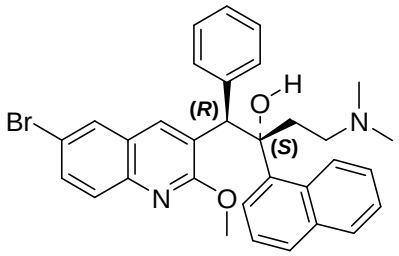
71

Figure 17: Phenoxyacetic acid derivative

1.2.1.7 DIARYLQUINOLINES

Koen *et al* has reported on the anti-mycobacterial properties of diarylquinolines (DARQs).[82] Chemical optimization of a lead compound led to a series of DARQs with potent *in vitro* activity against several mycobacteria, including *M.tb.* Molecules of the DARQ series have a MIC below 0.5 µg/ml against *M.tb* H37Rv. Antimycobacterial activity was confirmed *in vivo* for three of these compounds and the most active compound of the class, R207910 **72** (MIC_{99} = 0.06 µg/mL), is a pure enantiomer with two chiral centres (Figure 18).

.



R207910 **72**

Figure 18: Diarylquinoline derivative.

Compound R207910 **72**, has several properties, both *in vitro* and *in vivo*, that may improve the treatment of TB. In addition, it also appears to act on a new target providing an anti-mycobacterial spectrum different from those of current drugs. Its clinical potential is currently being tested on patients.

1.3 SUMMARY OF ANTIBIOTICS

In summary, the first-line antibiotics act either as bacteriostatics or as bacteriocides. Penicillins and cephalosporins act as bacteriocides. They are both β-lactams and their activity depends on disrupting the synthesis of the peptidoglycan layer of the bacterial cell walls by binding the β-lactam ring to the enzyme called DD-transpeptidase, an enzyme responsible for catalysing the formation of the peptidoglycan cross-linkages. This then causes an imbalance between cell wall production and degradation, which lead rapidly cell death. On the other hand tetracyclines and erythromycin are both bacteriostatics. Erythromycin binds to the 50S subunit of the bacterial 70S rRNA complex; this then inhibits protein synthesis, which is critical for cell life and replication. Tetracyclines bind to the 30S ribosome of the bacteria and thereby preventing attachment of amino acyl tRNA to the RNA ribosome complex. Due to mutations and other factors, the search for new drug entities is an ongoing process. As mentioned in section 1.1 first-line antibiotics contain fused aromatic rings, quinones and hydroxyl moieties. This framework has the ability to undergo redox cycling, (ROS) such as hydrogen peroxide and superoxide which damages the cell.

1.4 CANCER

While it seems likely that the rise of antibiotic resistance may lead to an increase of infection related deaths, cancer is currently the leading cause of death worldwide and accounted for 7.6 million deaths (13% of all deaths) in 2008.[83] Among the most deadly cancers are lung, stomach, liver, colon and breast cancer. It is projected that worldwide annual deaths from cancer will continue to rise to an estimated 13.1 million by 2030.[83] There are a number of prevention strategies that can be adopted in order to reduce the risk of cancer reaching the advance stage. These include early detection and curative treatment.

Cancer is a disease that occurs as a result of the suppression of apoptosis (programmed cell-death) which leads to increased cell-proliferation. Proteins that sense cellular damage or growth signals normally arrest the cell-cycle so that the damage can be repaired or, if that is impossible, apoptosis is induced. Malfunction of this system leads to cancer by allowing cells to proliferate when they should either be repaired or die. Most human carcinogenesis was shown to have abnormalities in some component of the retinoblastoma protein (pRb) pathway.[84] Mutations of the retinoblastoma gene result in a loss of functioning of the retinoblastoma protein, which leads to a wide range of human cancers, including osteosarcomas, small-cell lung cancer, lung cancer and breast cancer.

The cell-cycle is controlled by proteins called cyclins and their catalytic partners.[84] The miscoding mutations of a cyclin results in a loss of affinity for its catalytic protein. This can take place due to epigenetic inactivation by methylation in a variety of tumours. Other types of cancers can be due to chromosal rearrangement. In most human cancer cells, the cyclins that have been observed to mutate are cyclin-dependent kinase complex CDK4/6 cyclins. For example, it has been observed that a miscoding mutation of CDK4/6 resulted in a loss of cyclin-dependent kinase inhibitor p16 binding. There have been attempts to exploit this by treating cancer patients with inhibitors that block CDK activity. Indirubicin, flavopiridol and UCN-01 were the first CDK inhibitors tested in clinical trials in which benefits have been noted in some patients.[84]

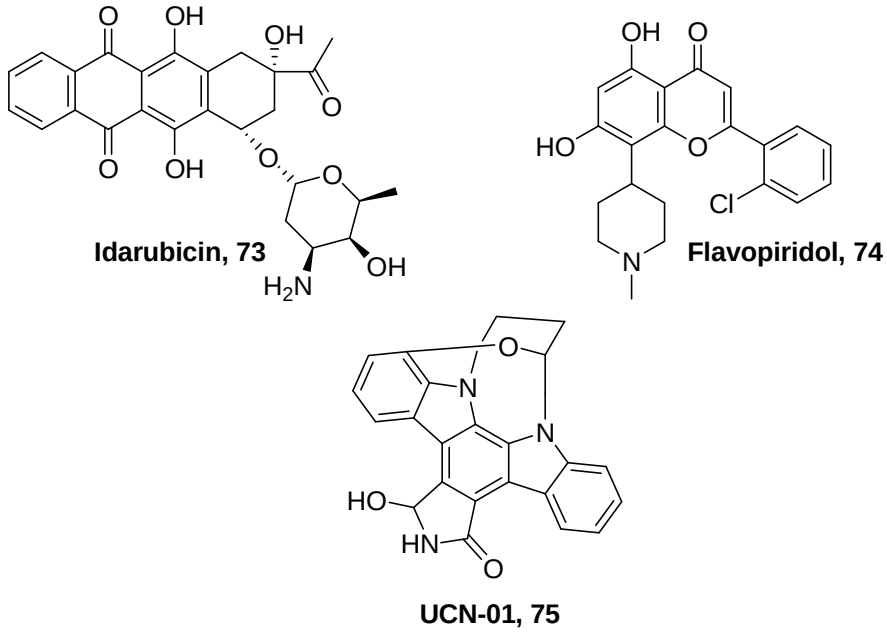


Figure 19: Structures of indirubicin, flavopiridol and UCN-01

1.5 THE CELL-CYCLE IN CANCER CELLS

To ensure a proper progression through the cell-cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one. As mentioned, this is controlled by cyclins and their corresponding catalytic enzymes. Critical regulators and check points of the cell-cycle progression are shown in Figure 20.

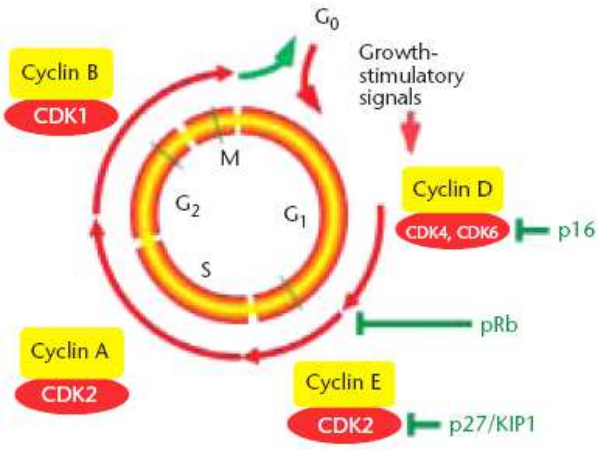


Figure 20: Critical regulators of a cell-cycle[96]

Growth factor signals stimulate the synthesis of D-type cyclins, which then form active complexes with CDK4 and CDK6 in the early G_1 phase. The primary substrate of CDK4/6 is the retinoblastoma protein (pRb), which in its active hypophosphorylated form inhibits cell-cycle progression by binding and repressing the activity of the transcription factor, E2F.[85] The transcription of several genes (including E-type cyclins) takes place in the G_1-to-S transition by hyperphosphorylating the pRb, which causes it to disassociate from E2F. The activation of the cyclin E/CDK2 complex drives progression from the G1 to S phase. The appropriate completion of deoxyribonucleic acid (DNA) synthesis in its S phase requires the activity of A-type cyclins together with CDK2. The cyclin B/CDK1 complex regulates entry to mitosis, whereas proteolytic degradation of B-type cyclins regulates the exit from mitosis. Other CDK/ cyclin complexes are regulated as follows: (i) inhibiting or activating phosphorylation; (ii) inhibition by specific CDK inhibitors (CDKIs) such as p16, p21/WAF1, p27/KIP1 or (iii) by targeted degradation, mediated for example by the SCF (Skp1 – Cullin – F-box protein) ubiquitin ligase. It has been observed that, in tumour or cancer cells, the cyclin dependent kinase inhibitory proteins are either absent or mediated.[85]

1.6 APOPTOSIS

Apoptosis is an active, energy-dependent, genetically programmed process that occurs widely during mammalian development. It offers a practical way of getting rid of old, damaged or dangerous cells. Apoptosis is mediated by two major pathways known as the mitochondrial and the death-receptor pathways. The various interactions between the two pathways are summarized in Figure 19.[84,853] The two pathways are intimately connected, and sometimes the death receptor-mediated apoptotic stimulus is amplified by the mitochondrial loop. It begins when a death-

ligand (such as tumour necrosis factor (TNF), Fas-ligand (FasL) or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L)) interacts with its corresponding receptor (TNF receptor, Fas receptor or KILLER/DR4 and DR5 respectively) leading to the activation of a caspase cascade pathway which includes caspase 8 and caspase 3 as shown in Figure 21. This results in the cleavage of proteins that are essential to cell viability.[85]

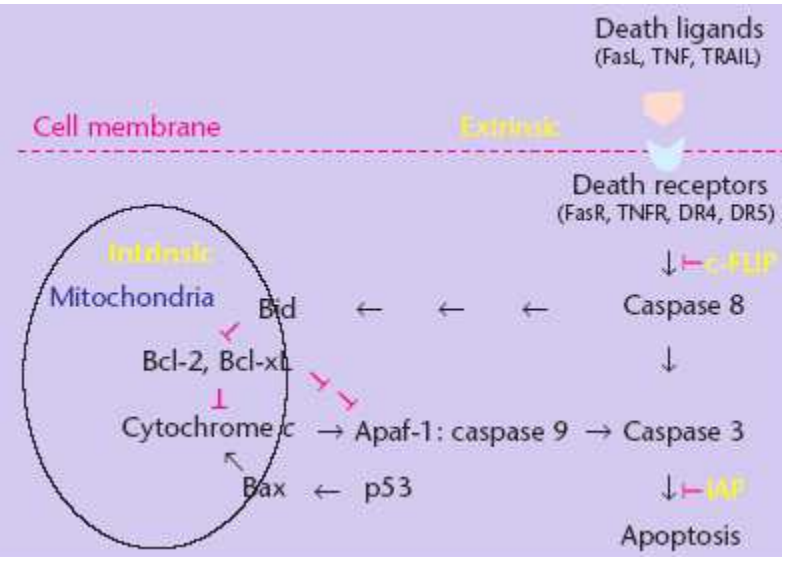


Figure 21: The mitochondrial and death-receptor pathway of apoptosis[96]

The death receptor pathway is an extrinsic one that is triggered by extracellular cues such as growth factor withdrawal, matrix detachment and cytokine-mediated killing. The mitochondrial cascade is an intrinsic pathway and might be triggered by intracellular cues such as DNA damage or osmotic stress and it involves caspase 9 as shown in Figure 21 above.

The first genetic component of the human cell-death pathway to be identified was the *B-cell CLL/lymphoma 2 (Bcl2)* gene. This pathway is mediated by Bcl-2 family proteins, which consist of 20 members that have been recognised and described in humans.[83] The relative ratios of anti-

and pro-apoptotic Bcl-2 family proteins dictate the ultimate sensitivity or resistance of cells to various apoptotic stimuli, including growth factor depletion, hypoxia, radiation and anticancer drugs. In response to apoptotic signals, pro-apoptotic Bcl-2 family proteins such as Bax translocate and alter the permeability of the mitochondrial membrane which leads to cytochrome C release and activation of the caspase cascade. Anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL counter these effects.[84]

1.7 CANCER IN AFRICA

Cancer is an emerging public health problem in Africa. About 715,000 new cancer cases and 542,000 cancer deaths occurred in 2008 on the continent.[82] Despite its growing burden, cancer still receives low public health funding in Africa and this might be due to lack of infrastructure and other pressing public health problems.[86] Lack of infrastructure (such as poor availability of diagnostic and screening services), low awareness of early signs, and limited availability of treatment are major challenges faced by the continent. When focusing on the African continent one has to take into account that the continent has a diverse population, culture, economic status and other sociodemographic characteristics, which affect the occurrence of cancer and its outcomes. For example, the Northern Africa region is dominated by Arabs, while the sub-Saharan region is dominated by an indigenous black population with South Africa having as much as a 9% white population.[87] These socio-demographics contribute to the variations of cancer in each region.

The dominant type of cancers in the continent are cervical, liver, Kaposi sarcoma and urinary bladder, and all are a result of an infectious agent.[87] The well-known infectious agents are viruses, bacteria and parasites. From the literature it has been reported that of the new diagnosed

cancers in females, 21% is due to cervical cancer.[87] This is the leading cause of death (21,600) in women in Eastern Africa 2008, accounting for about 25% of the total new cancer cases and deaths.[87] Countries like Malawi, Mozambique and Zambia have the highest toll of cervical cancer on a world scale. This is due to the high prevalence of human papillomavirus (HPV) infection and lack of screening.[83] A different picture emerges when looking at Southern Africa. Breast cancer was the most diagnosed cancer and the leading cause of cancer death among women (9000 cases and 4500 deaths) in Southern Africa.[87] It was found that breast cancer incidents were six times higher in whites than in blacks.[87] The same trend is also observed in Northern Africa, where breast cancer is dominant among females.[85] In the middle and Western Africa both breast and cervical cancers occurred with similar frequency. In sub-Saharan Africa cervical cancer still remains the leading cause of cancer deaths among women.[87]

In males the dominant type of cancer was found to be liver cancer, and this has accounted for 11% of all cases.[83] Liver cancer was the leading cause of deaths among men in Middle and West Africa.[88,89] When analyzing demographic data, Kaposi's sarcoma was found to be the leading cause of cancer deaths among men in Eastern Africa. Kaposi's sarcoma is a cancer commonly associated with infections of human herpes virus-8 and HIV.[88,89] In sub-Saharan and Northern Africa chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are the leading cause of deaths among men.[90,91] Contamination of staple foods, such as maize and ground nuts, with aflatoxin $B_{1,}$ a known cancer-causing agent produced by moulds during inadequate storage of crops is the contributing factor.[92,] In Northern Africa it was lung cancer that was more dominant among males and the leading cause of deaths.[88] However, the lung cancer incidents were only half as high as the rates in Southern Africa and this is due to smoking which accounts for 65% of the cases in South Africa. Egyptian men were found to have the

highest incidents of bladder cancer.[93] About 40% of the disease is caused by a parasite, *Schistosoma hematobium*. The infection occurs when people come into contact with early development stage of the parasite which is released by snail larvae. Prostate cancer was commonly diagnosed among men in Southern Africa.[85] The survival rate after diagnosis is much poorer on the continent, which might be due to the fact that most cases are diagnosed at the advanced stage. Incident and mortality rates are the two frequently used measures of cancer occurrence. They are usually expressed in 100,000 people per year and the mortality rate in Africa is high in both male and female when compared to North America.

1.8 CANCER TREATMENTS

The most feasible and cost effective approach to cancer control in Africa is prevention of exposure to cancer-causing agents or risk factors, including infections, tobacco use and obesity. The most commonly diagnosed cancers in Africa which are caused by infection are cervical, liver and bladder cancers. They are potentially preventable by vaccination, improved hygiene, sanitation and/or treatment. For example, the human papillomavirus (HPV) is a cancer causing infectious agent that can be prevented by vaccination. The vaccines are administered to adolescent girls.

Tobacco use accounts for 20% of cancer deaths world wide and 6% are from Africa. Tobacco use shortens life expectancy by 10-20 years.[85] The WHO established the Framework Convention on Tobacco Control (FCTC), this was due to the globalization of the tobacco epidemic. This includes raising the price of tobacco products, banning smoking in public places, restricting tobacco advertising and promotion, counter advertising and providing treatment and counselling for tobacco dependence. Obesity has also been associated with increased risks of several cancers,

including breast, colorectal, stomach, liver, kidney and uterine corpus. The prevalence of obesity and physical inactivity is increasing in some African countries thereby increasing obesity especially in urban areas. According to the Global School-Based Student Health Survey, more than 40% of 13-15 year-old teens in urban areas of Kenya and Zimbabwe spent three or more hours per day watching television and doing other sedentary activities.[86] Methods that are commonly used in the treatment of cancers are chemotherapy, radiation therapy, targeted therapy, transplantation therapy, biological therapies and photodynamic therapies. These are further discussed below.

1.8.1 CHEMOTHERAPY

Chemotherapy is the treatment of cancer cells with drugs. The latter kills the cancer cells or makes them to be less active. The medicines are used in combination and together are called chemotherapy regimes. It can be given before or after surgery in order to shrink the cancer cells. If given before surgery it is called neoadjuvant chemotherapy. An example of the medicine used for this type of cancer is Abraxane which is albumin bound paclitaxel and called the Taxane chemotherapy. Taxane chemotherapy (paclitaxel/Taxol® **77** and decotexel/taxotere® **78**) is one of the common chemotherapy drugs used for breast cancer (Figure 23).

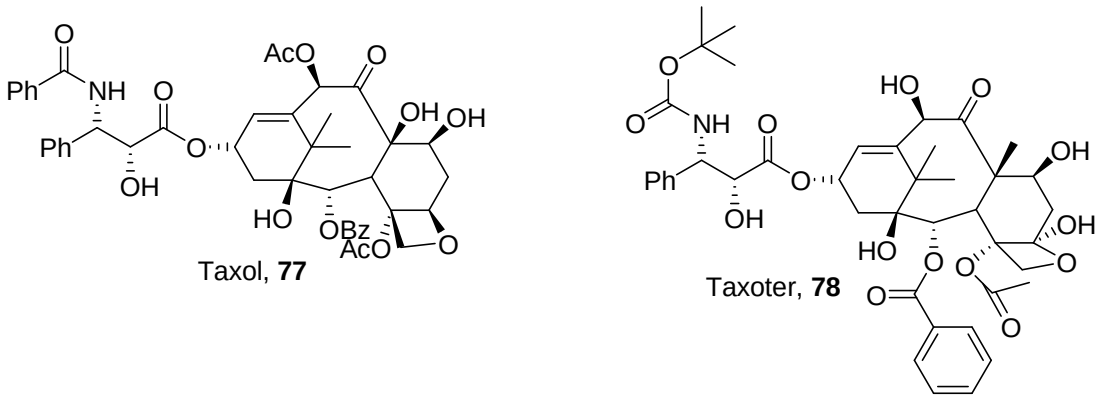
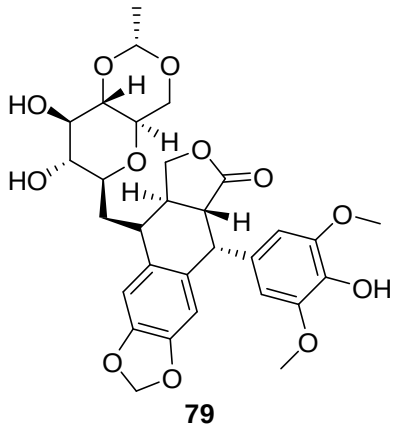


Figure 23: Structure of Taxane and Taxotere

It is given intravenously and it interferes with the ability of cancer cells to divide. These chemotherapy drugs are sometimes used in combinations with other drugs, for example Fluorouracil (5-FU) for breast cancer. Another example of a well-known anticancer drug is an etoposide also known as VP16 (Figure 24).



79

Figure 24: Etoposide

Etoposide is used for Kaposi's sarcoma, Ewing's sarcoma, lung cancer, testicular cancer, lymphoma as well as in bone marrow and blood stem transplant.[94] The mechanism is such that it forms a ternary complex with DNA and topoisomerase II enzyme, thereby breaking the DNA strand and promoting apoptosis.[94] This is the drug we have selected as a positive control for our cancer screening as will be outlined in chapter 7. A vinca alkaloid used in chemotherapy is Vinblastine (Figure 25). It is used for breast cancer, head cancer, neck cancer and testicular cancer.[95]

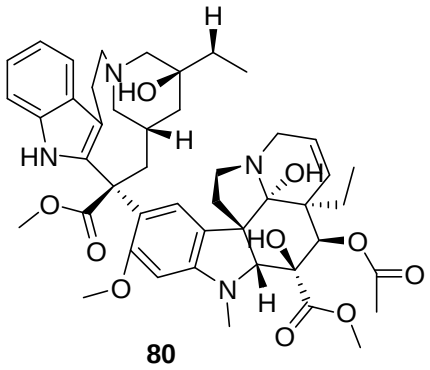


Figure 25: Vinblastine

It is a microtubule-disruptive drug and at low concentration it suppresses microtubule dynamics.[95] At high concentration it reduce microtubule polymer mass. It also enhances microtubule detachment from spindle poles, thereby inducing apoptosis. A quinolone alkaloid which is composed of planar pentacyclic ring structure Camptothecin **81** also possesses anticancer activity (Figure 26).[96] It inhibits the DNA enzyme called topoisomerase I and thereby inducing apoptosis.[96]

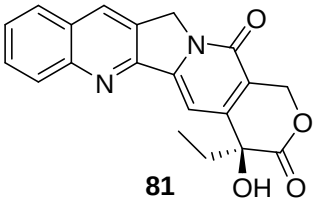


Figure 26: Camptothecin

1.8.2 RADIATION THERAPY

Radiation therapy is the use of high-energy radiation to kill cancer cells by damaging their DNA. The challenge with this type of treatment is that it can also damage normal cells, therefore treatment must be carefully planned to minimize side effects. This involves the use of either a radiation machine or a radioactive material placed near tumour cells or injected into the blood

stream, in theory using this method cancer cells whose DNA is damaged will die and be eliminated naturally by the body.[97]

1.8.3 TARGETED THERAPIES

Targeted therapy is the newer type of chemotherapy that use drugs or other substances that block the growth and spread of cancer by interfering with specific targets that are involved in tumour growth and progression (and is described in more detail in other parts of this Chapter).[98] They mainly focus on proteins that are involved in cell signalling pathways and thereby help stop cancer progression. This induces cancer cell death through the process called apoptosis.

1.8.4 TRANSPLANTATION

This involves organ or tissue transplants and is dominated by hematopoietic stem cell transplants (HSCTs). These include bone marrow and peripheral blood transplants and the organs are mainly the liver and the lung. The biggest challenge in this type of cancer treatment is preventing rejection by the host. One of the methods used to prevent this is severe immunosuppression at a great risk of a variety of infections.[99]

1.8.5 ANGIOGENESIS INHIBITORS

Angiogenesis is the formation of new blood vessels. Tumour cells need new blood vessels in order to grow and spread. This process involves the migration, growth and differentiation of endothelial cells which line the inside wall of blood vessels. Inhibitors prevent the formation of new blood vessels in cancer cells, thereby stopping or slowing the growth or spread of tumours. For example, Avastin®is (bevazumab) the angiogenesis inhibitor is a monoclonal antibody which binds to the vascular endothelial growth factor (VEGF). The latter is then unable to activate the

VEGF receptor. Monoclonal antibodies are laboratory produced antibodies that bind to specific antigens expressed by cancer cells.[100]

1.8.6 BIOLOGICAL THERAPIES

Biological therapy uses living organisms, substances derived from living organisms or synthetic versions of such substances to treat cancer. Sometimes vaccines and bacteria are used to stimulate the body's immune system to act against cancer cells. This therapy can be used to either treat cancer itself or for side effects of other cancer treatment.[101]

1.8.7 PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) combines a photosensitizer (drug) with a specific type of light in order to kill cancer cells. The photosensitizer is activated by light at a specific wavelength and produces oxygen that kills cancer cells. For example, Porfimer sodium and photofrin are used in PDT treatment as photosensitizers.[102]

1.9 SUMMARY OF CANCER THERAPY

In summary, cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (13% of all deaths) in 2008. This disease occurs as a result of the suppression of programmed cell-death, which results in increased cell-proliferation. Normally proteins that sense cellular damage or growth signals arrest the cell-cycle so that the damage can be repaired or, if that is impossible, apoptosis is induced. Malfunction of this system leads to cancer by allowing cells to proliferate when they should either be repaired or die. Most cancer deaths are due to late detection of the disease, due to a variety of reasons. Common human cancers include

osteosarcomas, small-cell lung cancer, melanoma cancer (a skin cancer) and breast cancer. Other types of cancers can be due to chromosomal rearrangement. As mentioned above the cost effective approach to cancer control is prevention to exposure. Cervical, liver and bladder cancers are the most diagnosed types of cancers in South Africa. They can be prevented by vaccination or improved hygiene, and can be treated. Methods that are mostly adopted in cancer treatment are; chemotherapy, radiation therapy, targeted therapy, transplantation therapy, biological therapies and photodynamic therapies.

In this work we screened our synthesised compounds against four cancer cell line namely; lung cancer, renal, breast cancer, melanoma cancer. As mentioned before the structural motif of a great number of biological compounds contain fused are aromatic rings, quinones and hydroxyl moieties. These moieties have the ability to undergo redox cycling, this process is when compounds catalytically cycle and generate ROS such as hydrogen peroxide and superoxide which damages the cell. ROS also inhibit active efflux which is responsible for moving antibiotics out of the cell.

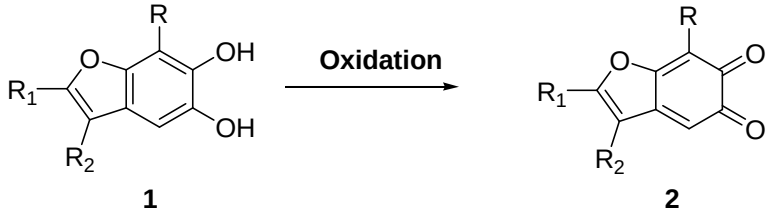
This study is divided into three sequential parts. The first part is the application of enzymes in synthetic chemistry (biocatalysis) to generate polyaromatic and substituted aromatic compounds. This is followed by derivitazation of selected compounds with isoniazid and thiosemicarbizide. The last section encompasses biological evaluation of the synthesised compounds as anti-bacterial and anti-cancer agents. Biocatalysis and the objectives of the project will be discussed in depth in Chapter 2.

LACCASES AS CATALYSTS FOR A SUSTAINABLE ROUTE TOWARDS BENZOFURANS

2.0 THE AIM OF THE PROJECT

This study was divided into three distinct parts. The first part is the application of enzymes in synthetic chemistry (biocatalysis) to generate polyaromatic and substituted aromatic compounds. The reason is to employ green methods in the synthesis of organic compounds as discussed in section 2.3. The methodology adopted was C-H activation which is efficient in terms of atom and step economy. The latter involves the introduction of a C-H heteroatom bond without the use of a functional group such as triflate, halide, tin or palladium catalyst. This is followed by derivatazation of selected compounds with isoniazid and thiosemicarbizide. This will be discussed in details in chapter 6 of this dissertation. The last section encompasses the biological evaluation of the synthesised compounds as anti-bacterial and anti-cancer agents.

The first objective identified was to investigate the use of the enzyme, laccase, in synthetic organic chemistry in order to develop novel as well as green synthesis methods for synthesis. As explained above green chemistry is environmentally friendly or sustainable chemistry. The specific commercial laccase, Suberase®, from Novozymes was selected for this investigation. In our investigation we used phenols and quinones as potential substrates for the laccase. Quinonoids have been noted in the literature to possess both anticancer and antibacterial activity due to their ability to undergo redox cycling which results in the generation of reactive oxygen species (ROS), Scheme 1.



1 **2**

Scheme 1: Oxidation of a phenolic structure to a quinonoid.

Laccase has the ability to oxidise phenols *in situ* to *o*-quinones. The quinones then react with 1,3-dicarbonyls via Michael addition. This will be discussed in detail in Chapter 3. On the other hand when a quinone reacts with a 1,3-dicarbonyl it is reduced, resulting in a hydroxyl group. The hydroxyl group can be oxidised back to the quinone in the presence of laccase. This will be discussed in more details in Chapter 5. The targeted products for this work are depicted in Figure 2 below.

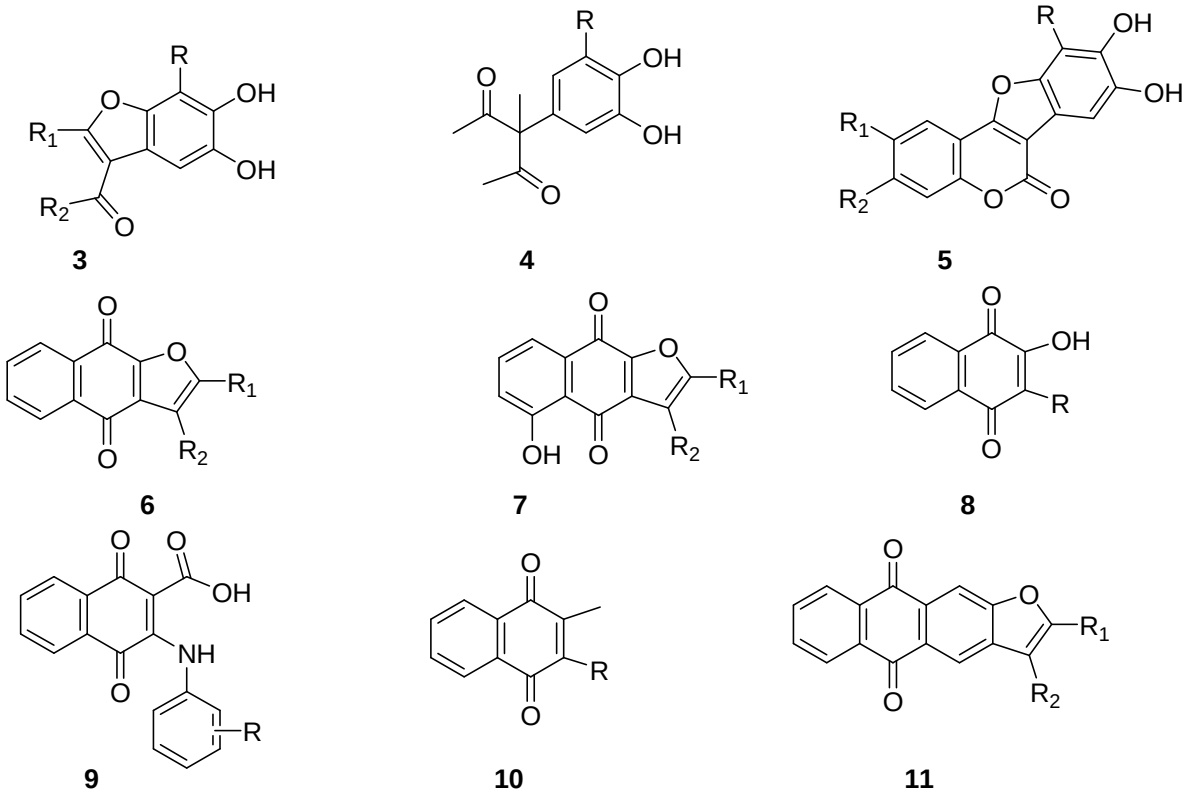
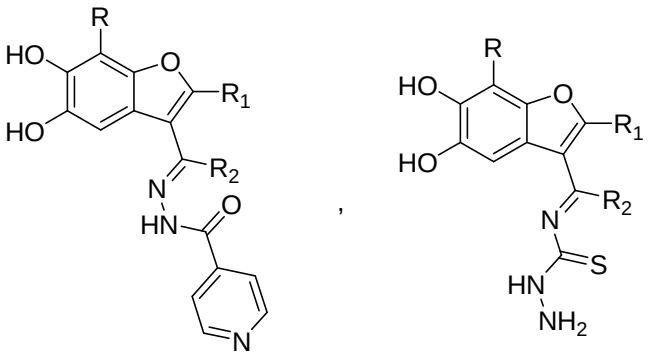


Figure 2: Targeted compounds.

The second part of the project was to select a number of benzofurans for structural modification with isoniazid, a well-known anti-TB drug, and thiosemicarbizide as shown in Figure 3. The idea was to increase the potency of the benzofuran derivatives against bacteria and cancer cell-lines

by introducing a dual effect. This is observed when a drug is biologically active against two targets due to its structural design and is discussed in details in Chapter 5.



12 **13**

Figure 3: Structures of benzofuran derivatives

Antibacterial resistance and cancer are major public health problems. The third objective of our study was to evaluate the synthesized compounds for both anticancer and antibacterial activity. Several of the compounds were also screened against a range of both Gram positive and Gram negative bacteria namely: *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative), *Bacillus substilis* (Gram-positive). The minimal inhibitory concentration (MIC) values were then determined. In particular we were targeting TB due to the fact that two African countries, South Africa and Nigeria, are in the top five countries with the highest toll of TB in the world. These were tested sequentially against *Mycobacterium smegmatis* and *M. tuberculosis*. Compounds were also screened for anticancer activity against 3 cancer cell-lines; that is renal (TK10), melanoma (UACC62) and breast (MCF7).

2.1 BIOCATALYSIS

Biocatalysis is the use of biological catalysts, such as enzymes, to perform chemical transformations. For this project the enzyme that was explored and utilized was laccase, as will be outlined later in this thesis.

2.1.1 LACCASES

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are oxidoreductases that are part of the family of multinuclear copper-containing oxidases.[121,122,] They are also known as a blue copper oxidoreductase, because most purified laccases exhibit a characteristic blue color due to their electronic absorption around 600 nm.[123] They catalyze the monoelectronic oxidation of substrates using molecular oxygen and release water as the only by-product in a four electron transfer process.[124,125] The first plant laccase activity was discovered in 1883 by Yoshida in the sap of a Japanese tree, *Rhus vernicifer*.[126] Plant laccases are believed to participate in the formation of polymer lignins via a radical-based mechanism.[127] They can also be found in *Rhus succedanea, Acer pseudoplatanus, Pinus taeda, Populus euramericana, Liriodendron tulipifera, Nicotiana tobacum, Lolium perenne* and *Zea mays*.[127] The discovery of fungal laccases by Bertrand then followed in 1896.[126] This type of laccase can be found in *Trametes versicola, T hirsute, T ochracea, T. viloosa, T. gallica, Cerrena maxima, Coriolopsis polyzona, Lentinus tigrinus and Pleurotus eryngii*. Laccase has also been found in various basidiomycetous and ascomycetous fungi.[129,130] There are also laccase-like proteins in bacteria such as *Azospirillum lipoferum, Bacillus sphaericus, Bacillus sp.* (*mnxG*), *Bacillus substilis (cotA), Escherichia coli (yacK), Marinomonas mediterranea (ppoA), Streptomyces antibioticus* and *Streptomyces griseus* (*epoA*).[131]

2.1.2 THE ACTIVE SITE

More than a hundred laccases have been isolated and characterised to date. Three laccases have been structurally characterized and they are *C. cinereus* and *T. versicolar* from *Melanocarpus albomyces*.[132] Most fungal laccases are monomeric glycoproteins with an average molecular mass of 50-130 kDa. The carbohydrate moiety constitutes 10-45% of the protein mass and is comprised of galactose, mannose and *N*-acetylglucosamine, This enhances the stability of the enzyme as well as protecting it against proteolysis and inactivation by free radicals. The active site of laccase contains 4 copper atoms which are as follows: one type 1 (T1) copper and a three nuclear cluster (T2/T3) consisting of 1 type 2 (T2) and two type 3 (T3).[132]

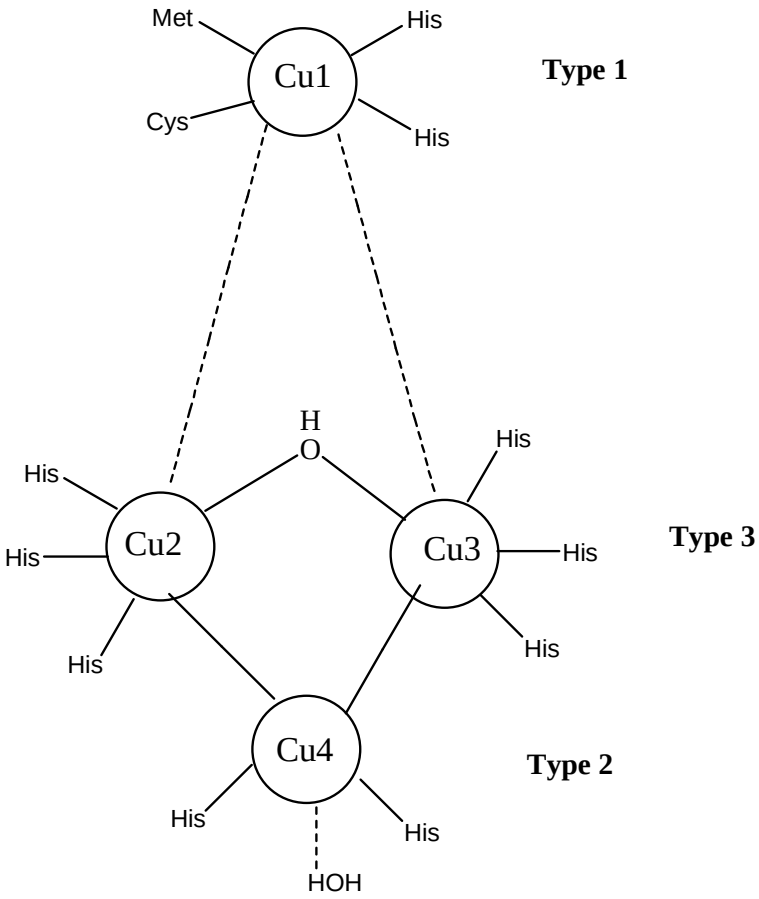


Figure 4: The active site of laccase encoded by the gene *cotA* from *Bacillus substilis*.

2.1.3 LACCASE SUBSTRATES AND APPLICATION

Laccase has a number of characteristics and applications. These range from ligninolytic activity of laccase in lignocellulose degradation to application in synthesis.[133] They have biotechnological, chemical and industrial applications, as well as applications in Green Chemistry. Their biotechnological applications include delignification of lignocelluloses and desulfurization and solubilization of coal.[134] In textile industries it is used for textile dye decolorization and remediation of coloured waste waters.[135] Their use in enzyme-based biosensors allows for monitoring pollutants, and they can also be used in the transformation and inactivation of toxic environmental pollutants. Laccase activity has been modified by the use of laccase-mediated systems.[136] Normally phenolic type laccase substrates also can include aromatic amines (Figure 2) and the reaction products are dimers and oligomers.[137]

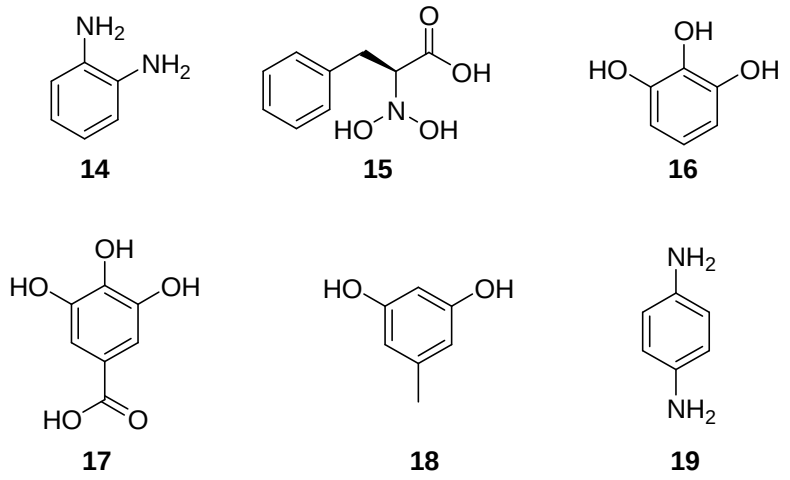
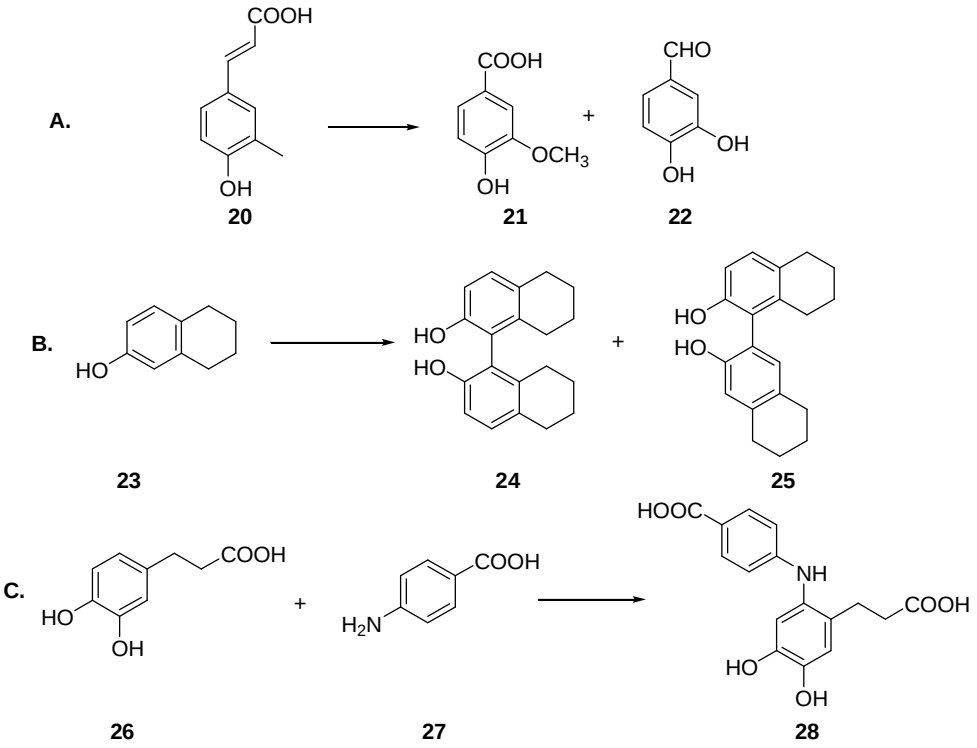


Figure 5: Structures of laccase substrates.

Scheme 2 show examples, applications and the examples include A. The biotransformation of ferulic acid **20** to vanillic acid **21** and vanillin **22**,[138] B. use in the oxidation of tetrahydro-2-naphthol to form a dimeric product as shown in Scheme **2** below and C. use in the coupling reaction of dihydrocaffeic acid and 4-amino benzoic acid.



Scheme 2: Coupling reaction of dihydrocaffeic acid and 4-amino benzoic acid.

Laccases are similar to other phenol-oxidizing enzymes, but differ in that their substrates can extend to non-phenolic subunits of lignin by the inclusion of a mediator such as 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) **29** (figure 3).[139a] Other mediators include hydroxybenzotriazole (HOBT) **30**, 2,2,6,6-tetramethyl-piperidin-1-yl)oxyl (TEMPO) **31**, hydroxyanthanilic acid (HAA) **32**.

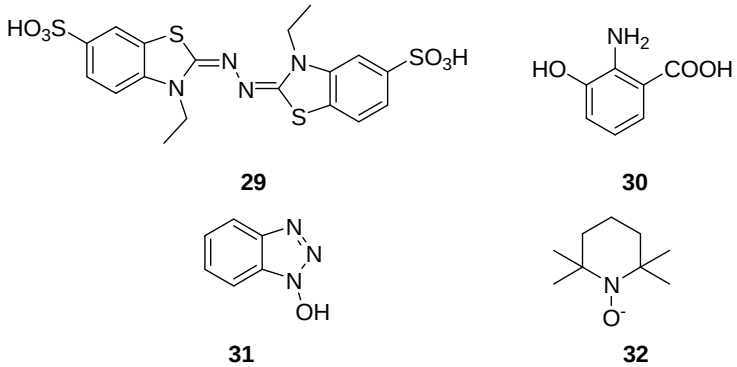
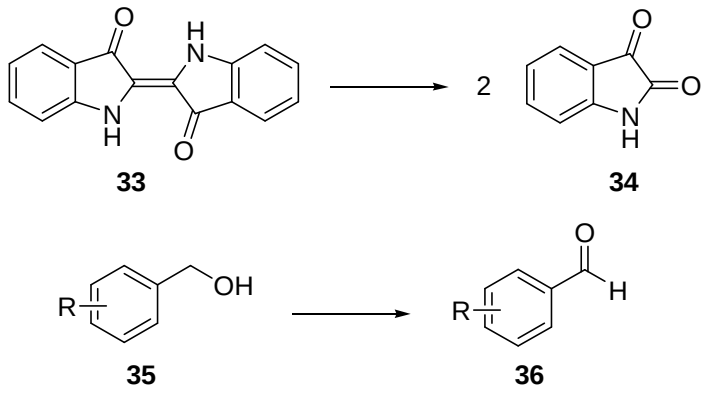


Figure 3: Laccase mediators

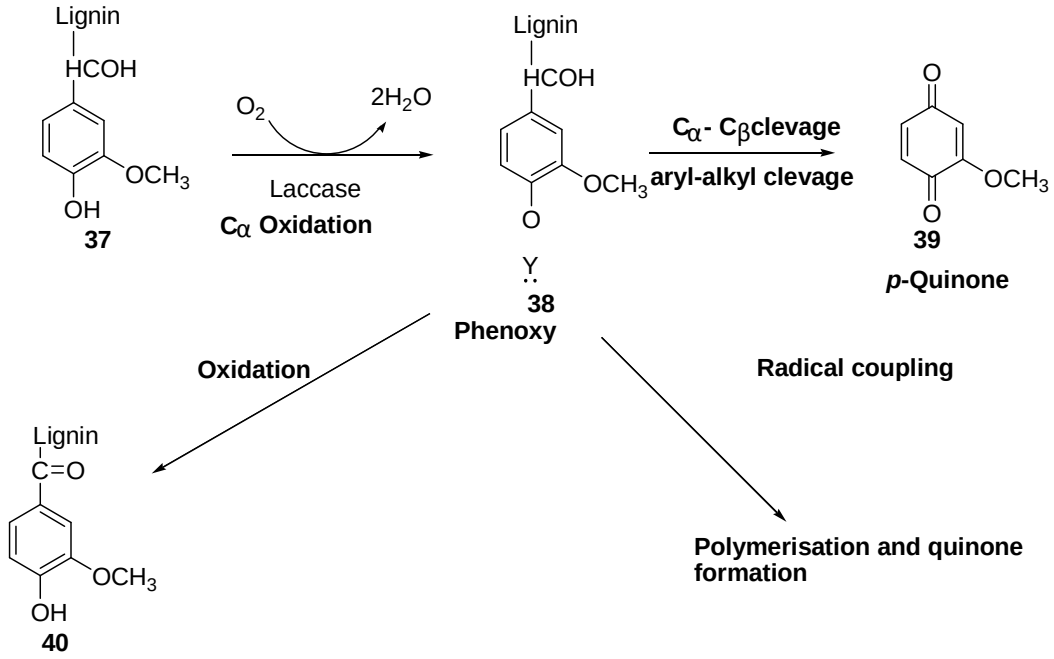
Below are the examples of oxidation of indigo to isatin and oxidation of benzyl alcohol to aldehydes, using laccase using laccase mediator substrates (Scheme 3).[136] The mechanism of the mediator substrate oxidation is suggested to occur via electron transfer (ET), radical hydrogen atom transfer (HAT)/ ionic oxidation depending on the structure.



Scheme 3: oxidation of indigo to isatin and oxidation of benzyl alcohol to aldehydes using Laccase Mediator substrates

2.1.3 MECHANISM OF ACTION OF LACASSE

Laccase only attacks the phenolic subunits of lignin, leading to Cα oxidation to give compound **38**, Cα-Cβ cleavage, and aryl-alkyl cleavage to give compound **39**, as shown in Scheme 4 below.[139]



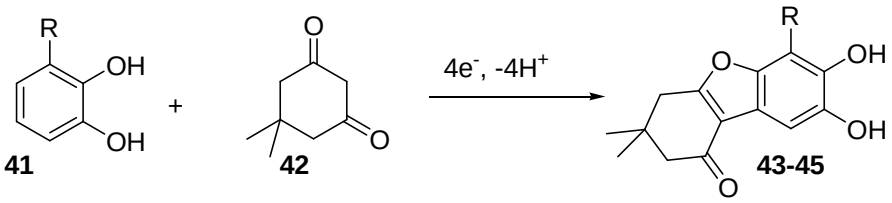
Scheme 4: Mode of action of laccase

As previously mentioned the active site of laccase contains 4 copper atoms and these are: one type 1 (T1) copper and a three nuclear cluster (T2/T3) consisting of 1 type 2 (T2) and two type 3 (T3).[139] The mechanism of laccase has been attributed to three major steps (i) Type 1 copper is reduced by accepting electrons from the reducing substrate. (ii) Electrons are transferred from Type 1 copper to the trinuclear T2/T3 coppers (iii) Molecular oxygen is activated and reduced to water.

The electron transfer from substrate to Type 1 copper is controlled by a redox potential difference. The Type 1 copper site has a shallow pocket whereas Type 2 and Type 3 appear to exclude all other oxidising agents except oxygen. Electron transfer from the substrate to the Type 1 copper site is the rate-determining step.

2.2 SYNTHETIC ASPECTS

Most of the published synthesis on Benzofurans involves the formation of annelated ring by intramolecular cyclisation of benzene ring.[155] These procedures involved multi-steps, rigorous reaction conditions and expensive catalysts.[155] At the beginning of this research the efficient synthesis for benzofurans was reported by Nematollahi and coworkers. It involved the electrochemical oxidation of catechols in the presence of 1,3-dicarbonyls (Scheme 5).[161a]

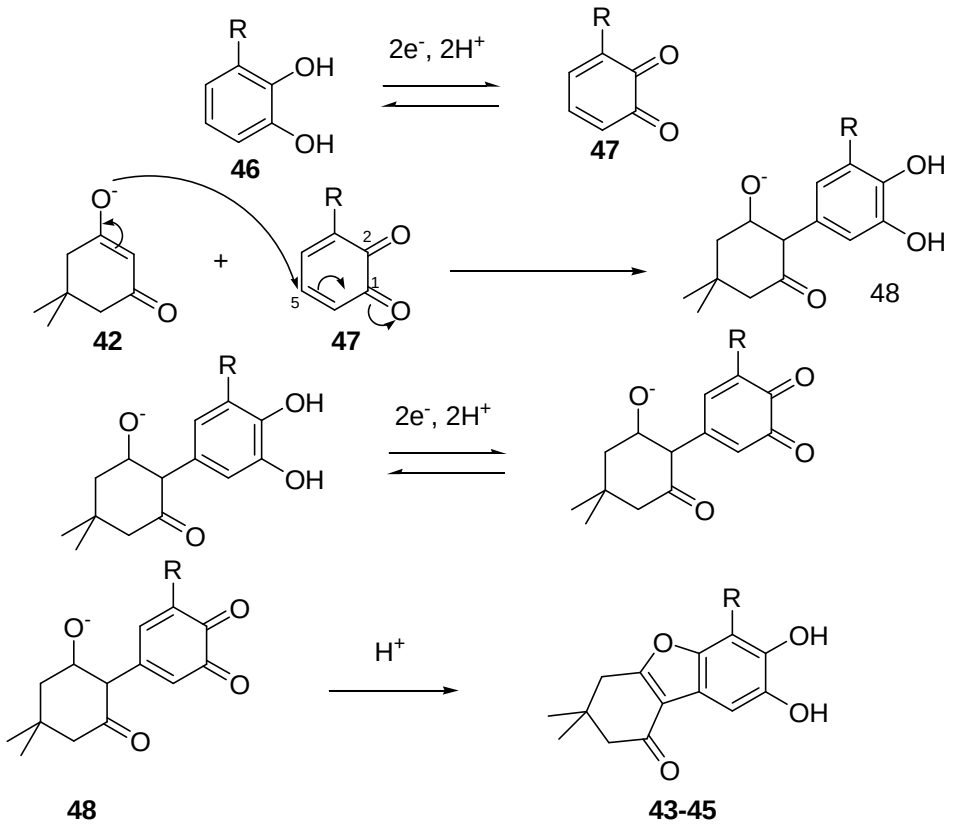


43, R = H, 83%
44, R = CH_3, 87%
45, R = OMe, 93%

Yield obtained after consumption of about 3000C electricity

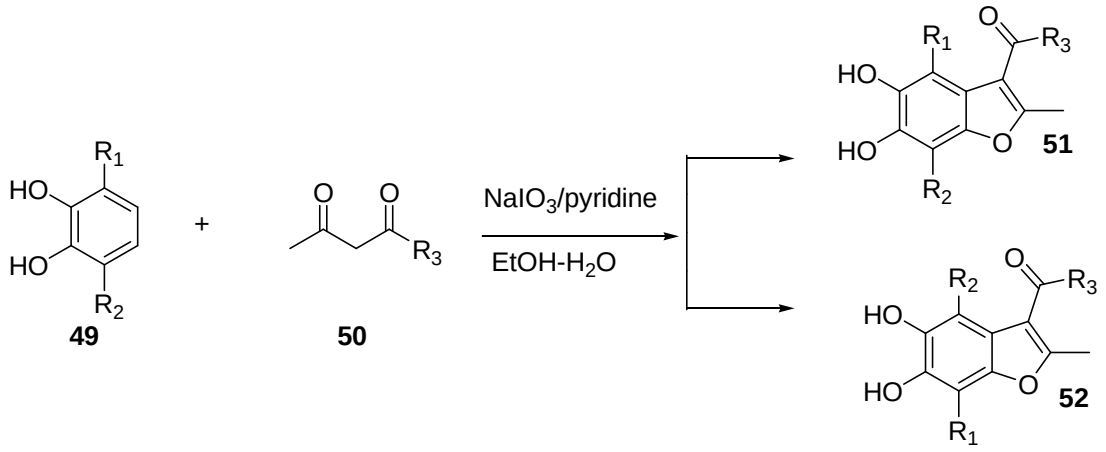
Scheme 5: Electrochemical synthesis of Benzofurans

This electrochemical study was achieved through the addition of a 1mM solution of catechol in aqueous solution containing 0.15M sodium acetate as a supporting electrolyte in cyclic voltammetry. The oxidation of a catechol occurs in the presence of 1,3-dicarbonyl to a quinone as shown in Scheme 6 below. The Michael acceptor **47** is attached at C-5 position yielding to a selective formation of benzofuran.



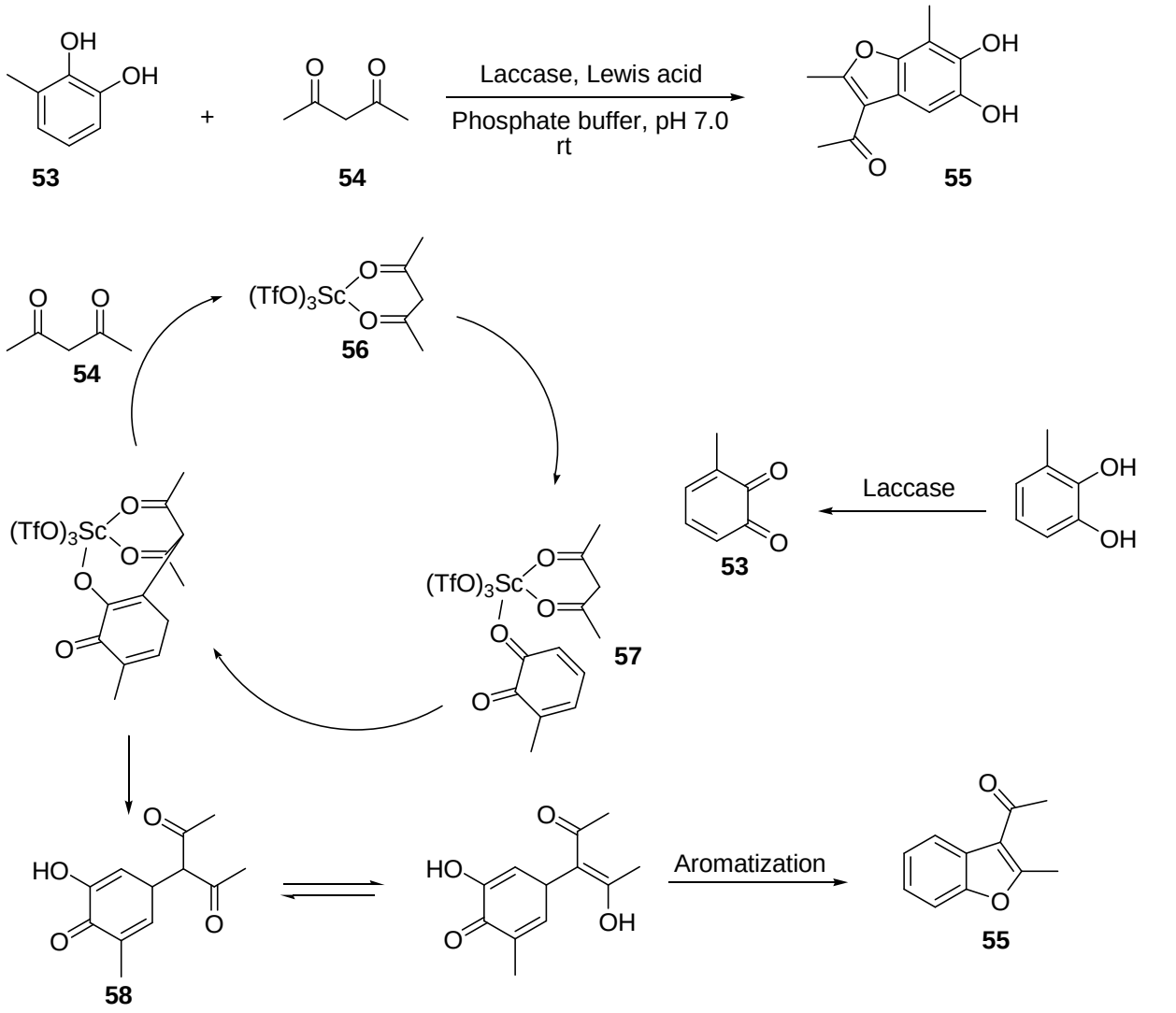
Scheme 6: Synthetic sequence for the electrochemical method

Another efficient method was reported by Wanzlic and Darbawar. This involved the oxidative coupling of catechol with 4-hydroxy-coumarins in the presence of ferricyanide.[161b] The chemical aspects were also investigated and with few positive outcomes. One procedure was reported by L-x Pei and coworkers, in their approach they used pyridine as a catalyst (Scheme 7).[154]



Scheme 7: Chemical synthesis of Benzofurans

This was achieved in ethanol, however if the is presence of water in ethanol it led to no reaction. Another disadvantage is that over reaction led to formation of polymers. The use of pyridine which is carcinogenic is also a disadvantage. The biocatalytical synthesis however remained scarce. The first biocatalytic study at accomplishing the preparation of polyhydroxylated benzofurans was reported in 2007 by Witayakran and coworkers (Scheme 15).[155] They used 20 mol% of sodium dodecyl sulfate (SDS) as their surfactant as well as scandium triflate ($Sc(OTf)_3$) as a catalyst in order to enhance Michael addition (Scheme 8).



Scheme 8: Biocatalytic synthesis of Benzofurans

Our methodology differs from all the published syntheses in that, it fulfills a number of green chemistry principles. It achieves selective, efficient transformation, at room temperature, in aqueous environment and without the use of an additional catalyst. Details of this methodology will be discussed in the chapters that follow. We also noted that there were no published reports on biocatalytic synthesis for substituted 1,3-diketones with catechols. To the best of our knowledge this is the first report to discuss the biocatalytic synthesis of Benzofuro[3,2-*c*]chromen-6-one skeleton from fluorinated catechols and acid catechols with 1,3-dicarbonyls. It is also our first report to discuss the biocatalytic synthesis of naphthoquinones containing a substituent at position 2 (menadione) with a range of structurally diverse 1,3-dicarbonyls and coumarins. For the first time we were able to biocatalytically synthesise derivatives of juglone and 1,4-naphthoquinones from 1,3-dicarbonyls.

2.3 BACKGROUND AND ORIGINS OF GREEN CHEMISTRY

Green chemistry refers to the design and formulation of processes that minimize environmental pollution and thereby reduce hazards and risks to human beings. Its main objective is to eliminate the hazardous impact of chemicals over a chemical products' life-cycle. The need for environmentally sustainable chemistry first became apparent to the public with a publication by Rachel Carson.[103] Her publication titled 'Silent Spring' (1962**)** helped spread public awareness of the hazards of environmental pollution and pesticides to the environment. In 1969 President Richard Nixon established the citizen advisory committee on environmental quality.[104] He subsequently appointed a White House Committee to determine whether an environmental agency should be established. The Environmental Protection Agency (EPA) was established in 1970[83] and the Office of Pollution Prevention and Toxics was established in the 1980**s**. The EPA

implemented the Green Chemistry Programme in 1993[105] which was followed by the establishment of the Presidential Green Chemistry Challenge award in 1995 by President Bill Clinton. The Green Chemistry Institute was launched in 1997 with the main objective to advance the broader chemistry enterprise and its practitioners for the benefit of the Earth and its people. Paul Anastas and John Warner published "The 12 Principles of Green Chemistry" in 1998**,** which codified how chemical process could be made greener. The California Green Chemistry initiative was established in 2000 and serves to develop policy options for green chemistry. In 2001 Paul Anastas was appointed as head of Research and Development at the EPA.[106]

2.4 The Twelve Principles of Green Chemistry

These principles were published by Anastas and Warner in **1998** in a book titled: "Green Chemistry: Theory and Practice".[106] They serve as the basis of creating and implementing chemicals and processes.

Principle 1: Prevention

It is better to prevent waste than to treat or clean up waste after it has been created.

Principle 2: Atom economy

Synthetic methods should be designed to maximize the incorporation of all material used in the process into the final product.

Principle 3: Less hazardous chemical synthesis

Synthetic methods should be designed to use and generate substances that possess little or no toxicity to humans and the environment.

Principle 4: Designing safer chemicals

Chemical products should be designed to affect their desired function while minimizing their toxicity.

Principle 5: Safer solvents and auxiliaries

The use of auxiliary substances (e.g. solvents, separation agents etc.) should be made unnecessary wherever possible and innocuous when used.

Principle 6: Design for energy efficiency

Energy requirements of chemical processes should be more recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.

Principle 7: Use of renewable feedstocks

A raw material or feedstock should be renewable rather than depleting wherever technically and economically practicable.

Principle 8: Reduce derivatization

Unnecessary derivatization (use of blocking groups, protection/deprotection, and temporary modification of physical/chemical processes) should be minimized or avoided if possible because such steps require additional reagents and generate waste.

Principle 9: Catalysts

Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.

Principle 10: Design for degradation

Chemicals should be designed so that at the end of their function they break down into innocuous products and do not persist in the environment.

Principle 11: Real-time analysis for Pollution and Prevention

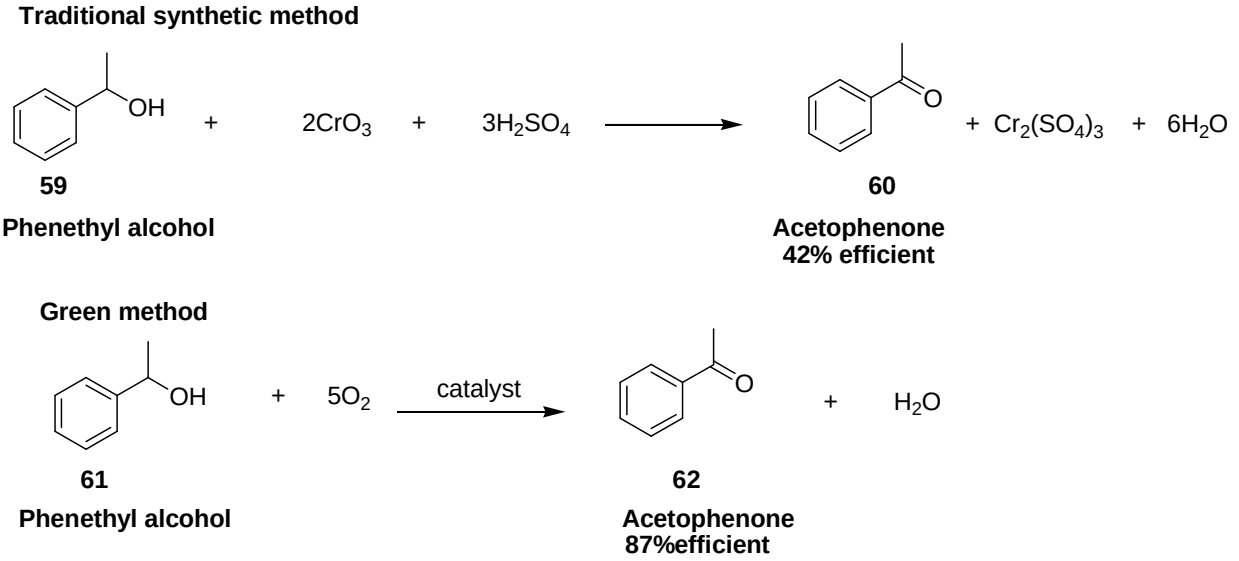
Analytical methodologies need to be further developed to allow for real-time process monitoring and control to limit the formation of hazardous substances.

Principle 12: Inherently safer chemistry for accident prevention

Substances and a form of a substances used in chemical processes should be selected to minimize the potential of chemical accidents including releases, explosions and fires.

2.5 The importance of Green Chemistry

Green chemistry focuses on meeting the needs of the present generation without compromising the needs of future generations. It also looks at methods for the prevention of primary pollution, not on remediation. It reduces waste, materials, hazards, risks, energy and cost. One of the good examples where a Green method is utilised is in the commercial synthesis of acetophenone (Scheme 9).[107]



Scheme 9: The commercial synthesis of acetophenone.

The traditional synthetic method makes use of toxic chemicals that are in the EPA Toxic Release Inventory (TRI). This route generates a byproduct-chromium (III) sulfate that is a TRI chemical known to be a human carcinogen and its atom efficiency is only 42% whereas the green method provides a more user friendly method with only water as a byproduct at 87% atom efficiency.

2.6 GREEN CHEMISTRY IN PHARMACEUTICAL INDUSTRIES

2.6.1 Background

The practice of Green Chemistry has become a widespread protocol in some industry sectors. In 2005 the Green Chemical Institute (GCI) and global pharmaceutical corporations established the GCI Pharmaceutical Roundtable.[104,105] This was done to encourage innovation while catalyzing the integration of green chemistry and green engineering in the pharmaceutical industry for sustainable business. Sustainability in green chemistry can be defined as a step towards processes that are cleaner and/or that make better use of beneficial resources. Members of the Roundtable include the American Chemical Society (ACS), The Green Chemical Institute (GCI), Amgen, AstraZeneca, Boehringer-Ingelheim, Bristol Myers Squibb, Codexis, Dr Reddy's, DSM Pharmaceutical products, Eli Lilly and company, Genentech (a member of the Roche group), GlaxoSmithKline, Johnson & Johnson, Merck & Co Inc, Novartis, Pfizer Inc., Roche and Sanofi.[106] In 2011 the medicinal chemistry subgroup of the roundtable was established.[106] The main reason for this group is to encourage the intergration of green chemistry and engineering principles into discovery research.

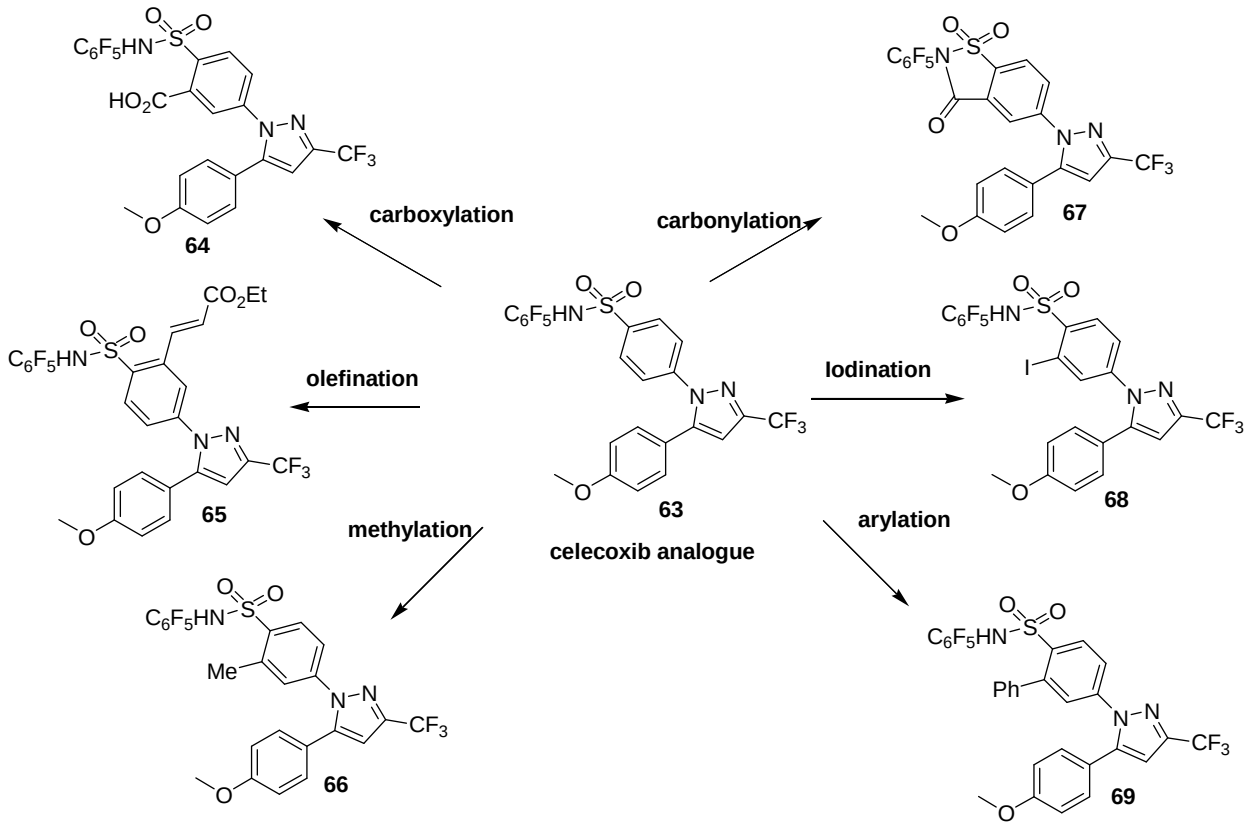
2.6.2 Drug Discovery

There are a number of considerations when designing drug molecules and these include molecular property calculations, structure-based drug discovery (SBDD), in silico models, quantitative structure-activity relationship (QSAR) etc.[109] The challenge with medicinal chemistry is that the chemistry selected when the compound is first synthesized can have a large influence on the chemistry of an ongoing project and a chemist might be under pressure to rapidly achieve a target for development of a process. However, the principles of green chemistry can always be implemented after synthetic route design. There are a number of

synthetic strategies that pharmaceutical companies have adopted or would like to adopt when applying green principles and are discussed below.

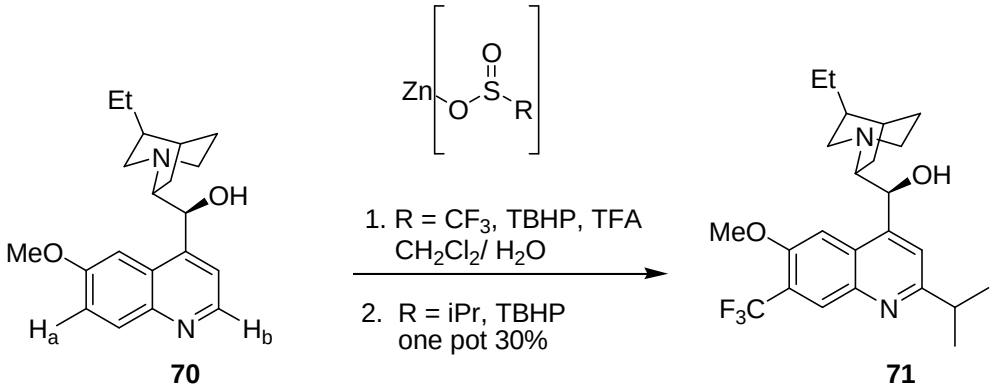
2.6.2.1 C-H Activation

This methodology is efficient in terms of both atom and step economy. It has become prominent in the past decade and is currently considered to be one of the very topical areas of research. It involves the introduction of a C-C or a C-heteroatom bond without requiring a functional group such as a halide, triflate, boron, tin and other metalation processes. For example, Prof Jin-Quan Yu, in collaboration with Pfizer, demonstrated C-H functionalization for late stage derivitization of the anti-inflammatory drug, Celecoxib, which has a sulfonamide moiety (Scheme 10).[110] A wide range of new analogues were accessed in a single transformation that involves olefination, carboxylation, carbonylation, iodination, arylation and methylation (Scheme 10). Prior to the development of this methodology it would have required many steps, solvents, energy and generation of waste streams.



Scheme 10: Yu's exemplification of C-H functionalization directed by a sulfonamide moiety.

Another example where these mild and novel methods are applied is in the late stage derivitization of drug-like heterocycles. Zinc sulfinate reagents were adopted for fluoroalkylation and alkylation of unactivated C-H bonds on aryl, heteroaryl and aliphatic substrates by the Baran group.[110] This one-pot sequential functionalization of a complex molecule, dihydroquinine, undergoes regioselective *trifluoromethylation* followed by *isopropylation* to regioselectively afford the doubly derivatized product in a 30% yield (Scheme 11).

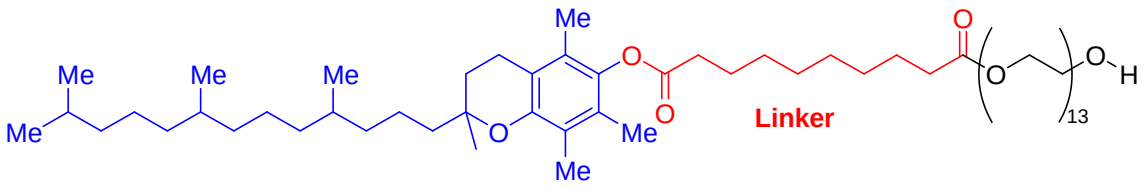


Scheme 11: Baran's sequential functionalization of dihydroquinine.

This methodology is atom efficient, reduces time and energy, uses only Zn as a catalyst and the reaction is performed in the environmentally friendly solvent water. Although C-H activation is not applicable in all circumstances, chemists are encouraged to consider this and other synthetic improvements when designing routes to targets.

2.6.2.2 Aqueous Chemistry

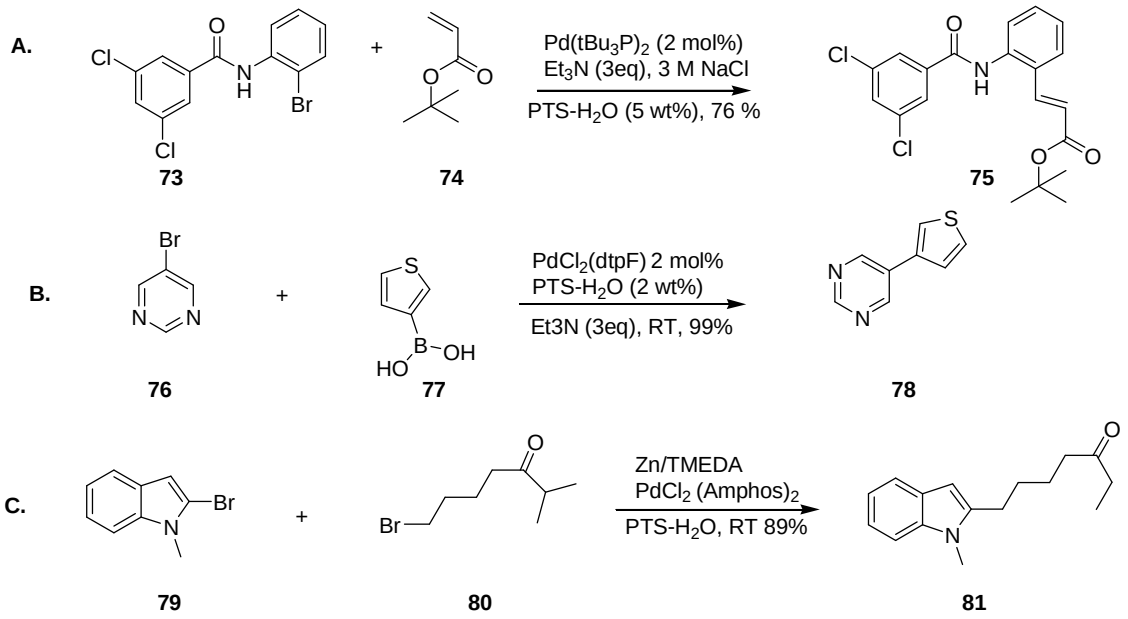
Palladium-mediated reactions have been well known for the past two decades within the chemistry community. This was more evident by the award of the 2010 Nobel Prize to Heck, Suzuki and Negishi who pioneered the palladium-mediated processes that now bear their name.[111] Advances have been made in the past years in order to adopt green methods in palladium catalyzed reactions.[112] For example Lipshutz and co-workers developed a micellar catalysis using environmentally benign designer surfactants as shown in Scheme 12. The concept of the micellar catalysis relies on the ability to solubilize organic based reactants in aqueous media by the addition of a surfactant.



72

Scheme 12: A representative of a Lipshutz surfactant.

The substrate becomes trapped within a nano-micelle within the water and the chemistry takes place within this compartmentalization. This approach has been utilised in a number of reactions including a designed surfactant linking a vitamin E derivative through a linker (succinic or sebacic acid) to a polyethylene glycol chain of varying lengths. Other success stories include transition metal mediated cross-couplings (from halide precursors and through C-H activation), olefin metathesis, 1,4-conjugate addition of hydride and Mitsunobu type reactions. With regards to Negishi reactions (Scheme 13, A), addition of tetramethylethylenediamine (TMEDA) and as an additional catalyst which is critical due to the conversion of organozinc reagents to pure aqueous reagents. In the absence of TMEDA and additional catalyst, there is an incompatibility of conventional organozinc reagents to purely aqueous conditions which is not in favor of the reaction.



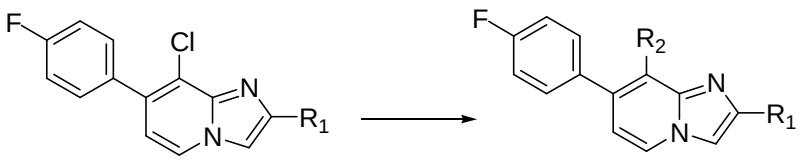
Scheme 13: Surfactant methodology in common Palladium-mediated couplings.

These methods often lead to reactions that have high regio and geometric selectivity, can occur at room temperature, reduces energy, have high efficiency, occur under remarkably mild conditions and avoid organic solvents.

2.6.2.3 Telescoping

Telescoping refers to multiple synthetic steps that are performed in one pot.[114] Historically this application was more focussed on multicomponent reactions as opposed to sequential orthogonal reactions. This application reduces time, energy, solvent, as well as product extraction and work up time. This has been succesfully adopted for application of methyliminodiacetic acid (MIDA) boronates toward the preparation of drug-like arrays. Protected borates were developed by Burke.[115] A one-pot sequential reduction-amination Suzuki-Miyaura cross coupling (SMCC),

heterocycle formation SMCC and Buchwald-Hartwig-SMCC were also achieved (scheme 14).[116-118]

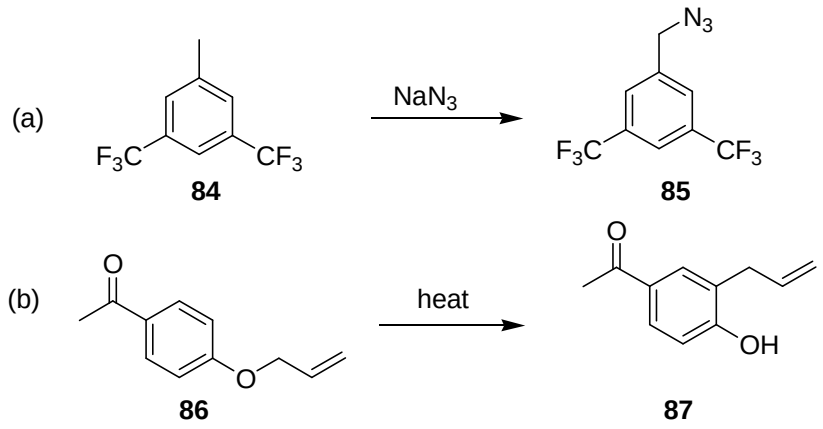


82 **83**

Scheme 14: A one-pot sequential reduction-amination Suzuki-Miyaura cross coupling (SMCC)

2.6.2.4 Flow Chemistry

The new and developing area of flow chemistry, which involves continuous reactions rather than batch reactions, is favorable in the pharmaceutical industries since it can result in higher yields, increased safety, less solvent usage, reuse of solid supported catalysts, less waste generation and the possibility of automation.[119] Flow chemistry is now found at all stages of drug discovery and development processes. [120] It is used for safety, as only limited quantities of unstable intermediates were present at any one time in, for example, the azide synthesis.[120] Azide synthesis is toxic and can cause an explosion due to the presence of hydrazoic acid produced from sodium azide, Flow chemistry shortens the duration of the synthesis and prevents potential reactive intermediates produced.[120] Also it is safer for reactions where extreme pressure and temperature are required. From the literature it has been noted that 40-50% of all reactions recently hypothesized in pharmaceutical industries are suitable for continuous flow chemistry, and that it requires 10% less solvent usage.[119] An example where reactions takes place above the boiling point is in the use of a channel flow reactor for the synthesis of an azide which mitigates the toxicity and explosion risks associated with hydrozoic acid produced from sodium azide (Scheme 15a). Another example is the Claisen rearrangement reaction that takes place at 230°C as shown in (Scheme 15(b)).



Scheme 15: Reactions enabled by operation in thermal tube flow reactors.

A challenge arises when the product precipitates in the channel reactor. In order to address this challenge, co-solvents are introduced in flow chemistry in order to promote solid formation post reaction. This is not feasible for all reactions, but for most reactions it does prevent clogging. For heterogeneous mixtures, continuous stirred tank reactors (CSTRs) have been a solution. The Schotten Bauman acylation and crystallization adopts this method in three disposable continuous stirred tanks. This type of reaction solves the challenges that chemists face when scaling up.

The chapters that will follow will discuss the laccase-based synthesis of the desired compounds in more detail, including Benzo[*b*]furans, Benzo[3,2-c]chromen-6-ones, quinone derivatives, as well as an imine/Schiff base family.

BENZO[B]FURANS

3.0 INTRODUCTION

Benzofurans are attractive to chemists because they possess a broad range of biological activities. Anti-allergic and anti-inflammatory activities have been exhibited by 5-benzofuranol **1**, while benzofuran fused benzocarbazoles **2** (Figure 1) exhibit antitumor and antibiotic activities.[140, 141] Benzofurans, of which machicendiol **3** is an example, are used in the treatment of asthma, rheumatism and ulcers.[142] Amiodarone hydrochloride **4,** an ideal antiarrhythmic drug, contains a 2,3-substituted benzofuran moiety.[143] It has also been noted that benzofurans that possess an hydrazide group exhibit a broad spectrum of pharmacological and biological properties, such as analgesic, anticancer and anti-inflammatory properties that may be due to a the azomethine linkage.[144] It has been observed that compound **5**, a Schiff-base bearing several methoxy groups has pronounced antimicrobial activities.[145]

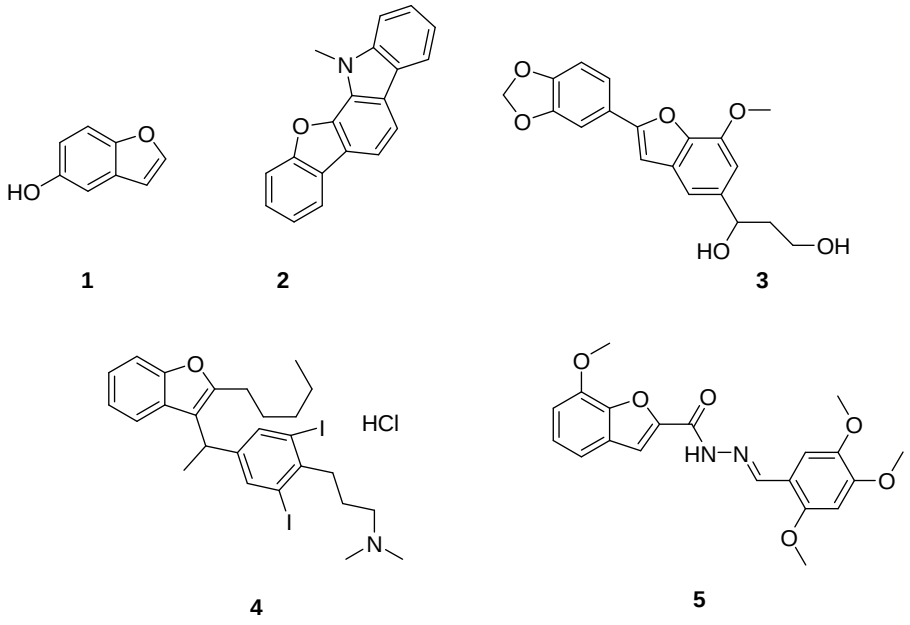


Figure 1: Biologically active benzofuran derivatives

Benzofurans **7** and **8** possess anticancer activity, while both **10** and **11** exhibit antibacterial activity as shown in Figure 2. Usnic acid **6** and **9** are particularly interesting as they exhibit either anticancer activity or antibacterial activity depending on the isomer synthesized. This metabolite showed activity against the wild-type p53 breast cancer cell line MCF7 (IC_{50}/GI_{50} = 18.9 µM), and the lung cancer cell line H1299 (IC_{50}/GI50 = 22.3 µM). This was one of the motivations for screening our synthesised benzofuran derivatives for both anticancer and antibacterial activity as discussed in Chapter 8.

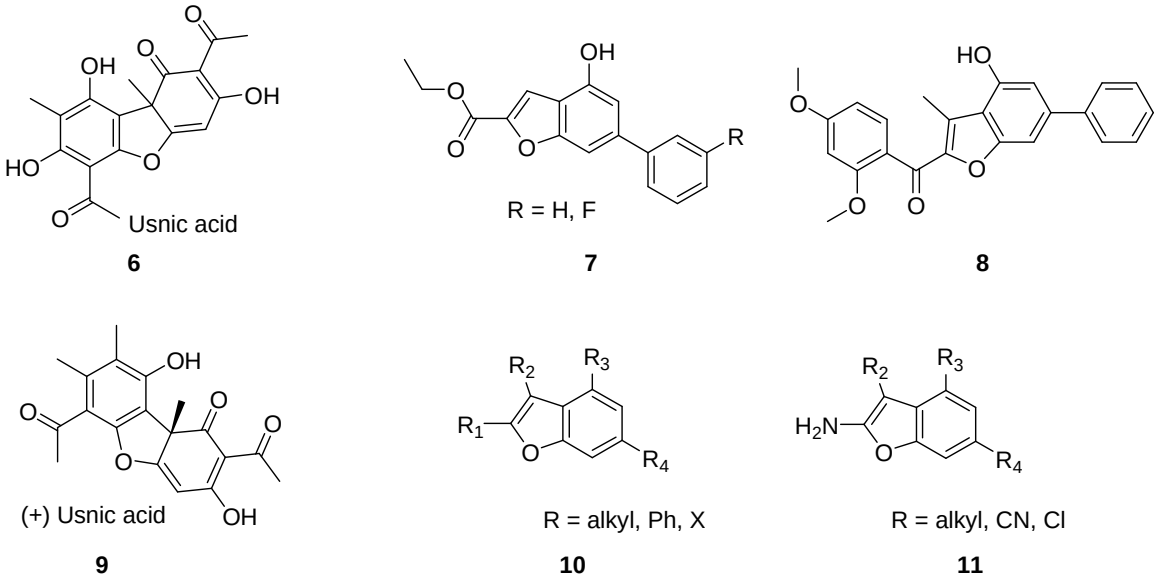
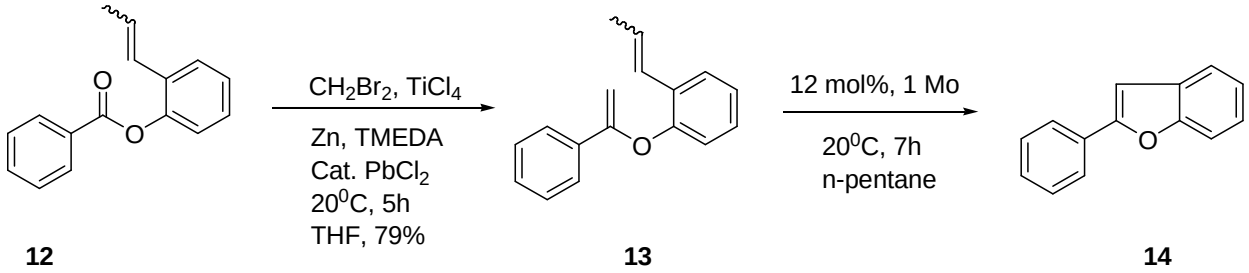


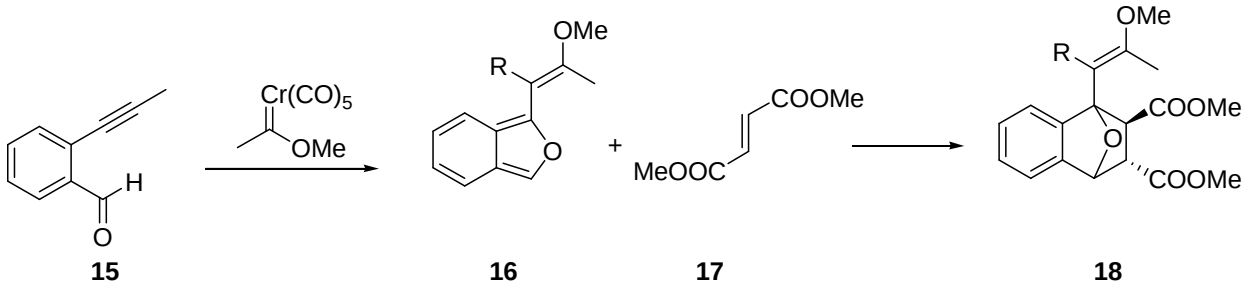
Figure 2: Benzofurans exhibiting anticancer and antibacterial activity.

The strategies for the construction of benzofurans varies from the ring closing metathesis reaction. In 1978 Takia reported that a reagent prepared from Zn, TiCl4 and CH_2Br_2 can olefinate carbonyl in a manner that is similar to the Tebbe-like reagents as shown in Scheme 1 below.



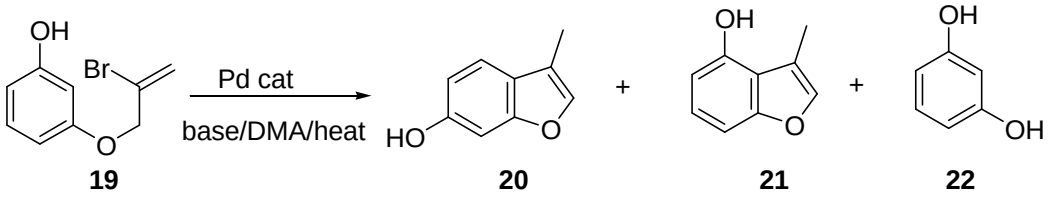
Scheme 1: Olefination of a carbonyl to a benzofuran

Another example is the coupling of conjugated dienynes with Fisher carbene complexes, anion-accelerated palladium-mediated intramolecular cyclization. In the synthesis of benzofurans Fischer carbene complexes are coupled with aldehydes **15** which results in furan derivatives **16** as shown in Scheme 2. This ring system can undergo Diels-Alder reactions to give compound **18**.



Scheme 2: Diels-Alder reaction in the synthesis of a benzofuran

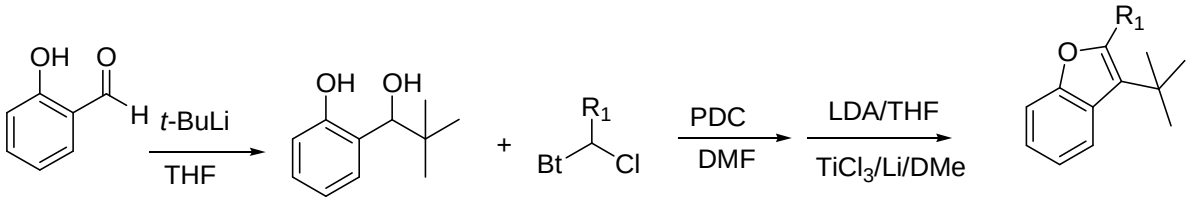
This reaction sequence serves as a cornerstone for preparation of pharmaceutically important structures, for example estrogen like structures.[146-159] Biologically important furopyridines can be prepared by this approach in high yields. The detailed mechanism is such that it involves cyclisation of vinyl bromide by heating bromide with CS_2CO_3 in dimethyacetamide (DMA) in the presence of catalytic amounts of Herrmann's palladacyclic catalyst (HC) as shown in Scheme 3.



19 **20** **21** **22**

Scheme 3: Palladium-catalyzed intramolecular coupling of aryl halides with phenols

The main side product of this reaction, which was not observed in the aryl coupling process, is resorcinol. The exact mechanism for this side chain elimination is not clear. Another example from literature is utilization of *o*-hydroxyphenyl ketones or *o*-(1-hydroxy-2,2-dimethylpropyl)phenol **23** with 1-benzotriazol-1-ylalkyl chloride **25** . These reactions undergo a two or three steps mechanism as shown in Scheme 4.

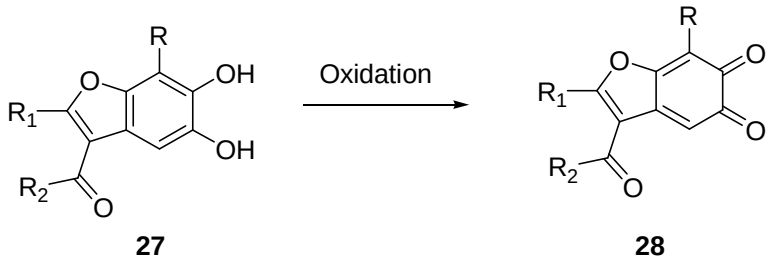


23 **24** **25** **26**

Scheme 4: Synthesis of benzofuran derivatives from *o*-(1-hydroxy-2,2-dimethylpropyl)phenol **23** and 1-benzotriazol-1-ylalkyl chloride **25**

The mechanism is similar to the one described in Scheme 3 with the exception of oxidizing an alcohol to a ketone using Sir Ewart Jones reagents (pyridium chlorochromate in DMF).

The goal of our research was to develop green methods of synthesis of the 5,6-dihydroxylated benzo[*b*]furans **27** using laccase biocatalysis (Scheme 5).



27 **28**

Scheme 5: Oxidation of a phenol to a 1,2-quinonoid

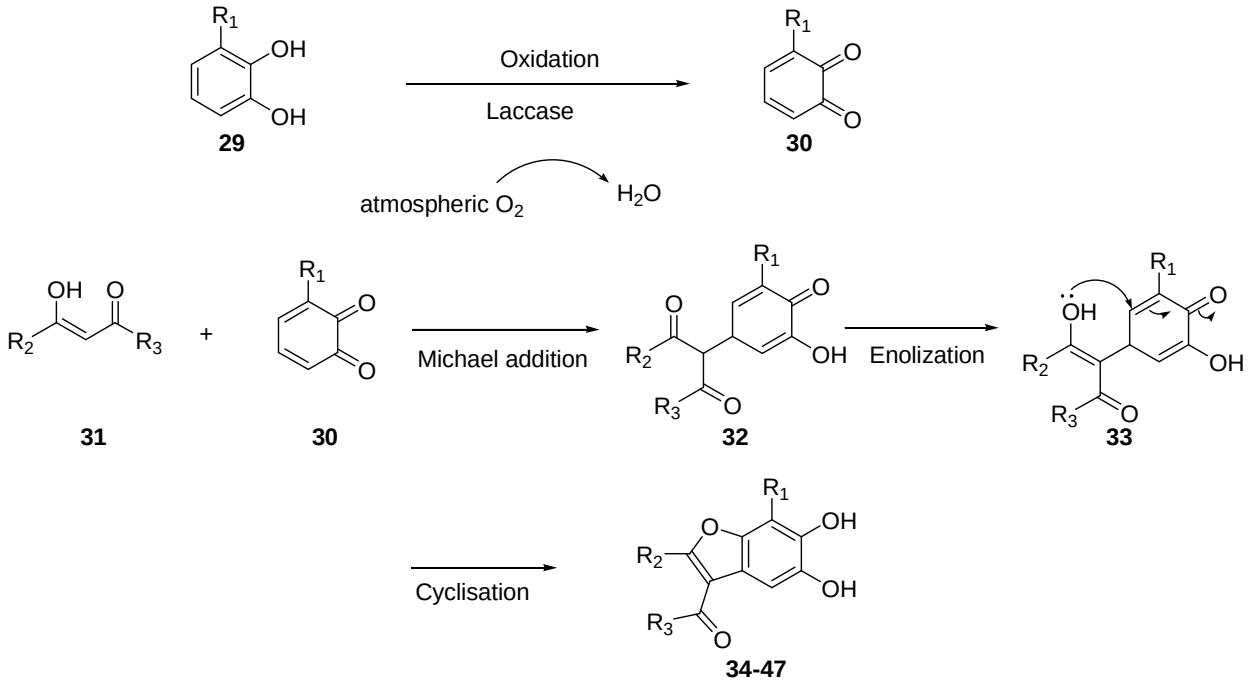
Since quinonoid compounds display potent biological properties such as antibacterial, anticancer, antifungal and antimalarial activity,[160] we anticipated that the 5,6-dihydroxylated benzo[*b*]furans **27** could have similar pharmacological activities because the *ortho* phenolic substituents (hydroxyl groups) could be oxidized to the corresponding *o*-quinone **28** within the cell. This study, which we published in 2013,[161] was the first on the synthesis of a variety of 5,6-dihydroxylated benzo[*b*]furans **27** and catechol derivatives using the commercial laccase, Suberase®, as well as the first report on the anticancer activity of the synthesised compounds (see the Appendix). The published manuscript is included in the dissertation, and is appended after the references.

3.1 RESULTS AND DISCUSSION

3.1.1 SYNTHESIS

Our interest was in both cyclised and un-cyclised 1,3-diketones. We explored a number of different methods as shown in Scheme 6 below.

The method used for the synthesis entailed reacting one equivalent of the catechol with one equivalent of the 1,3-dicarbonyl at room temperature using the laccase Suberase® in a vessel open to air at pH 7.15 (Scheme 6). A pH of 7.15 was chosen because it would make the reaction medium sufficiently basic to deprotonate the alpha-proton from the 1,3-dicarbonyls and thus facilitate the Michael addition reaction with the *in situ*-generated *o*-quinone. This is then followed by oxidation, which is then followed by cyclisation.



Scheme 6. Synthesis of the 5, 6-dihydroxylated benzo[*b*]furans **34-47**.

The catechols (**29a–e**) and the 1,3-dicarbonyls (**31a–e**) used in this study are depicted in Figure 3.

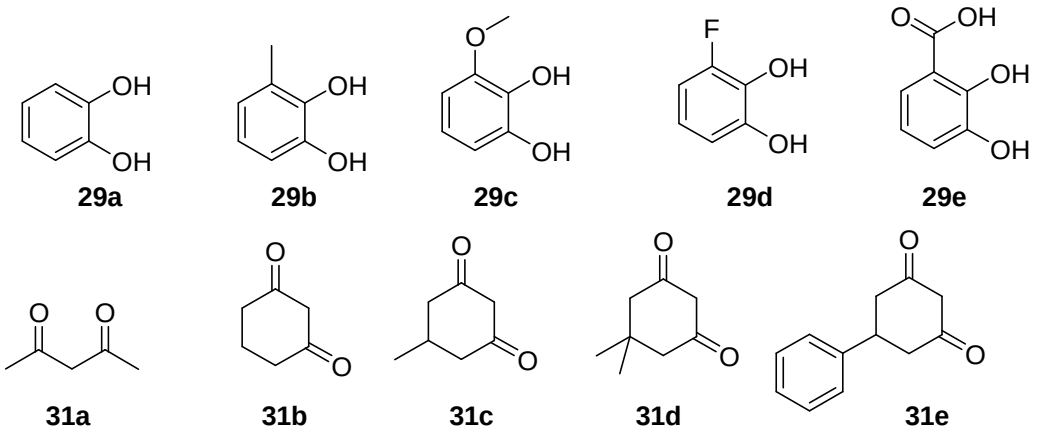


Figure 3. The catechols **29a**–**c** and 1,3-dicarbonyls **31a**–**f** used in this study.

The first approach, Method A, entailed reacting the catechols **29a**–**e** with the 1,3-dicarbonyls **31a**–**e** at room temperature (rt) at pH 7.15 for 24 h. The second approach, Method B, entailed conducting the reaction under the same conditions but for a longer time (44 h) to determine whether a longer reaction time would improve the yield of the product. In the third approach, for Method C, DMF was added to the reaction mixture as a co-solvent to improve the solubility of the organic substrates. The number of equivalents of the 1,3-dicarbonyl was also increased so that a ratio of 1,3-dicarbonyl to catechol was 4:1. These reactions were conducted for 42 h. We were able to successfully synthesise compounds **34**-**47** shown in Figure 4.

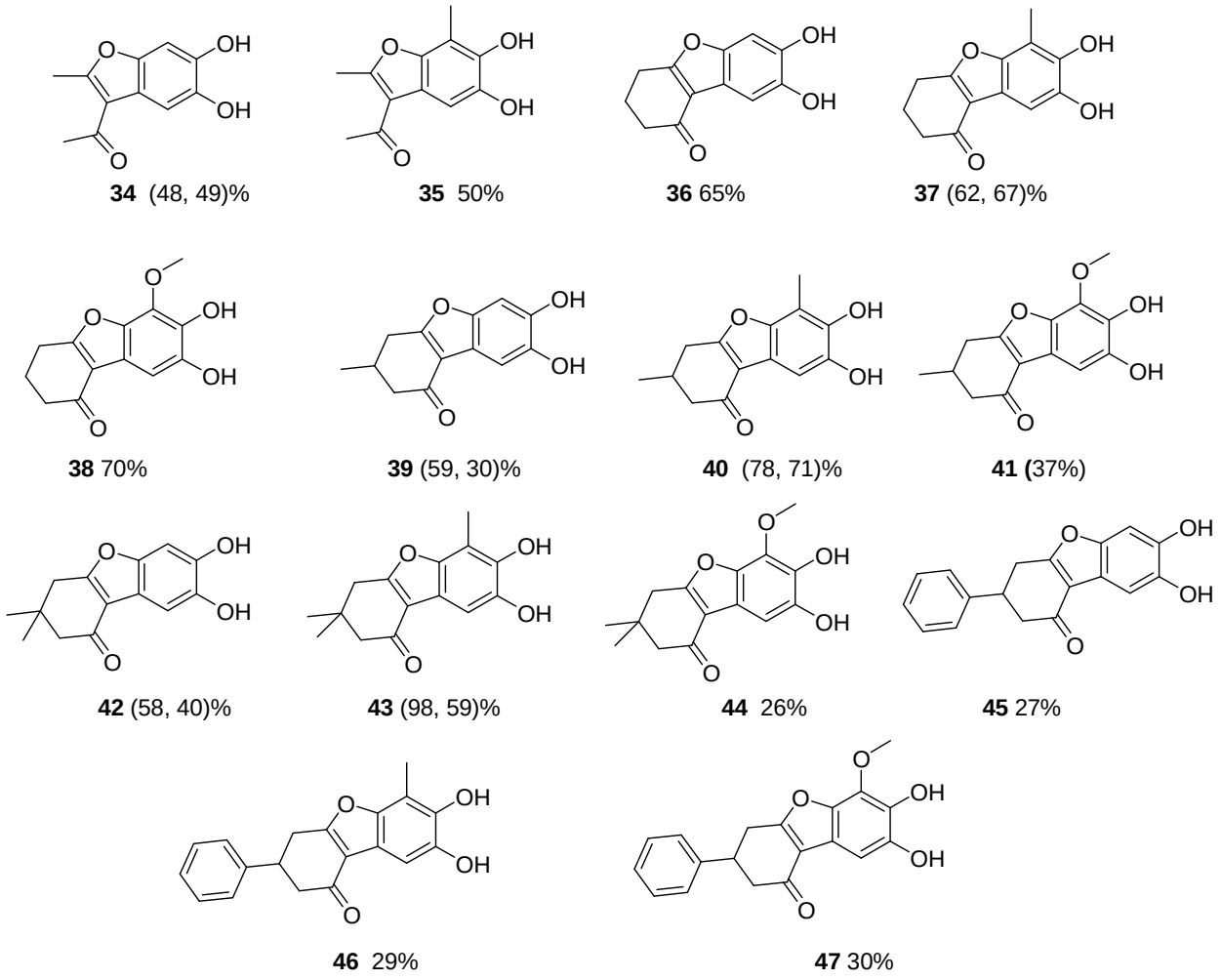


Figure 4: Structures of the 5, 6-dihydroxylated benzo[*b*]furans **34-47** synthesised at rt.

For fluorinated catechols **29d** the desired product was not formed. We were also unable to form benzofuran derivatives with the acid catechol **29e**. Interestingly, we observed in the ^{1}H NMR spectrum the effect of chirality in the neighbouring hydrogens for compounds with a stereogenic centre as shown Figure 5 below. The reason for this is that Ha and Hb are in different environments or diastereotopic due to the presence of the stereogenic centre.

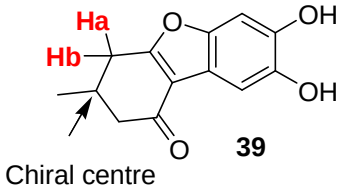


Figure 5: Structure of 7,8-Dihydroxy-3-methyl-3,4-dihydrodibenzo[*b*,*d*]furan-1(2*H*)-one

The ^{1}H nuclear magnetic resonance (^{1}H NMR) for compound **39**, for example, shows two doublet of doublets at 2.30 (1H, dd, *J* 4.6, 12.8 Hz, CH_a) and 3.01 (1H, dd *J* 4.6, 12.8 Hz, CH_b)

The results of the investigations with Methods A to C are depicted in Table 1.

Table 1. Synthesised benzo[b]furans **34-47, 49, 50** (yield in parentheses) at rt in phosphate buffer at pH 7.15

Entry	Catechol	Dicarbonyl	Reaction time (h)	Method	Product (%Yield)	Yields of other enzymatic	Yield of other syntheses
1	**29a**	**31a**	24	A	**34**(48)	60[153]	47[154]
2	**29a**	**31a**	44	B	**34**(49)		
3	**29b**	**31a**	24	A	**35**(50)	76[155], 62[153]	
4	**29a**	**31b**	24	A	**36**(65)	87[156]	22[157]
5	**29b**	**31b**	24	A	**37**(62)	70[156]	
6	**29b**	**31b**	44	B	**37**(67)		
7	**29c**	**31b**	24	A	**38**(70)	89[156]	
8	**29a**	**31c**	24	A	**39**(59)	85[156], 39[158]	
9	**29a**	**31c**	42	C	**39**(30)		
10	**29b**	**31c**	24	A	**40**(78)	71[156]	
11	**29b**	**31c**	42	C	**40**(71)		
12	**29c**	**31c**	24	A	**41**(37)	97[156]	
13	**29a**	**31d**	24	A	**42**(58)	91[156]	90[159],90[160],82[161],66[162]
14	**29a**	**31d**	42	C	**42**(40)		
15	**29b**	**31d**	24	A	**43**(98)	92[156]	87[161]
16	**29b**	**31d**	42	C	**43**(59)		
17	**29c**	**31d**	24	A	**44**(73)	95[156]	93[161],80[162]
18	**29c**	**31d**	44	B	**44**(77)		
19	**29a**	**31e**	24	A	**45**(76)	96[156], 44[158]	
20	**29a**	**31e**	42	C	**45**(50)		
21	**29b**	**31e**	24	A	**46**(80)	91[156]	
22	**29c**	**31e**	24	A	**47**(43)	97[156]	
23	**29c**	**31e**	42	C	**47**(15)		
24	**29b**	**48**	24	A	**49**(80)		
25	**29c**	**48**	24	A	**50**(55)		

Method A –Suberase® (8.0 mL), catechol (2.0 mmol), 1,3-dicarbonyl(2.0 mmol, 1 eq), phosphate buffer (20.0 mL, 0.10 M, pH 7.15), stirring time at rt = 24 h.
Method B – Suberase® (8.0 mL), catechol (2.0 mmol), 1,3-dicarbonyl (2.0 mmol, 1 eq), phosphate buffer (20 mL, 0.10 M, pH 7.15), stirring time at rt = 44 h
Method C – Suberase® (4.5 mL), catechol (0.60 mmol), 1,3-dicarbonyl (2.4 mmol, 4 eq), phosphate buffer (4.0 mL, 0.10 M, pH 7.15) and DMF (2.0 mL), stirring time at rt = 42 h.

From these results it can be seen that 5,6-dihydroxylated benzo[*b*]furans can be accessed using all three synthesis methods. For Method A the highest yield that was obtained is 98%

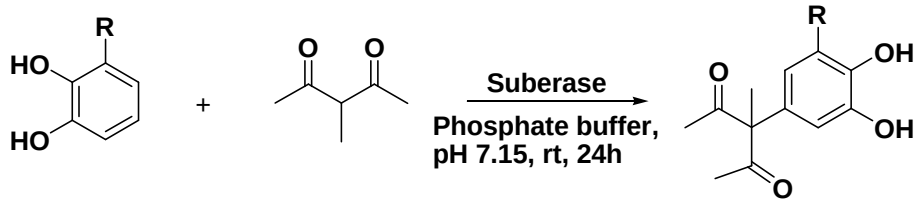
for **43** (Entry 15, Table 1) and the lowest is 37% for **41** (Entry 12, Table 1). For Method B the highest yield was 77% for **44** (Entry 18, Table 1) and the lowest 49% for **34** (Entry 2, Table 1). In the case of Method C, the highest yield was 71% for **40** (Entry 11, Table 1) and the lowest 15% for **47** (Entry 23, Table 1). When comparing the yield of product using Method A to that obtained using Method B, it can be seen that there was not a significant increase in yield even though the reaction time has almost doubled (Entries 1 and 2, 5 and 6, 17 and 18, Table 1). It was therefore concluded that there was a limit to the quantity of product that can be formed in the reaction. The presence of DMF (Method C) may have deactivated the laccase, Suberase®, resulting in a lower yield of the product. As explained by Müller *et. al* the higher volume of organic solvent increases the solubility of a substrate but significantly decreases the overall reaction rate by deactivating the enzyme.[127] The optimum conditions for synthesising these 5,6-dihydroxylated benzo[*b*]furans using Suberase® were thus that used under Method A.

The biocatalytic synthesis of 5,6-dihydroxylated benzo[b]furans has been reported previously, with the first enzymatic synthesis of compound **35** being reported in 2007 by Witayakran *et al*.[153] using *Trametes villosa* laccase in phosphate buffer (pH 7.0) with a Lewis acid, scandium tris(trifluoromethanesulfonate), and sodium lauryl sulfate at 20ºC and afforded it in 76% yield [155] as shown in Table 1. In 2009, Witayakran *et al*. also reported on a laccase–lipase co-catalytic system for the synthesis of compounds **34** and **35** using phosphate buffer (pH 7.0) with lipase.[153] Although lipase is used to catalyse the hydrolysis of esters and bromohydrin esters it was used in their study as a co-catalyst, wherein the Michael addition step was enhanced by lipase addition providing improved yields. Compound **34** was obtained in 60% yield using *Candida rugosa* lipase and laccase from *T. villosa* at 23°C. Compound **35** was obtained in 62% yield using

Candida antarctica (CALB) lipase and laccase from *T. villosa* at 23ºC.[153] We have synthesised compounds **34** and **35** without the use of scandium tris(trifluoromethanesulfonate) or lipase and achieved a 49% yield of compound **34** (Entry 2, Table 1) and a 50% yield of compound **34** (Entry 3, Table 1) Hajdok *et al.*[154] was the first to report on the synthesis of compounds **36–47** using laccase initiated oxidative domino reactions. One method entailed using a commercial laccase from *Trametes versicolor* in an acetate buffer (pH 4.38) at room temperature while the other method used laccase from *Agaricus bisporus* in phosphate buffer (pH 5.96) also at rt. The latter method was found to be better since it gave the product in higher yield and purity with yields ranging from 71 to 97%. The yields of compounds **34–35** using our methods are 37–98%, however all the purity yields are above 90%. The commercial fungal laccase, Suberase® from *Myceliophthora thermophila*, was overall less effective than the laccase from *A. bisporus* which was used by Hajdok et al.[154] Compounds **39** and **45** (entry 8 and 19 respectively, Table 1) have also been synthesised by employing tyrosinase and laccase from *A. bisporus* and were obtained in 39 and 44% yields, respectively.[156] Our method was higher yielding since compound **39** was obtained in 59% yield and compound **45** in 76% yield (Entries 8 and 19, respectively, Table 1). There have also been literature reports on the chemical syntheses of 5,6-dihydroxylated benzo[b]furans. The first report of compound **34** was a chemical synthesis which afforded **34** in 47% yield using pyridine and sodium metaiodate in ethanol at 20ºC.[154] Our methods afforded similar yields for compound **34** (48 and 49%, Entries 1 and 2, respectively, Table 1). Duthaler and Scherrer reported on the chemical synthesis of compound **36** which was obtained in 22% yield using sodium acetate in water.[157] We were able to obtain compound **36** in 65% yield (Entry 4, Table 1). Electrochemical syntheses have also been reported for the synthesis of 5,6-dihydroxylated benzo[b]furans. The first report on the synthesis of **42** was by Grujic´ *et al.*[159] In

1976 and afforded it in 90% yield. Later Tabaković *et al.*[160] also reported on the electrochemical synthesis of **42** in 90% yield in water.[160] Another electrochemical synthesis of **42** using sodium acetate in water by Nematollahi *et al.*[161] afforded it in 82% yield. The electrochemical synthesis of **42** by Davarani et al.[162] only afforded a 66% yield. We could only obtain a 58% yield for **42** (Entry 13, Table 1). Nematollahi *et al.*[161] also reported on the electrochemical synthesis of **43** and **44** (entry 15 and 17) which were obtained in 87 and 93% yields, respectively, also using sodium acetate in water as a reaction medium. The electrochemical synthesis of **43** and **44** in a sodium acetate solution by Davarani *et al.*[162] afforded these compounds in slightly lower yields, 81 and 80%, respectively. Compound **43** was obtained in 98% yield (Entry 15, Table 1) which is higher than the yields obtained by electrochemical synthesis, while compound **44** was obtained in 77% yield (Entry 18, Table 1) which was lower than that obtained by electrochemical syntheses.

Two reactions, using derivatives with the 1,3-diketone containing an extra methyl substituent, gives insights into the oxidative catalytic mechanism of laccase on benzofurans. In this approach, as expected we formed an intermediate that could not cyclize due to the presence of methyl at position 2 as shown in Scheme 2 below. This then supports the reaction mechanism in the synthesis of benzofurans.

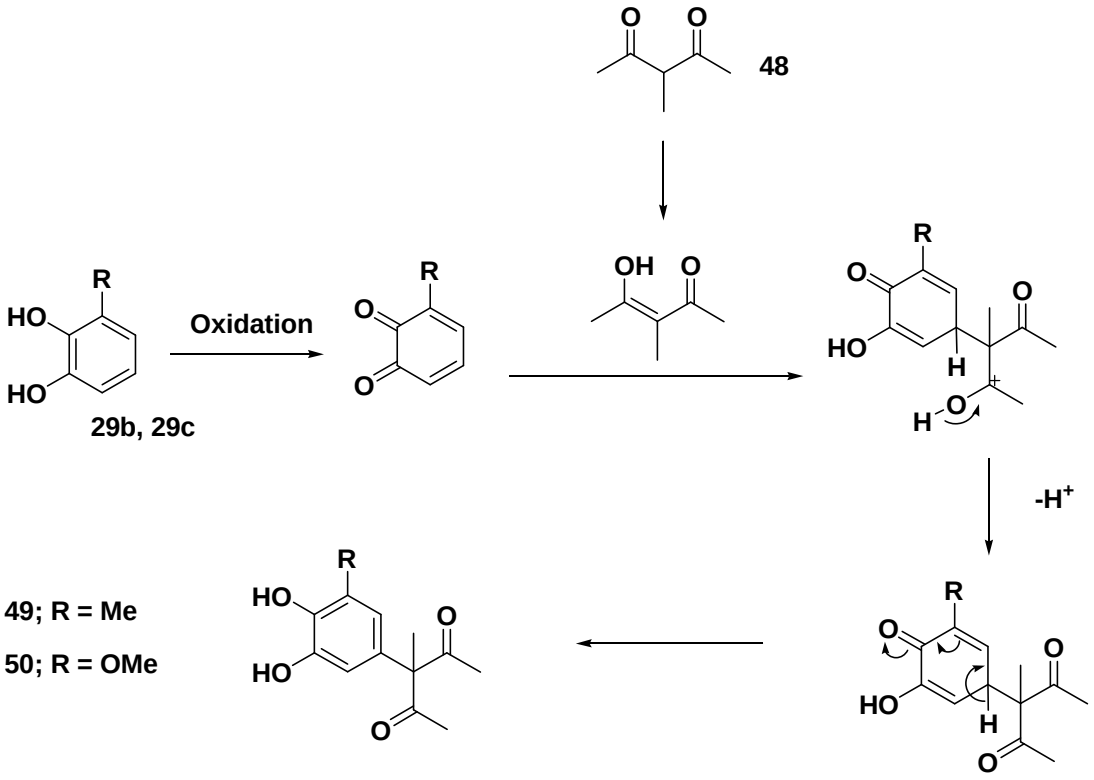


29b, 29c **48** **49-50**

49=R = Me, 80%
50=R = OMe, 55%

Scheme 7

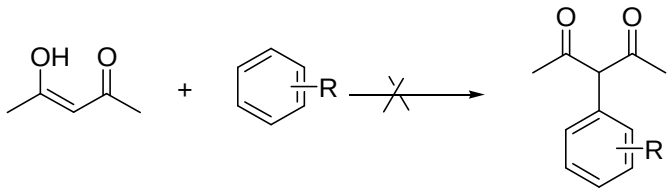
The proposed mechanism involving an *o*-quinone intermediate of **29b** and **29c** is shown in Scheme 5 below. The mechanism is such that the catechol is oxidised to an *ortho*-quinone. The quinonoid undergoes a Michael addition reaction with a substituted 1,3-diketone. However since it is substituted the second enolization cannot take place and instead a subsequent re-aromatization takes place as shown in Scheme 8 below.



Scheme 8: A proposed mechanism for C-C bond formation for the catechol derivatives **29b** and **29c.**

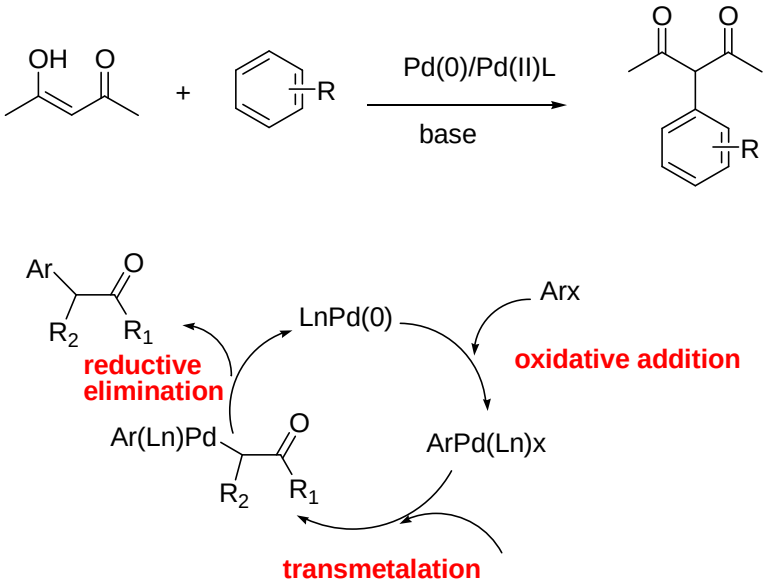
These reactions were conducted using Method A which was used for the synthesis of the 5,6-dihydroxylated benzo[*b*]furans **34–47**. In this case, the aim was to synthesize one carbon-carbon bond using the 1,3-dicarbonyl **48** without subsequent cyclisation. This reaction is of crucial

significance as cross coupling of an enolates cannot take place with unactivated sp^2-hybridized electrophiles without a transition-metal catalyst (Scheme 6).



Scheme 9: Showing unactivated sp2-hybridized electrophiles not reacting with enolates.

These types of reactions can only take place through the enolate arylation reaction and it proceeds via the classical cross-coupling mechanism which includes; oxidative addition, transmetalation and reductive elimination as shown in Scheme 7 below.



Scheme 10: Showing activated sp^2-hybridized electrophiles reacting with enolates.

We have successfully demonstrated that the enzyme Suberase® can be utilised in cross coupling of enolates to unactivated sp^2-hybridized electrophiles without the use of a transition-metal catalyst.

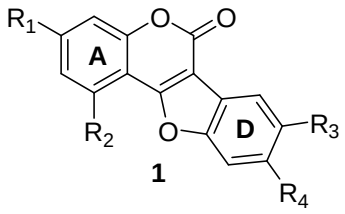
3.2 CONCLUSION

The fungal laccase from *M. thermophila,* which is commercially available as an inexpensive preparation known as Suberase®, can be used in the catalytic synthesis of benzofurans under mild and environmentally friendly reaction conditions. A pH of 7.15 was chosen because it would make the reaction medium sufficiently basic to deprotonate the alpha-proton from the 1,3-dicarbonyls and thus facilitate the Michael addition reaction with the *in situ*-generated *o*-quinone. This is then followed by oxidation which is then followed by cyclisation to give us our desired 5,6-dihydroxylated benzo[*b*]furan. Evidence for this catalytic mechanism comes from the use of 1,3-diketone blocked at carbon 2, where it was evident that under these conditions we were unable to cyclise the intermediate to a benzofuran and rather achieved a C-C monocoupled product. The yields achieved in the present study are, in some cases, similar to or better than that obtained by other enzymatic, chemical, or electrochemical synthesis. This method has eliminated the use of the Lewis acid, scandium tris (trifluoromethanesulfonate), and lipase which was used in previous laccase methods.A proposed mechanism involves an *o*-quinone intermediate and the mechanism is such that the catechol is oxidised to a quinonoid. The quinonoid undergos a Michael addition reaction with a substituted 1,3-diketone. However since it is substituted the second reduction cannot take place. This opened the question as to what other reactants could be included to yield a wider range of products. We successfully demonstrated that the enzyme Suberase® can be utilised in cross coupling of enolates to unactivated sp2-hybridized electrophiles without the use of a transition-metal catalyst.

BENZOFURO[3,2-*C*]CHROMEN-6-ONE

4.0 INTRODUCTION

As discussed in Chapter 3 natural products with a benzofuran skeleton are well known for their broad range of biological activities.[163] Coumestan **1**, which contains a benzofuro [3,2-*c*]chromen-6-one skeleton, belongs to an important class of natural oxygenated aromatic products (**Figure 1)**, the flavonoid category of phytoestrogens.[163] These compounds exhibit antibacterial, anticancer antifungal, antihepatotoxic, and phytoalexin activities.[163,164] In China compounds with benzofuro[3,2-*c*]chromen-6-one skeleton are used in the treatment of septic shock.[165] They are found in a variety of plants including split peas, pinto beans, lima beans, alfalfa and clover sprouts.



1

Figure 1: The structure of a coumestan.

HCV, a major human pathogen that causes non-A and non-B hepatitis, is also associated with the development of chronic disease such as steatosis, liver cirrhosis and hepatocellular carcinoma. HCV infection is estimated to be four to five times more prevalent than the Human Immunodeficiency Virus-1 (HIV-1).[166] Although there is currently no vaccine for HCV, five

coumestan analogues, Wedel lactone **2**, LQB16 **3**, LQB34 **4**, LQB93 **5** and LQB96 **6** have been reported to inhibit HCVNS$_5$B which is required for viral RNA replication in HCV (Figure 3).

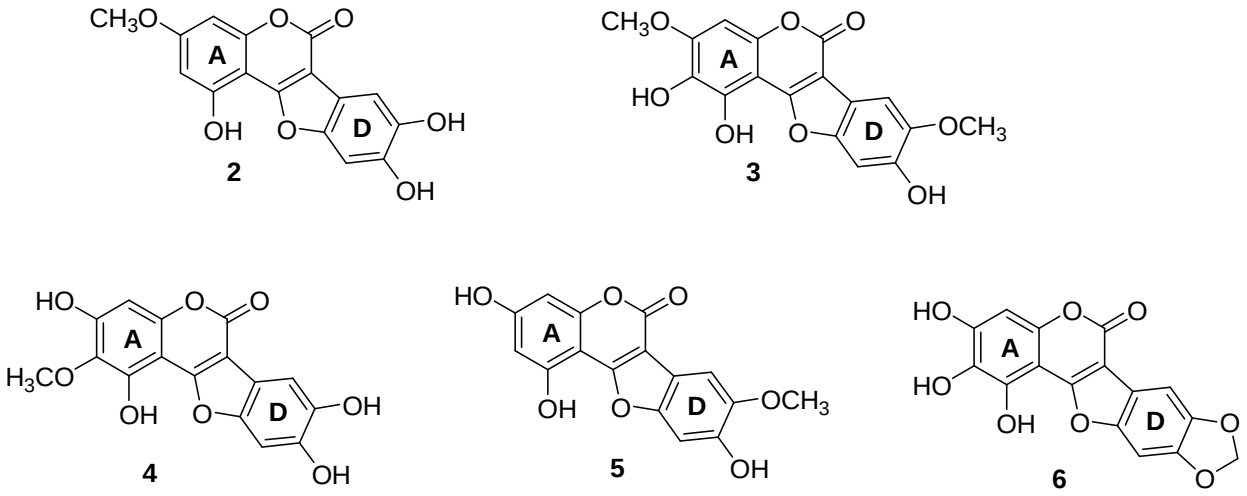


Figure 2: Five coumestan analogues that inhibit HCVNS$_5$B.

In the literature it has been reported that benzofuro[3,2-*c*]chromen-6-ones can be synthesized by electrochemical oxidation, tyrosinase-catalyzed oxidation, as well as chemical oxidation using ferricyanide. In light of the biological activity exhibited by the coumestans and the structural similarity (*ortho* phenolic substituents) to the 5,6-dihydroxylated benzo[*b*]furans **7** (Chapter 3) we were interested in developing a green, efficient one-pot synthesis using laccase as the catalyst.

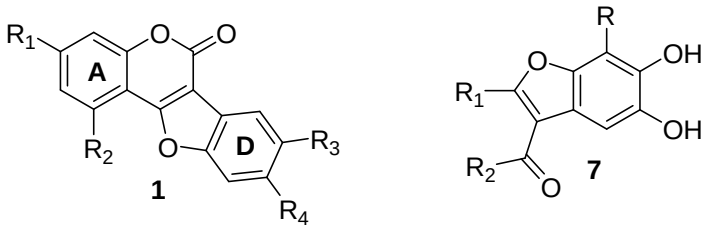


Figure 3: Showing a structure of a coumestan and benzo[*b]*furans

4.1 RESULTS AND DISCUSSION

4.1.1 SYNTHESIS

The catechols **8a–e** and the coumarins **9a–d** used in this study are depicted in Figure 5. The method used for the synthesis entailed reacting one equivalent of the catechol **8a–e** with one equivalent of the coumarin **9a–d** shown in Scheme 1 using Suberase® in a vessel open to air at pH 7.15. A pH of 7.15 was chosen because it would make the reaction medium sufficiently basic to allow for deprotonation of the acidic hydrogen of the coumarin and thus facilitated the Michael addition reaction with the *in situ*-generated *o*-quinone.

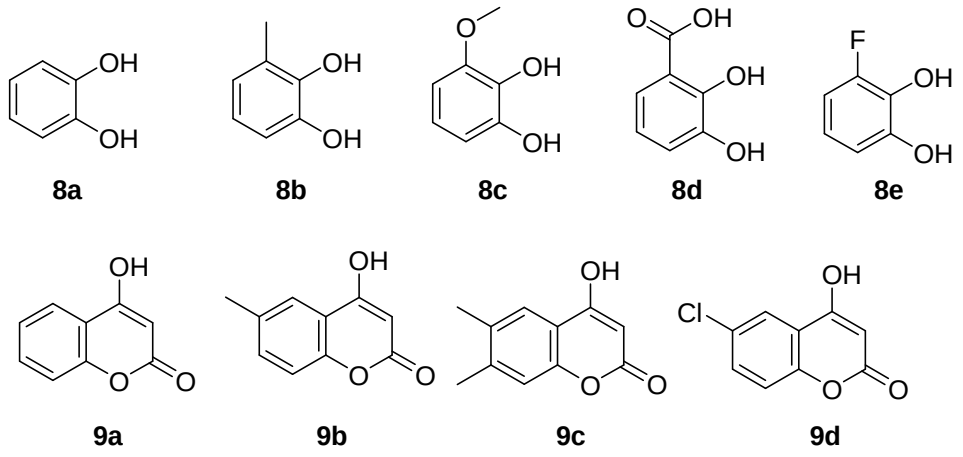
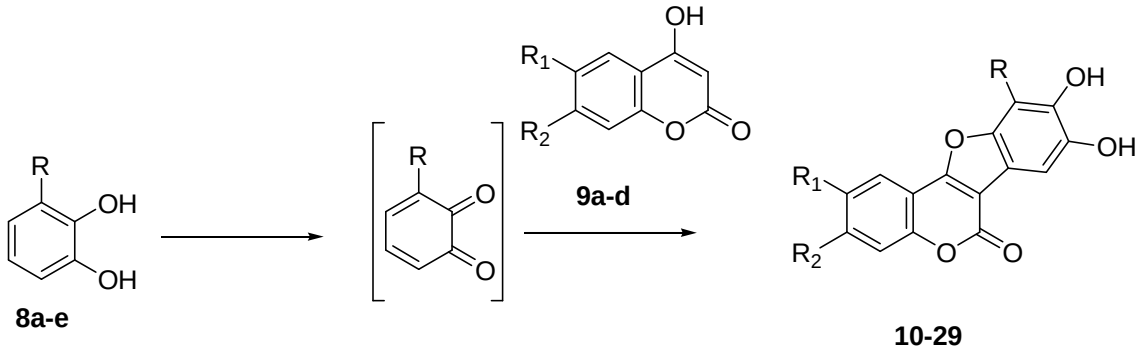


Figure 4: The catechols **8a**–**e** and coumarins **9a–d** used in this study.



Scheme 1. Synthesis of the 5,6-dihydroxylated benzo[*b*]furans **10-29.**

From the twenty compounds that were successfully synthesized (Figure 6), five are known (compound **10**, **11**, **12**, **15 and 16**) and 15 are novel (compounds **13, 14, 17-29**). Bhalerao *et al* have reported a synthesis for **15** in 94% yield using a mushroom tyrosinase enzyme at ambient temperature in phosphate buffer (pH 6.8).[166] Leutbecher *et al*. reported the synthesis of compound **15** in 94% yield with *Trametes versicolor* laccase in acetate buffer (pH 4.37) at 20°C.[167] They also reported the synthesis of **16** in 96% yield after 49 h using *Agaricus bisporus* laccase in acetate buffer (pH 6.0) at 20°C.[167] Bhalerao *et al* also demonstrated the synthesis of **18** in 96% yield after 1 h using mushroom tyrosinase at ambient temperature in phosphate buffer (pH 6.8).[166] Compound **13** could also be synthesized in 99 % by using *T. versicolor* laccase in acetate buffer (pH 4.37) at 20°C to.[166] The synthesis of **16** could be accomplished by using sodium acetate and potassium hexacyanoferrate(III) in water for 1 hr to afford a 96% yield.[170] Compound **15** could be synthesized via heterocyclic chemistry with sodium acetate; potassium hexacyanoferrate(III) in water 1 h through Michael addition reaction; 96%**.**[170]

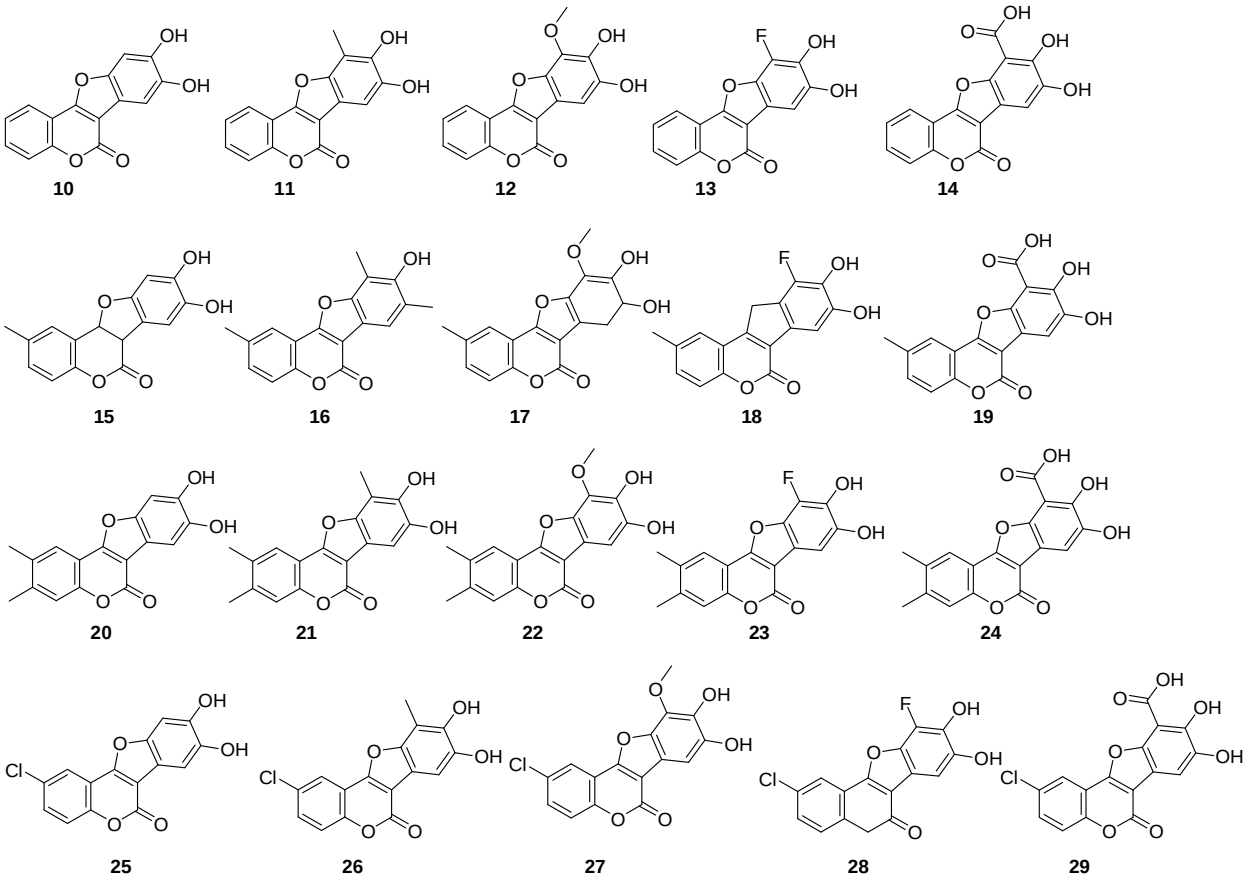


Figure 5: Structures of the biocatalytically synthesized benzofuro[3,2-*c*]chromen-6-ones **10**-**29**.

In the first approach, Method A was used, which entailed reacting the catechols **8a**–**e** with the heterocyclic 1,3-dicarbonyls **9a-d** at room temperature (rt) at pH 7.15 for 24 h. All the compounds could be synthesized at rt except for the acid derivatives **14**, **19**, **24** and **29**. As a consequence, it was decided to increase the reaction temperature. The second approach, Method B, thus entailed reacting **8d** with catechols **9a-d** at 40°C for 24hr. The results of the reactions are shown in Table 1.

For compounds with a fluorinated catechol, we observed splitting of the peaks in the ^{13}C NMR spectra, indicating carbon-fluorine coupling. The splitting is obviously only observed in the spectra of compounds prepared from the fluorinated substrate, 3-fluorocatechol. The percentage

purity for all the synthesized compounds was more than 90%. The yields of the compounds are indicated in Table 1.

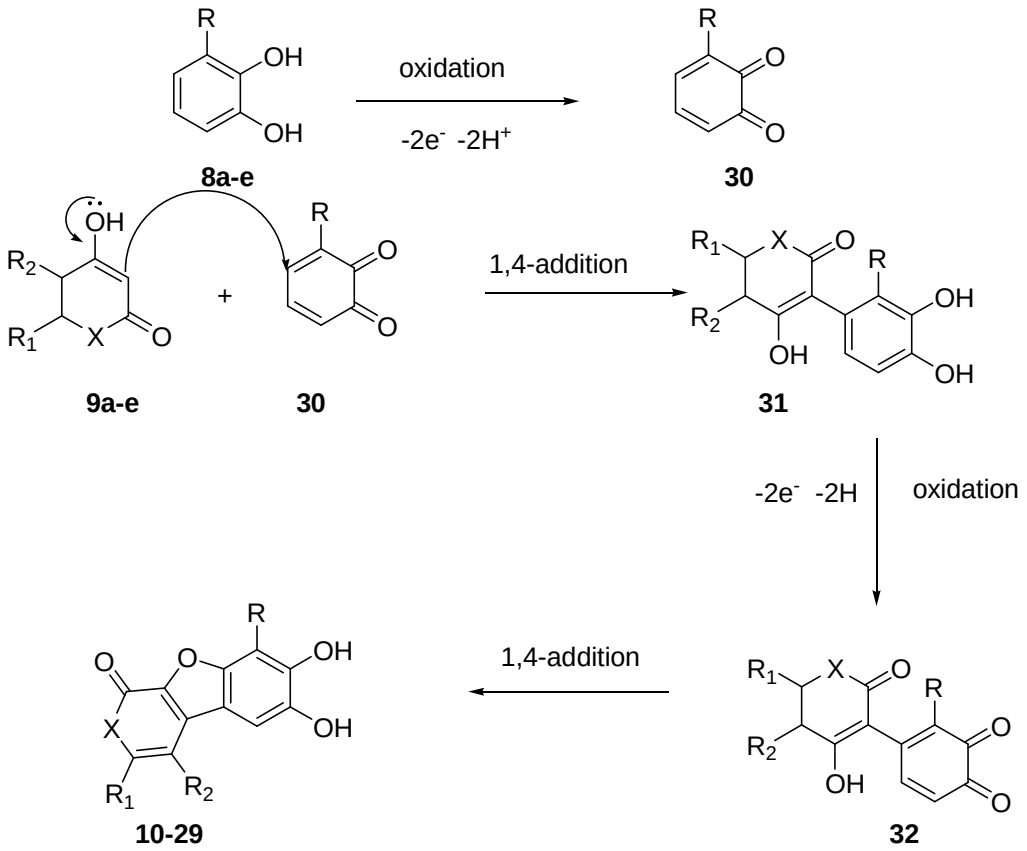
Table 1. Synthesised benzofuro[3,2-*c*]chromen-6-one **10-29** (yield in parentheses) at rt in phosphate buffer at pH 7.15.

Entry	Catechol	Dicarbonyl	Reaction time(h)	Method	Product (%Yield)	Yields of enzymatic	Yields of other syntheses
1	**8a**	**9a**	24	A	**10**(86)	96[166] 85[167]	95[168],96[169]
2	**8b**	**9a**	44	A	**11**(76)	99[166]	
3	**8c**	**9a**	24	A	**12**(74)	64[166]	98[168]
4	**8d**	**9a**	24	A	**13**(72)		
5	**8a**	**9b**	24	A	**14**(78)		
6	**8b**	**9b**	44	A	**15**(79)	94[166], 94[167]	
7	**8c**	**9b**	24	A	**16**(78)	96[167]	
8	**8d**	**9b**	24	A	**17**(72)		
9	**8a**	**9c**	42	A	**18**(78)		
10	**8b**	**9c**	24	A	**19**(74)		
11	**8c**	**9c**	42	A	**20**(74)		
12	**8d**	**9c**	24	A	**21**(70)		
13	**8a**	**9d**	24	A	**22**(73)		
14	**8b**	**9d**	42	A	**23**(78)		
15	**8c**	**9d**	24	A	**24**(78)		
16	**8d**	**9d**	42	A	**25**(70)		
17	**8e**	**9a**	24	B	**26**(78)		
18	**8e**	**9b**	44	B	**27**(76)		
19	**8e**	**9c**	24	B	**28**(74)		
20	**8e**	**9d**	42	B	**29(**76)		

Method A - Suberase® (8.0 mL), catechol (2.0 mmol), coumarin (2.0 mmol, 1 eq), phosphate buffer (20.0 mL, 0.10 M, pH 7.15), stirring time at rt = 24 h.
Method B - Suberase® (4.0 mL), catechol (1.0 mmol), coumarin (1.0 mmol, 1 eq), phosphate buffer (8.00 mL, 0.10 M, pH 7.15), stirring time at 40°C = 24 h.

Proposed mechanism of formation for the coumestans

It is assumed that the first step of these domino reactions is the laccase-catalysed oxidation of a catechol with O_2 to give *o*-benzoquinone as shown in Scheme 4. The *o*-benzoquinone **30** then undergoes a 1,4- addition with the coumarin **9a-e** under basic conditions to afford **31,** which is a transitory intermediate.



Scheme 4: A proposed mechanism for the synthesis of benzofuro[3,2-*c*]chromen-6-one derivatives **10-29**

After a second laccase-catalyzed oxidation, a final intramolecular oxa-1,4-addition, involving the formation of a tricycle, takes place. This reaction is highly regioselective since the initial 1,4-addition exclusively occurs at the more electrophilic carbon atom of the corresponding *o*-benzoquinone. Interestingly, for benzofuro[3,2-*c*]chromen-6-one, both the acid **8d** and the fluorinated **8e** catechols reacted to afford **23** and **24**. This was not observed for the 1,3-diketones discussed in Chapter 3 where the fluorinated and acid catechols did not react.

4. 1. 2 SPECTROSCOPIC EVIDENCE THAT SUPPORTS CYCLIZATION

In order to confirm that a furan ring has been formed through the second oxa-1,4-Michael addition we selected compound **15** as an example for careful scrutiny by NMR. If the reaction

had undergone a C-C coupling addition we would then expect to see 1,2,4-trisubstituted benzene ring **33** as shown in Figure 7 below. However if the further cyclisation, had occurred then we will expect to obtain a product displaying two aromatic protons in the ^{1}H NMR spectra **15**.

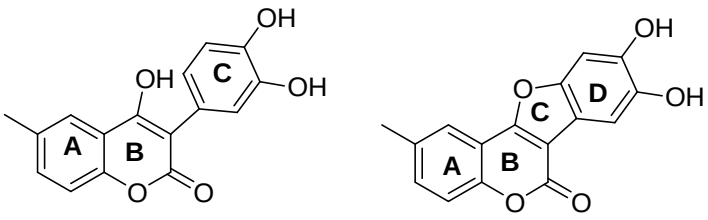


Figure 6: Two possible structures

Both compounds would have the same number of protons and carbons. However **33** has an additional OH group making a total of 3 OH protons that are expected in the ^{1}H NMR spectra. We observed from the 1H NMR spectra that we have 2 OH protons that are at 9.61ppm (2H, s, 2 x OH). This can only be possible if we have formed **15** not **33**. In addition, if we had synthesized compound **3**3we would expect to see 3 aromatic signals in the ^{1}H NMR from C ring in the aromatic region, whereas if compound **15** has been synthesized, we expect 2 aromatic signals in the 1H NMR spectra from ring D and these signals should be singlets, which we can indeed see at 7.21 ppm (1H, s, ArH) and 7.28 (1H, s, ArH). Therefore ^{1}H NMR confirms that we have synthesized **15**. We then proceeded to examine the ^{13}C NMR and mass spectroscopy data for further analysis. Sixteen signals matched that of **15** in structure; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 20.4, 98.9, 104.9, 105.3, 112.0, 113.9, 116.8, 120.6, 132.1, 134.3, 144.7, 146.5, 149.4, 150.5, 157.6, 157.7. Mass spectroscopy also confirmed that it was **15** ($M+H^+$ Found: 283.0512, $C_{16}H_{11}O_5$ requires $M+H^+$, 283.0606). Although **33** would have the same mass spectrometry data the NMR data would have differed from that of **15**. We can then conclude that the reaction

undergoes a 1,4-Michael addition followed by a second oxa-Michael addition. For compounds with a fluorinated catechol, we observed splitting of the peaks in the ^{13}C NMR spectra.

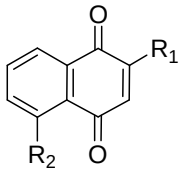
4.2 CONCLUSION

Twenty benzofuro [3,2-*c*]chromen-6-one compounds could be synthesized by exposure of catechols and coumarins to laccase under basic conditions at rt. Unlike in the synthesis of 5,6-dihydroxybenzo[*b*]furans in Chapter 3 we could access all the acid derivatives of benzofuro[3,2-*c*]chromen-6-ones (**14, 19, 24** and **29**) when the temperature was raised to 40°C. For compounds with the fluorinated catechol **10e** we observed a confirmatory splitting of the peaks in the ^{13}C NMR spectra which is also observed in the spectra of the fluorinated substrate, 3-fluorocatechol. Five compounds are known (compound **10**, **11**, **12**, **15 and 16**) while fifteen are novel (compound **13, 14, 17-29**).

NAPHTHOQUINONE DERIVATIVES

5.0 INTRODUCTION

A 1,4-naphthoquinone is a two planar benzene ring system, fused together with two carbonyls in one ring that are in a *para*-position to each other (Figure 1). These compounds can undergo electrophilic aromatic substitution like benzene, although, they are more reactive than benzene, and also have the ability to undergo 1,2- or 1,4 addition reactions that are enabled by the carbonyl functionalities.



1

Figure 1: Structure of a 1,4-naphthoquinone.

Herein, we report on the synthesis of 1,4-naphthoquinone derivatives, by using biocatalytic procedures as one of the key steps. A number of the synthesised derivatives were screened for both anti-cancer and anti-bacterial activities, and the biological screening results will be discussed in Chapter 7. The two classes of compounds that were investigated were substituted 1,4-naphthoquinones **2-5** (a two ring system) and anthraquinone **6** (a three ring system) as shown in Figure 2 below.

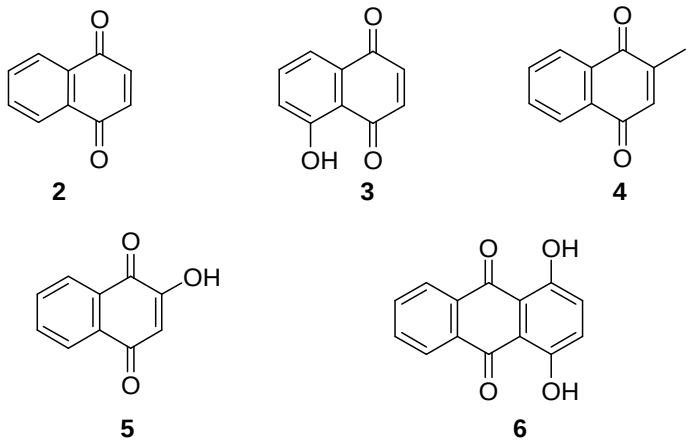


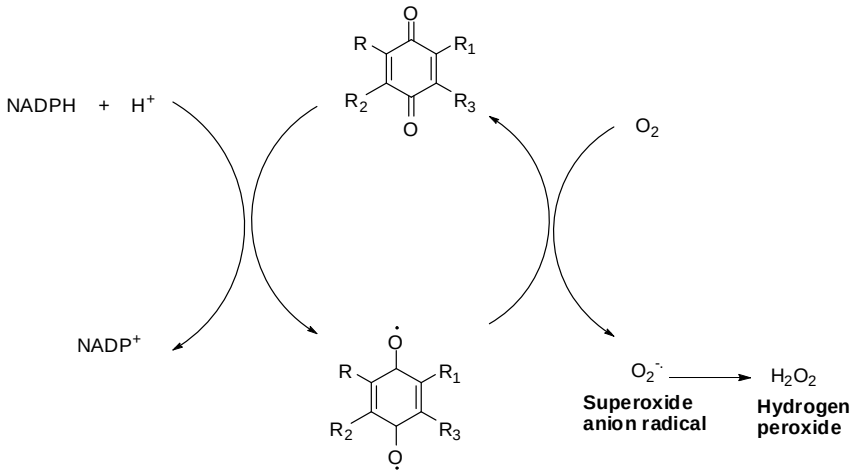
Figure 2: Structures of 1,4-naphthoquinones (**2-5**) and 1,4-dihydroxyanthracene-9,10-dione (**6**).

In summary we wanted to determine whether 1,4-naphthoquinones substituted at the 2-position with either an electron-donating or an electron-withdrawing group could be used in biocatalytic reactions to synthesise 1,4-naphthoquinone derivatives. The idea was to increase conjugation and ring size when compared to those of the 5,6-dihydroxylated benzo[*b*]furans and benzofuro[3,2-C] chromen-6-ones discussed in Chapters 3 and 4, respectively. By increasing the number of fused rings the lipophilicity also increases, which could result in an enhancement of absorption through biological membranes.

5.1 BACKGROUND

The search for novel drugs that are effective against both cancer and bacteria is a continuing process.[171] The molecular framework of a great number of pharmaceuticals and biological compounds contain a quinone moiety.[172] It has been noted from literature that the quinone moiety possesses both anticancer and antibacterial activity.[173] This is due to their ability to undergo redox cycling which results in the generation of reactive oxygen species (ROS) as shown in Scheme 1.[171] Redox cycling is a process in which compounds catalytically cycle and generate ROS such as hydrogen peroxide and superoxide which then damages the cell.

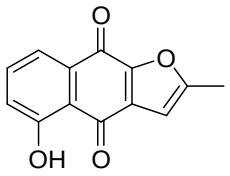
ROS also inhibits the drug efflux mechanism in drug resistant bacteria, which allows intracellular accumulation of potent drug molecules.[171]



Scheme 1: Redox cycling of NADH or NADPH and a quinone to produce ROS.[163]

The anticancer activity of quinones is also believed to be due to alkylation when quinones are activated inside the cells. This alkylation process involves covalent attachment to proteins, DNA or other targets, which leads to the inhibition of cell growth. For example, the furanonaphthoquinone **7** is an example of a biologically active 1,4-naphthoquinone.[171] It has been shown to possess antibacterial activity against drug resistant bacteria. *Chlamydia* is a genus of bacteria, including the species *C. trachomatis, C. pneumonia and C. psittaci* that are obligate intracellular parasites that are resistant to existing drugs, [171] (*C. pneumonia and C. psittaci* have recently been reclassified under the *genus Chlamydophila*). The furanonaphthoquinone **7** derivative depicted in Figure 3 has been screened against 14 strains of methicillin resistant *Staphylococcus aureus* (MRSA) and 12 strains of methicillin susceptible *Staphylococcus aureus* (MSSA) as a control.[171] Koyama found that the MIC of **7**

against MRSA was 5.36 µg/ml and the MIC against MSSA, 11.98 µg/ml. This shows that compound **7** exhibits stronger antibacterial activity against MRSA.

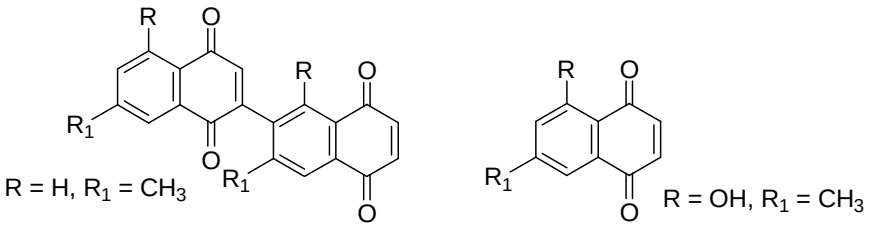


7

Figure 3: A furananophthoquinone derivative exhibiting activity against MRSA.

When compound **7** was screened against Fluconazole-resistant *Candida albicans* (FRCA) it exhibited the same degree of antibacterial activity as in MSSA and MRSA.

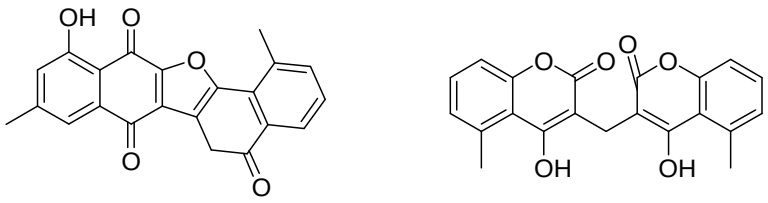
Other naphthoquinones, for example the compounds, Diospyrin **8** and Methyljuglone **9** (Figure 4), isolated from the South African medicinal plant *Enatalensis*, were found to be effective against *Mtb*. They inhibit several antibiotic resistant as well as antibiotic susceptible strains of *Mtb*.



8 **9**

Figure 4: Compounds isolated from the plant *Enatalensis*.

Crassiflorone, a new 1,4-naphthoquinone, from the stem bark of the *Diospyros crassiflora* tree, has been shown to exhibit antimicrobial activity.[174] The Grassiflorone derivatives **10** and **11** (Figure 5) were studied using a microdilution assay against six microbial cultures.[175,176]



10 **11**

Figure 5: Crassiflorone derivatives

The cultures were the bacteria *Escherichia coli* (LMP0101U), *Shigella dysenteriae* (LMP0208U), *Salmonella typhi* (LMP0209U), and *Staphylococcus aureus* (LMP0206U), and the yeasts *Candida krusei* (LMP0311U) and *Candida albicans* (LMP0204U). The antimicrobial activity was evaluated on the basis of MIC. Compound **11** showed the greatest activity against *S. dysenteriae* (MIC 4.88 µg/ml) and *S. typhi* (MIC 4.88 µg/ml), whilst compound **10** showed weaker activity (MIC 78.12 µg/ml and 19.53 µg/ml, respectively).

Other important examples of quinones that are known for inhibition of special proteins such as bacterial topoisomerase II, DNA gyrase (antibacterial), mammalian topoisomerase I and II (antitumor), HIV integrase and HIV protease (antiviral) are the important anticancer drugs doxorubicin **12** and mitoxanthrone **13** (Figure 6).[177-179] These anthracyclines are well known for their anticancer activity.[174] The mechanism of action is believed to involve topoisomerase II inhibition. Naphthoquinone analogues such as shikonin **14**, naphthazarin **15**, plumbagin **16**, and *β*-lapachone **17** are believed to also inhibit similar enzymes.[180]

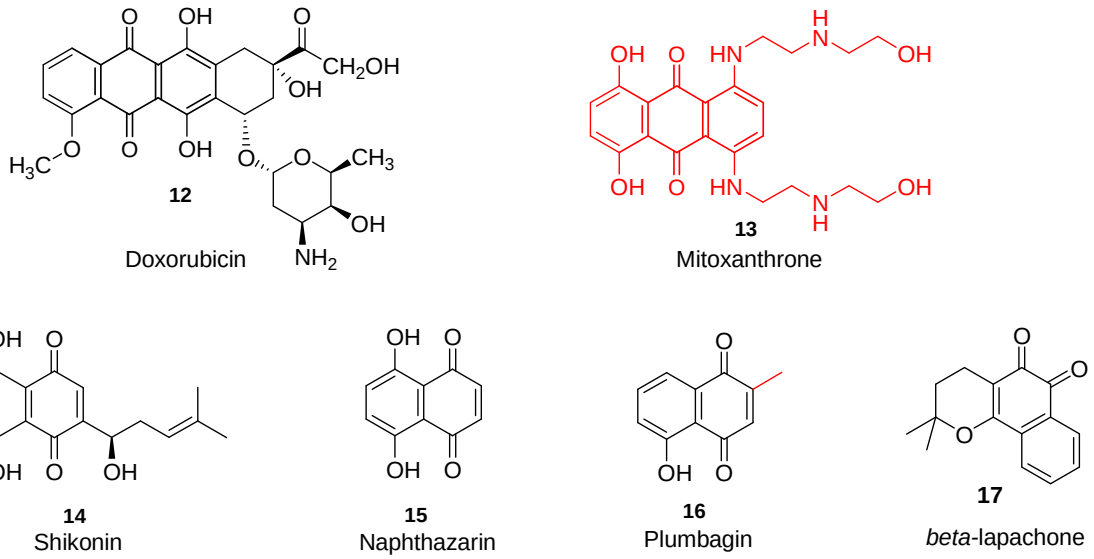


Figure 6: Naphthoquinone derivatives exhibiting anticancer activity.

5.2 RESULTS AND DISCUSSION

5.2.1 SYNTHESIS OF MENADIONE ANALOGUES FROM 1,3-DIKETONES

In this section we proceeded by reacting naphthoquinones containing a substituent at position 2 and reacted these substrates with 1,3-diketones. Menadione **18** was initially selected as the monosubstituted 1,4-napthoquinone to react with a range of structurally diverse diketones **19a-h** (Figure 7).

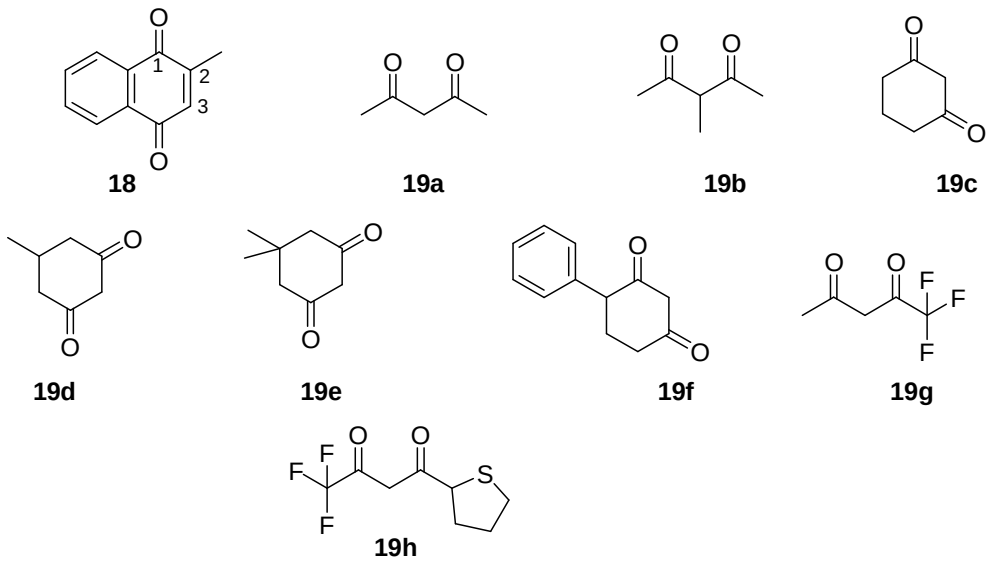
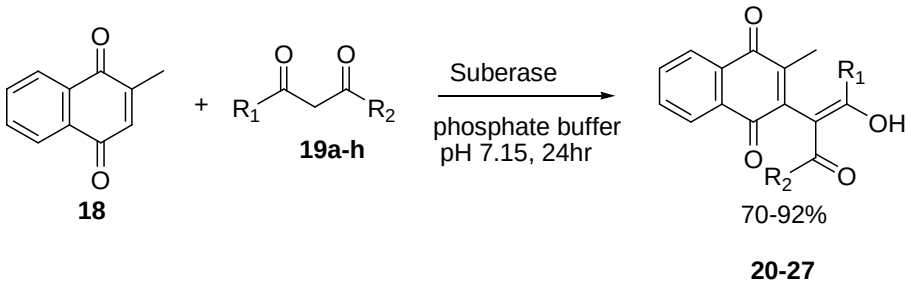


Figure 7: Menadione **18** and the 1,3-diketones **19a-h**.

The synthesis of C-C coupled menadione derivatives was achieved by reacting laccase (Suberase) with one equivalent of the 1,3-diketone (1.0 mmol) and one equivalent of menadione in a mixture of DMF (1.0 mL) and phosphate buffer (0.10 M, pH 7.15) in a test tube. The reaction mixture was stirred in an open vessel (providing oxygen) at 40°C. DMF was added to the reaction mixture to improve the solubility of the organic substrates in the reaction medium. The mixture was vigorously stirred under air until the substrates were consumed as judged by TLC. The product was extracted with ethyl acetate, purified by column chromatography and, if not pure, recrystallization. Characterization of the compounds was achieved by ^{1}H NMR, ^{13}C NMR and mass spectrometry.



Scheme 2: Biocatalytic synthesis of menadione derivatives

The results of the investigation are depicted in Table 1 below.

Table 1. Synthesised C-C coupled menadione analogues **20–27** (yield in parentheses) from diketones at 40°C in phosphate buffer at pH 7.15.

Entry	Naphthoquinone	Diketone	Reaction time (h)	Product (%Yield)
1	**18**	**19a**	24	**20** (76)
2	**18**	**19b**	24	**21** (82)
3	**18**	**19c**	24	**22 (**88)
4	**18**	**19d**	24	**23 (**88**)**
5	**18**	**19e**	24	**24** (84)
6	**18**	**19f**	24	**25** (92)
7	**18**	**19g**	24	**26** (70)
8	**18**	**19f**	24	**27** (83)

Method - Suberase® (4.0 mL), diketone (1.0 mmol), naphthoquinone/naphthohydroquinone (1.0 mmol, 1 eq), phosphate buffer (8.0 mL, 0.10 M, pH 7.15), stirring time at 40°C = 24 hrs

From these results it can be seen that the menadione analogues can be accessed using the above method. The synthesis afforded yields of products ranging from 70-92%. The highest yield that was obtained is 92% for **25** (Entry 6, Table 1) and the lowest is 70% for **26** (Entry 7, Table 1). The structures of the products **20**-**27** are depicted in Figure 8 below.

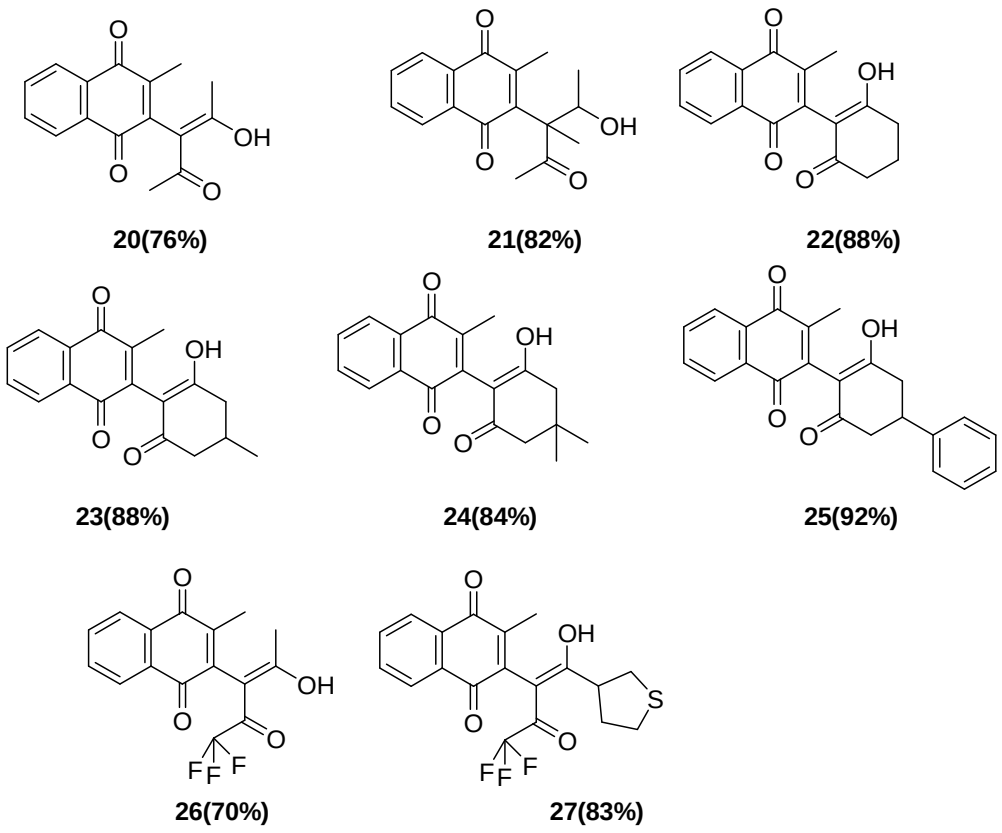
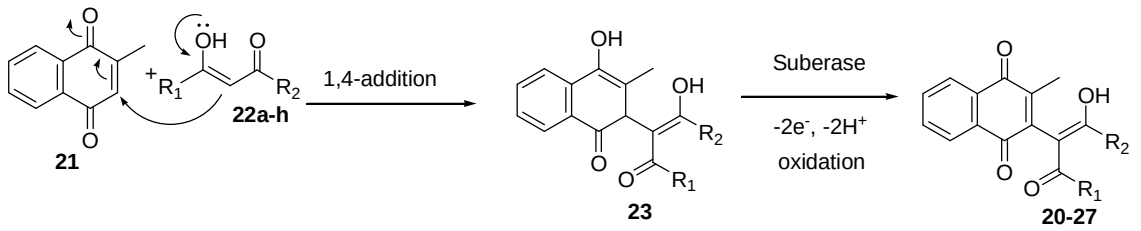


Figure 8: Structures of the menadione analogues **20**-**27** synthesised from 1,3-diketones.

5.2.2 MECHANISM OF FORMATION OF THE MENADIONE ANALOGUES FROM 1,3-DIKETONES

Analysis of quinones **20**-**27** using ^{1}H NMR, ^{13}C NMR and mass spectroscopy showed that the attack of the nucleophilic 1,3-dicarbonyl **22a-h** takes place at C-3 of the monosubstituted 1,4-naphthoquinone. C-3 is the most electrophilic carbon suitable for the 1,4-addition. Addition cannot take place at the C-2 position due to the higher energy transition state and the presence of the methyl group as shown in Scheme 3 below.

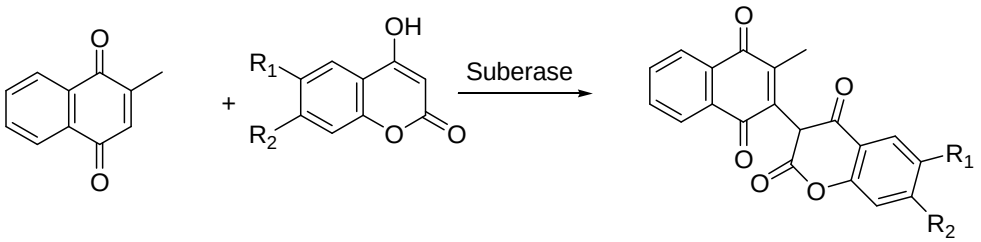


Scheme 3: Proposed mechanism for the formation of the menadione analogues from 1,3-diketones.

Several studies of monosubstituted quinones with nucleophiles have been previously reported.[170] One of them examined the influence of the first substituent on the course of the reaction. For example the reaction of methylquinone with anilines 2:1 to give 2,5 and 2,6-disubstituted products in a 2:1 ratio.[178] It has also been reported that the reaction between electrochemically generated formyl-*p*-benzoquinone and 3-hydroxy-1H-phenalen-1-one starts with the formation of a 2,5-disubsituted bis-adduct which then undergoes cyclization to the corresponding benzofuran derivatives. The synthesised compounds were easily accessible, and we observed almost complete conversion of the starting materials as determined by TLC monitoring and analyses of the reaction mixtures. We then decided to investigate whether the coumarins would react in a similar manner to the 1,3-diketones with menadione.

5.2.3 SYNTHESIS OF MENADIONE ANALOGUES FROM COUMARINS

Menadione **21** successfully reacted with the coumarins **32a-d** to give the expected mono-substituted menadione analogues.



21 **22a-d** **33-36**

33 $R_1 = R_2 = H$
34 $R_1 = Me$, $R_2 = H$
35 $R_1 = R_2 = Me$
36 $R_1 = Cl$, $R_2 = H$

Scheme 4. Synthesis of menadione analogues from coumarin.

The synthesis of the C-C coupled menadione analogues was accomplished by following the same method as that used for the reaction with the diketones to produce compounds **33-36**.

The results of the reaction of menadione **21** with each of the coumarins is shown in Table 2 below.

Table 2. C-C coupled menadione analogues **33–36** (yield in parentheses) synthesized from coumarins at 40°C in phosphate buffer at pH 7.15..

Entry	Naphthoquinone	Coumarin	Reaction time (h)	Product (%Yield)
1	**21**	**32a**	24	**33** (86)
2	**21**	**32b**	24	**34** (80)
3	**21**	**32c**	24	**35 (**85)
4	**21**	**32d**	**24**	**36 (**88**)**

Method - Suberase® (4.0 mL), diketone (1.0 mmol), naphthoquinone/naphthohydroquinone (1.0 mmol, 1 eq), phosphate buffer (8.0 mL, 0.10 M, pH 7.15), stirring time at 40°C = 24 h.

From these results it is evident that the synthesis afforded products in high yields, ranging from 80-88%. The highest yield was obtained for **36** (88% (Entry 4, Table 2) and the lowest for **34** (80% (Entry 2, Table 2). The structures of the synthesised products are shown in Figure 9 below.

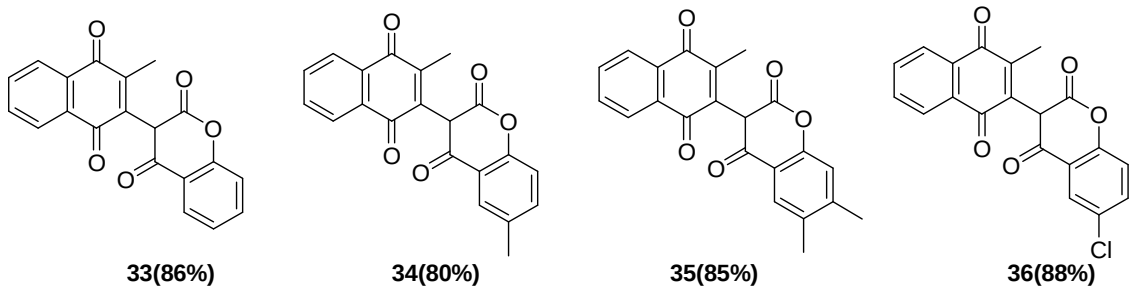
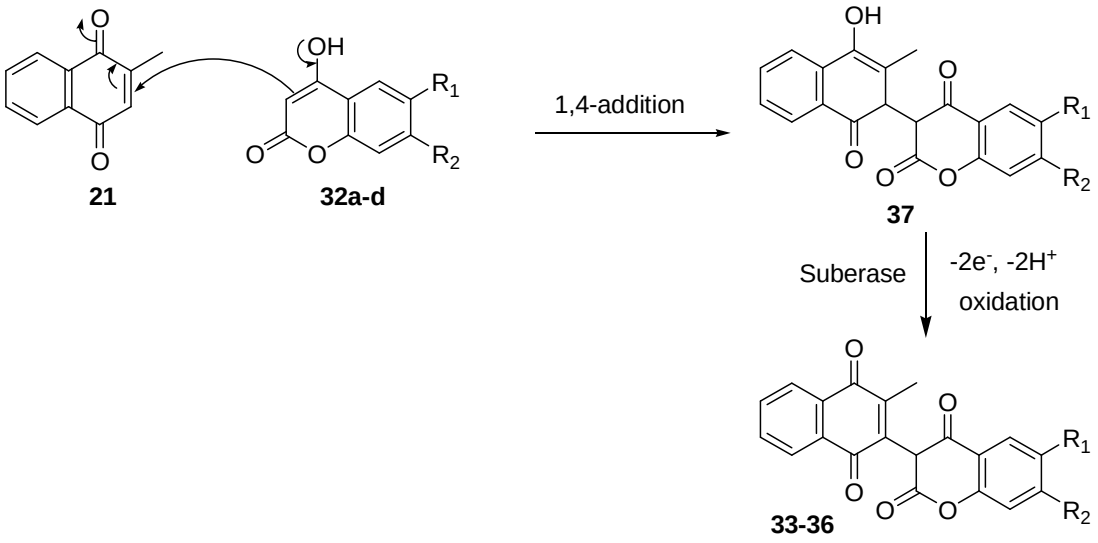


Figure 9: The structures of the menadione analogues **33-36** synthesised from coumarins.

The synthesis of menadione analogues from coumarins was achieved by oxidation of **37** which is formed after coumarin **32a-d** reacts with menadione **21** as shown in Scheme 5 below.



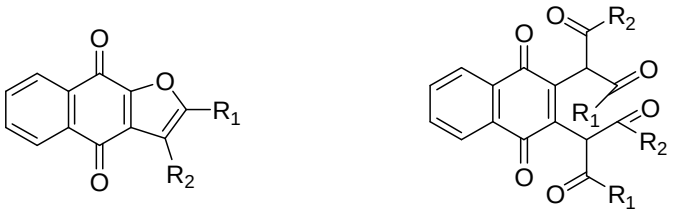
Scheme 5. Proposed mechanism for the synthesis of menadione analogues from coumarins.

The final step is oxidation resulting in the formation of the 2-monosubstituted *p*-naphthoquinone. In this transformation ring cyclisation does not occur as it did for the formation of the benzofurans and chromenes in Chapters 3 and 4 respectively.

Similar to the diketones discussed in Section 5.2.2 we could access the coumarin derivatives easily with no side products. This prompted us to investigate the reaction of the unsubstituted 1,4-naphthoquinones with 1,3-diketones to determine whether the initial 1,4-addition reaction would be followed, after oxidation, with a second oxa-Michael reaction, resulting in furanonaphthoquinone formation.

5.3 FORMATION OF 2,3-DIALKYLATED NAPHTHOQUINONES, THROUGH CONSECUTIVE C-C COUPLING REACTIONS.

In this study we expected the formation of a furanonaphthoquinone **38** (Figure 10) from the reaction of a 1,4-naphthoquinone with a diketone. Our interest in furan formation was inspired by the biological results of compound **7** shown in Figure 3**.** However, we observed that consecutive C-C bis-coupling reactions occurred resulting in the 2,3-dialkylated naphthoquionones ring structure **39** instead of the anticipated ring cyclisation which would have afforded **38** (Figure 10). The formation of **39** was initially not obvious from the NMR spectra, but was confirmed through mass spectrometry.



38 **39**

Figure 10: The targeted compound **38** and the actual naphthoquinone derivative **39** obtained.

5.3.1 SYNTHESIS OF 1,4-NAPHTHOQUINONE AND JUGLONE ANALOGUES FROM 1,3-DIKETONES

The reactants and substrates for this investigation are depicted in Figure 11 below.

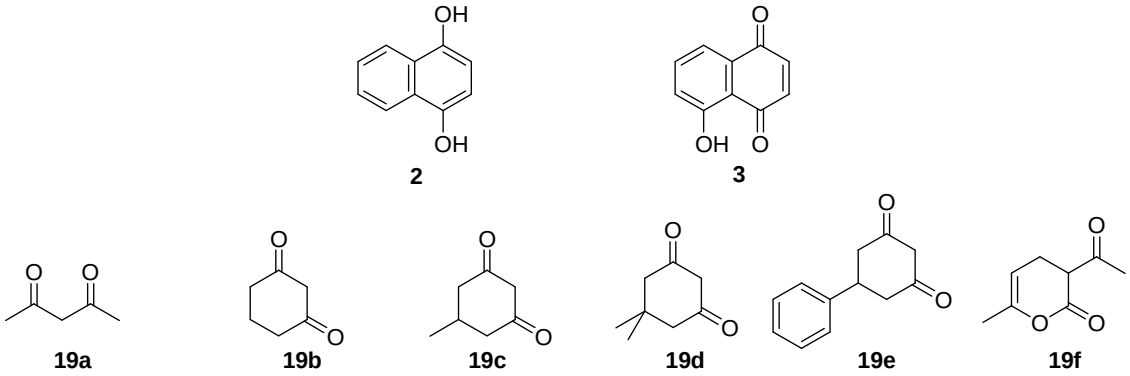
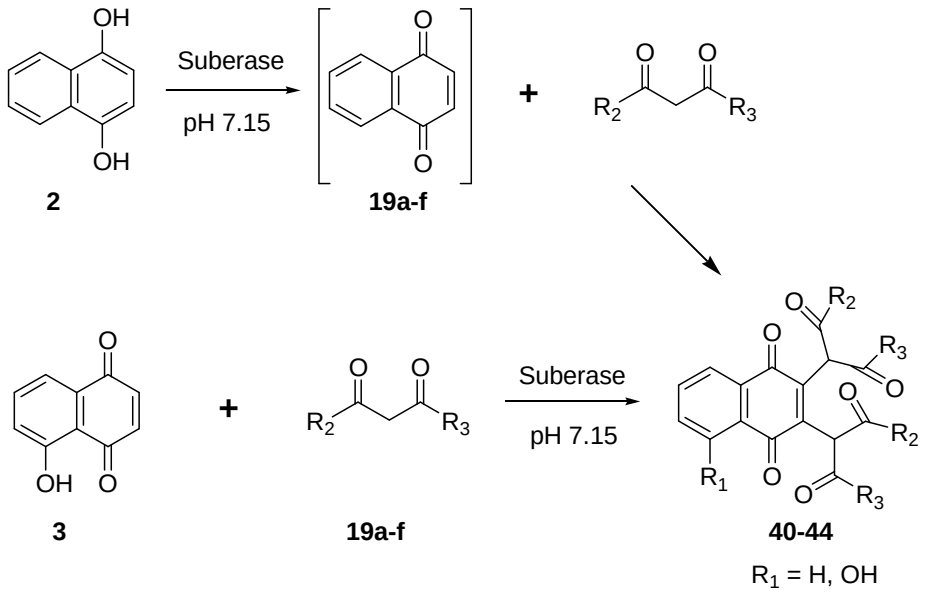


Figure 11. 1,4-Naphthohydroquinone **2**, juglone **3** and the 1,3-dicarbonyls **21a–f** used in this study.

The synthesis of C-C bis-coupled 1,4-naphthquinone derivatives was achieved by reacting one equivalent of the 1,4-naphthohydroquinone **2** with one equivalent of the 1,3 dicarbonyl compounds **19a-f**.

The reaction for the synthesis of C-C bis-coupled products from the reaction of 1,3-diketones with 1,4-naphthoquinones is depicted in Scheme 6 below.



Scheme 6. Synthesis of C-C bis-coupled 1,4-naphthquinone derivatives

Table 3 shows the results that were obtained when 1,4-naphthohydroquinone **2** and juglone **3** were each reacted with a 1,3-diketone in a 1:1 ratio.

Table 3. The synthesised C-C coupled 1,4-naphthoquinone derivatives **40-44** (yield in parentheses)

Entry	NQ/NHQ	Dicarbonyl	Reaction time (h)	Product(% Yield)
1	**2**	**19i**	24	**40** (50)
2	**3**	**19i**	24	**41** (52)
3	**2**	**19c**	24	**42** (55)
4	**3**	**19d**	24	**43** (50)
5	**3**	**19e**	24	**44** (53)

Method - Suberase® (4.0 mL), diketone (1.0 mmol), naphthoquinone/naphthohydroquinone (1.0 mmol, 1 eq), phosphate buffer (8.0 mL, 0.10 M, pH 7.15), stirring time at 40°C = 24 h; NQ = naphthoquinones; NHQ = naphthohydroquinone.

The products were obtained in moderate yields (50-53%) as can be seen from the results in Table 3. After discovering that the expected furan was not formed it was decided to increase the quantity of the diketone to improve the yield of the bis-coupled product. Instead of a 1:1 diketone:naphthoquinone ratio, a a 2:1 ratio was used. It was then observed that the yields increased as shown in Table 4 below.

Table 4. Synthesised C-C coupled naphthoquinone derivatives using a 2:1 stoichiometric ratio of diketone to naphthoquinone.

Entry	Naphthoquinone	Dicarbonyl	Reaction time (h)	Product (%Yield)
1	**2**	**20e**	24	**42** (60)
2	**3**	**20d**	24	**43** (65)

3	**3**	**20e**	24	**44** (73)

Method - Suberase® (4.0 mL), diketone (2.0 mmol), naphthoquinone/naphthohydroquinone (1.0 mmol), phosphate buffer (8.00 mL, 0.10 M, pH 7.15), stirring time at 40 °C = 24 h; ratio of diketone:naphthoquinone is 2:1.

It is evident from the results in Table 4 that an increase in the amount of the 1,3-diketone resulted in a higher yield of product. The structures of the products are shown in Figure 12 below.

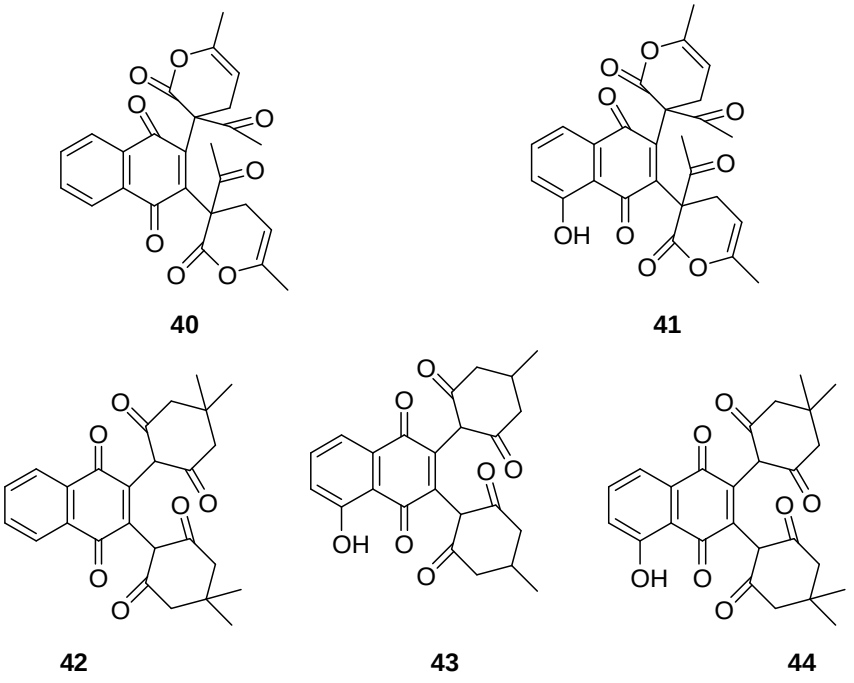


Figure 12: Structures of the C-C bis-coupled 1,4-naphthoquinones **40**-**44** synthesized at 40ºC.

This outcome was a surprise because in the literature most of the reactions between a 1,3-dicarbonyl and a *p*-quinone affords the benzofuran as the product.[170-172] However, Hadjok *et al* reported a similar result to ours for benzoquinone.[180] They reacted a 1,3-diketone with a hydroquinone in a 1:1 ratio and the product formed was also a bis-adduct. They also observed that even when the stoichiometry is changed to a 2:1 ratio the resulting product is still a bis-adduct, but the yield was better. In another example from literature a reaction of a *p*-

benzohydroquinone with a 1,3-diketone resulted in a mixture of both mono- and bis-adduct.[181] An imidazole reacted with a benzoquinone (1:1 ratio) led to the formation of mono-substituted, 2,3-disubstituted and 2,5-disubstitued products. Another interesting example is the domino processes between *p*-benzoquinone that is electrochemically generated from a hydroquinone and 2 equivalents of a cyclic 1,3-dicarbonyl (3-hydroxy-1H-phenalen-1-one). Under specific reaction conditions a bis-adductformed. This adduct could not be isolated and underwent cyclisation to a benzofuran derivative.[182]

In this project, these compounds were the most challenging class of compounds to purify as they seemed to be unstable during purification by silica gel chromatography. In some cases despite observing one spot on TLC, after column purification the product was still impure as could be seen by ^{1}H NMR spectroscopy.

We hypothesised that the acidic nature of silica gel (silicon dioxide) used in silica gel chromatography allowed for decomposition.

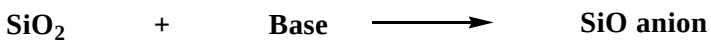


Figure 14: Reaction of silicon dioxide under basic conditions

Mass spectrometry results indicated that the reaction mechanism was that of a C-C bis-coupling. In Figure 15 are two possible naphthoquinone derivatives.

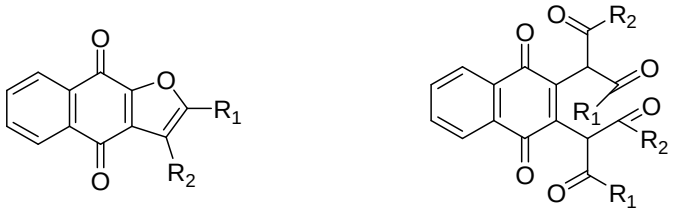


Figure 15: Structures of two possible naphthoquinone derivatives

From the ^{1}H NMR spectra it could not be determined whether a furan ring had formed based on the splitting pattern of peaks. However, when integration was taken into consideration it was evident that a C-C bis-coupling had occurred.

5.4. SYNTHESIS OF 1,4-NAPHTHOQUINONE AND JUGLONE ANALOGUES FROM COUMARINS

We were then interested to determine whether the same reaction would occur with a coumarin **32a-d** when it reacted with a naphthoquinone. The substrates and reactants used are shown in Figure 16 below.

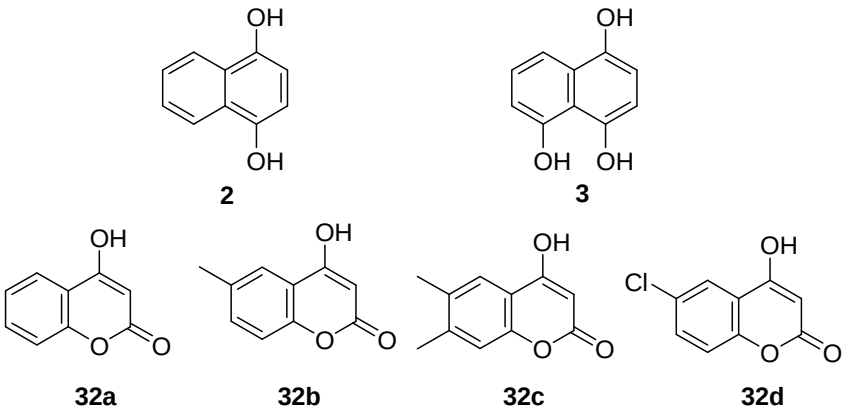
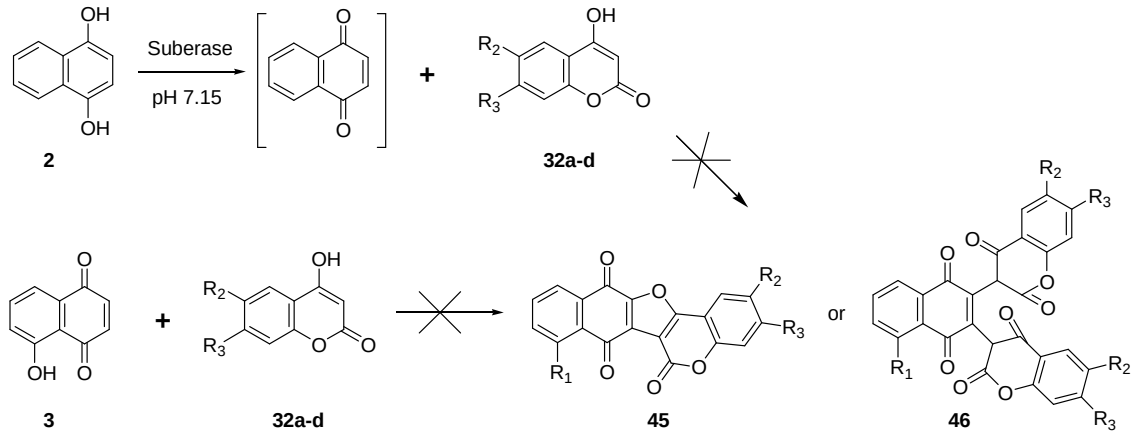


Figure 16: The 1,4-naphthohydroquinone **2**, juglone **3** and coumarins **32a-d**.

As previously mentioned the benzopyrone skeleton **32a-d** is a natural product like benzofuran and has shown to possess both anticancer and antibacterial activities.[139] We followed the same method as discussed in Section 5.4.1 with an intention of forming either compounds **45** or **46** (Scheme 7).



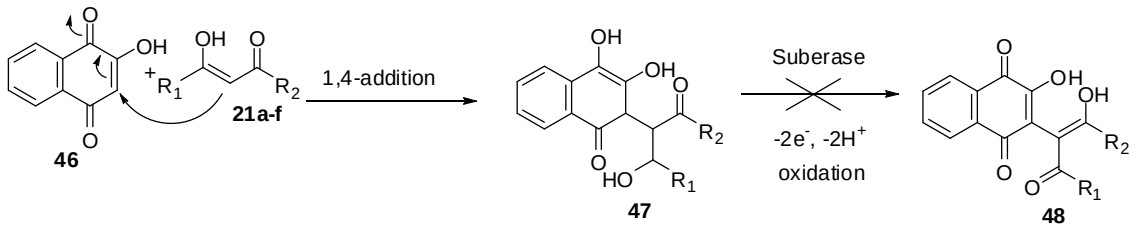
Scheme 7. Synthesis of coumarin derivatives

The attempted synthesis was such that 1,4-naphthohydroquinone **2** (1.0 mmol) and juglone **3** (1.0 mmol) was added to laccase (Suberase) with one equivalent of the coumarin (1.0 mmol) in a mixture of DMF (1.0 mL) and phosphate buffer (0.10 M, pH 7.15) in a test tube. The reaction mixture was stirred in an open vessel (providing oxygen) at 40°C. Further attempts were at room temperature with DMF, and then without DMF.

The reactions (Figure 16) were conducted initially at room temperature and then at 40ºC. These reactions were unsuccessful since neither a novel compound nor the desired product was formed. From TLC it appeared that a number of new spots had formed but after column chromatography no characterisable product could be isolated. A possible reason for the unsuccessful reaction is that the coumarin may not have been sufficiently soluble in the reaction mixture when compared to the 1,3-diketone.

5.5 LACCASE-MEDIATED SYNTHESIS OF 2-HYDROXY-1,4-NAPHTHOQUINONE ANALOGUES UTILIZING 1,3-DIKETONES AS MICHAEL DONOR SUBSTRATES

In Section 5.2.1 we explained how we achieved the synthesis of menadione analogues. We were interested in a substrate having an electron-withdrawing group at C-2 of the quinone and lawsone (2-hydroxy-1,4-naphthoquinone) was a suitable substrate. The reaction of lawsone **46** with diketones **21a-f** was investigated (Scheme 9). In the presence of lawsone **46** it was anticipated that a 1,4-addition would be the first step which would then be followed by oxidation to afford **48** as shown in Scheme 9 below.

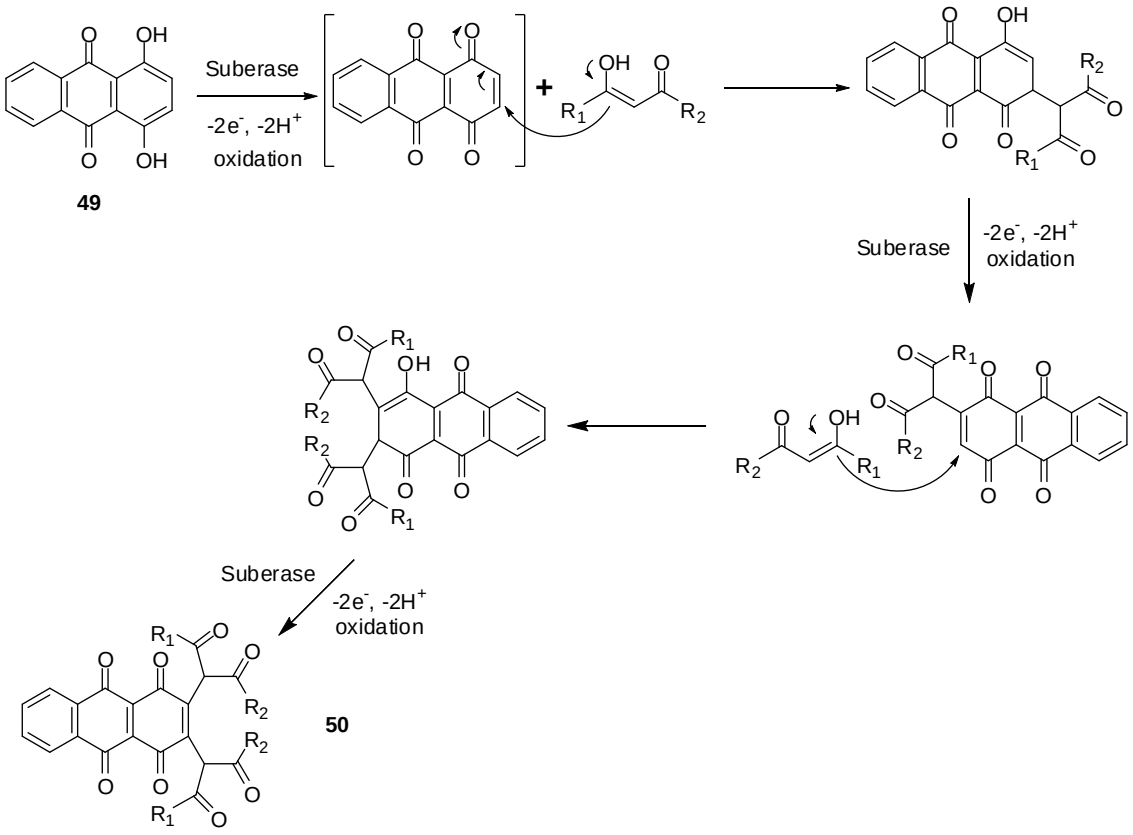


Scheme 9: Proposed mechanism for the formation of C-C bis-coupled 1,4-naphthoquinones.

The reaction was unsuccessful at both room temperature and 40°C. The co-solvent, DMF, was used at both temperatures for the reaction, the pH was also adjusted from 7.15 to 4.50 in order to increase the acidity in the reaction mixture but still we were unable to obtain the desired product using Suberase.

5.6 SYNTHESIS OF ANTHRACENEDIONE ANALOGUES FROM 1,3-DIKETONES

Our interest in synthesising anthracenedione analogues was inspired by drugs such as doxorubicin and mitoxantrone. We were interested in investigating the reaction of the 1,4-dihydroxyanthracene-9,10-dione **49** with each of the 1,3-diketones **21a-f**. A possible product of the reaction is shown in Scheme 10 below.

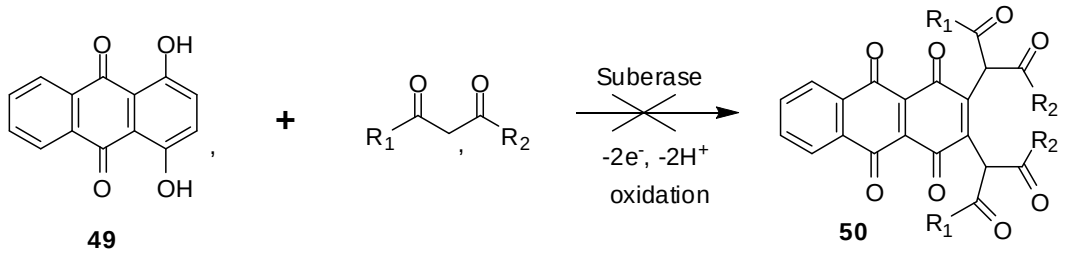


Scheme 10: Proposed mechanism for the formation of possible C-C bis-coupled 1,4-dihydroxyanthracene-9,10-dione analogues from1,3-diketones.

Initially we used Method C, this involves the addition of DMF to the reaction mixture as a co-solvent to improve the solubility of the organic substrates. The number of equivalents of the 1,3-dicarbonyl was also increased so that a ratio of 1,3-dicarbonyl to catechol was 4:1. These reactions were conducted for 42 h. To react 1,4-dihydroxyanthracene-9,10-dione **49** with the 1,3-

diketones **21a-f**. After 24 h it was observed that only starting materials were present in the reaction vessel. Extending the reaction time to 72 h and subsequently elevating the reaction temperature to 40ºC failed to generate any products. The reaction was also investigated at 40 ºC at pH 4.5 while all the other conditions were kept the same.

After much experimentation we could not get the 1,4-dihydroxyanthracene-9,10-dione **49** to react with a diketone (Scheme 11).



Scheme 11. Synthesis of 1,4-dihydroxyanthracene-9,10-dione analogues from 1,3-diketones.

It might be due to the fact that the 1,4-dihydroxyanthracene-9,10-dione **49** is not sufficiently soluble in the reaction mixture or it could be too large for the active site of the laccase, Suberase, to oxidise to the corresponding quinone.

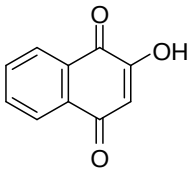
5.7 CONCLUSION

All the synthesised compounds in this chapter are novel: 7 menadione derivatives **22-29** from 1,3-diketones, 4 menadione derivatives **32-35** from coumarins and 5 C-C bis-coupled 1,4-naphthoquinones derivatives **40-44** from 1,3-diketones.

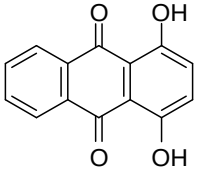
Our interest in naphthoquinones was inspired by the the quinone moiety which posseses a range of biological activities including both anticancer and antibacterial activities.[161]

The reaction of menadione with the 1,3-diketones and coumarins at 40°C at pH 7.15 using DMF as a co-solvent affords C-C coupled naphthoquinone derivatives substituted at position

3. The reaction of 1,4-naphthohydroquinone and juglone (well-known compounds with antibacterial activity) with 1,3-diketones produced unexpected C-C bis-coupled 1,4-naphthoquinone derivatives. These compounds showed some instability and degraded on a silica-gel column. The degradation was attributed to the acidic nature of silica (silicon dioxide) which can catalyse the degradation. Lawsone **46** and 1,4-dihydroxyanthracene-9,10-dione **49** did not react with the 1,3-diketones.



46



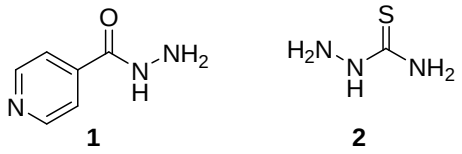
49

Figure 19: Substrates for which no reaction was observed with Suberase.

SCHIFF-BASE DERIVATIVES

6.0 INTRODUCTION

In light of the previous chapters, we were interested in synthesising derivatives of the first-line anti-TB drug, isoniazid **1**, as well as thiosemicarbazide **2** derivatives (Figure 1) from the previously synthesised 5,6-dihydroxylated benzo[*b*]furans (Chapter 3). The goal of these derivatizations was to develop drugs directed at multiple targets and to improve the biological activity against both cancer and bacteria. The detail of the synthetic approach is in the section that follows.



1 **2**

Figure 1: Structures of isoniazid **1** and thiosemicarbazide **2**.

The emerging area of multi-target drug discovery is one of increasing interest to members of the drug discovery community.[181] The potential advantages of drugs that modulate several targets are that there could be an improved balance of efficacy in addition to safety when compared to single target agents.[181] A number of drugs have been successfully synthesised that possess dual inhibitory activity, which is observed when a drug is biologically active against two targets due to its structural design. For example, Donepezil **3**, Galanthamine **4**, and Rivastigmine **5** (Figure 2) are drugs that can be used to treat Alzheimer's disease which is an age-dependent progressive brain disease that slowly destroys memory and thinking skills and eventually the ability to carry out the simplest tasks.[182-183] The main cause is not known yet but the disease is associated with a decrease in hippocampal acetylcholine (Ach)

in the cholinergic system. Acetylcholinesterase (AChE) is responsible for the hydrolysis of acetylcholine in the synaptic cleft.[184] The three drugs **3**-**5** (Figure 1) are AChE inhibitors and are therefore helpful in increasing the level of acetylcholine in the damaged cholinergic neurons. This, however, increases the butyrylcholinesterase (BuChE) activity that causes hydrolysis of acetylcholine in a new way.[185]

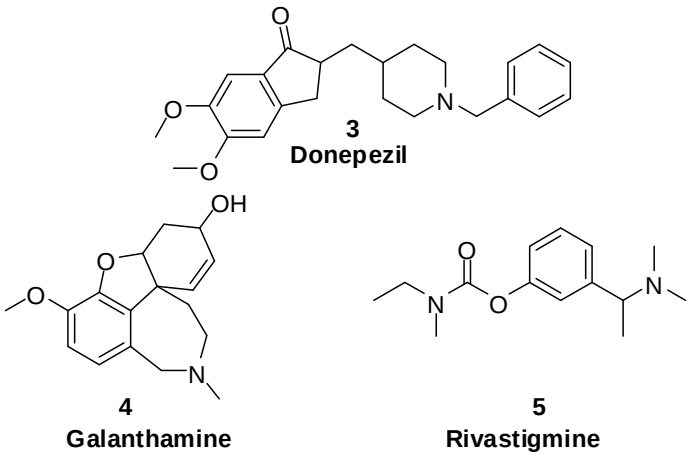


Figure 2: Three well known AChE inhibitors.

As a result the design and synthesis of dual AChE/BuChE inhibitors were considered.[186,187] Since benzyl pyridinium derivatives **6** have previously been reported to be excellent acetylcholinesterase inhibitors, while coumarin moiety **7** has been previously reported to possess anticholinesterase activity they were linked (Figure 3) in order to investigate the dual effect.[188,189]

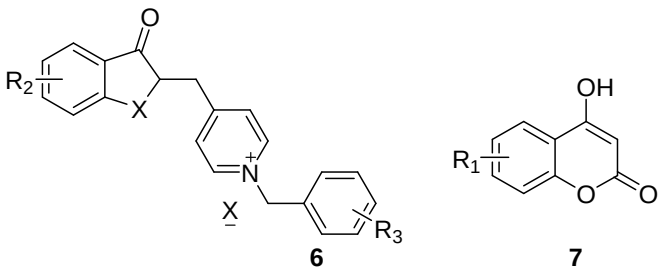


Figure 3: Structural framework of potent anticholinesterase **6** and antiacetylcholinesterase **7** inhibitors.

An assay for inhibitory activity was then performed using Donepezil **3** as a positive control. The derivatives were able to inhibit both acetylcholinesterase and butyryl cholinesterase. Compound **8** showed superior ability (0.11 nm) when compared to the control **3** (14 nm).

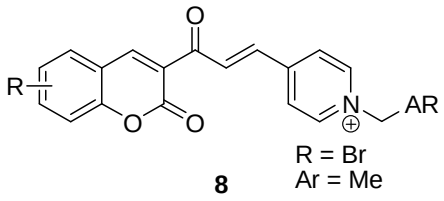


Figure 4: Structure of a potent derivative.

Another example of multi-target drugs is that of histamine receptors. Histamine is an organic compound that is involved in a local immune response and also regulates physiological activities. The histamine receptors belong to class A of the superfamily of the G-protein-coupled receptors (GPCRs).[190] These receptors are involved in the inflammatory response and the response to foreign pathogens, they are produced by basophils and mast cells. H_1R mediates numerous pathophysiological responses to histamine such as vasodilation via nitric oxide release, increase in capillary permeability, and contraction of smooth muscles in the gut and bronchi.[190] The first H_1R blockers were described as 'antihistamines' more than 70 years ago and are now widely used in the treatment of allergic conditions.[191] H_2R is mostly

associated with simulation of gastric acid and ulcers.[192] The development of H_2R in the 1970s revolutionised the treatment of gastric and duodenal ulcers.[192] The development of a combined H_1R and H_2R antagonist **9** was also accomplished (Figure 5).

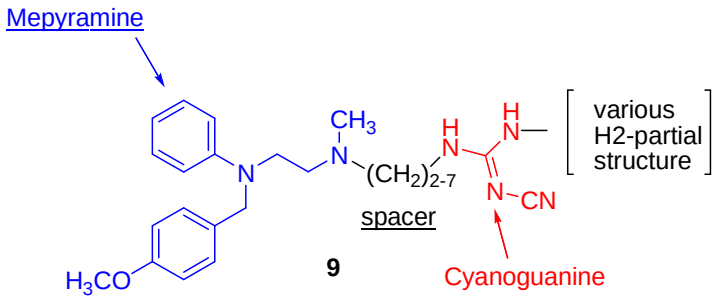


Figure 5: The structure of a spacer-linked combined H1/H2 receptor antagonist **9**.

The result is a combination of Mepyramine (H_1R antagonist) through a spacer to cyanogaunine (H_2R antagonist) and results in dual activity.[190] It is used as an as anaesthetic in operations to prevent hypertensive reactions to drugs. It can also be used in cancer chemotherapy and for the treatment of skin diseases.[191-198]

6.1 ISONIAZID DERIVATIVES

Schiff-base compounds possess a number of biological activities. For example, **10** is an intestinal antiseptic while **11** and **12** (Figure 6) exhibit antibacterial activity against both *S. aureus* ATCC 29213 and *M. tb* H37Rv.[198]

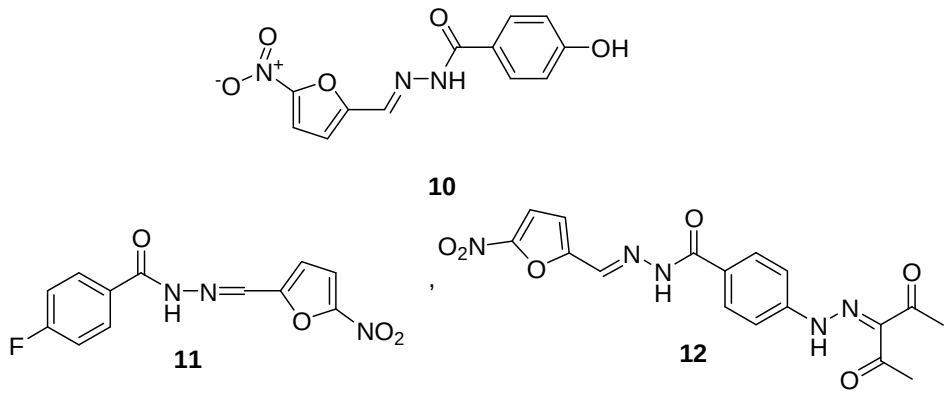


Figure 6: Schiff-base compounds exhibiting biological activity.

Isoniazid **8**, also known as isonicotinylhydrazine, is a first-line prodrug for TB treatment. This prodrug activates the KaTG enzyme to form the INH-nicotinamide adenine dinucleotide (NAD) adducts as previously discussed in Chapter 1. The emergence of multi-drug and new TB cases led to renewed research interest in new anti-TB treatments as well as the idea of derivatization of the clinically available drugs.[199] Isoniazid derivatives are considered an important class of compounds for the development of new drugs, and a number of isoniazid derivatives have been successfully synthesised through the Schiff-base reaction of an isoniazid with Carl-Mannich bases. [200,201] The latter are *β*-amino-ketones that are formed in the reaction of an amine, formaldehyde/aldehyde and an enolizable carbonyl compound. All the compounds were then evaluated for antimicrobial activity against *Bacillus substilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans and Candida gabrata*.[202] Amoxicillin, an antibiotic, was used as the positive control in the study. Compounds **13-18** (Figure 7) exhibited better growth inhibitory activity against all strains when compared to amoxicillin as shown in Table 1 below.

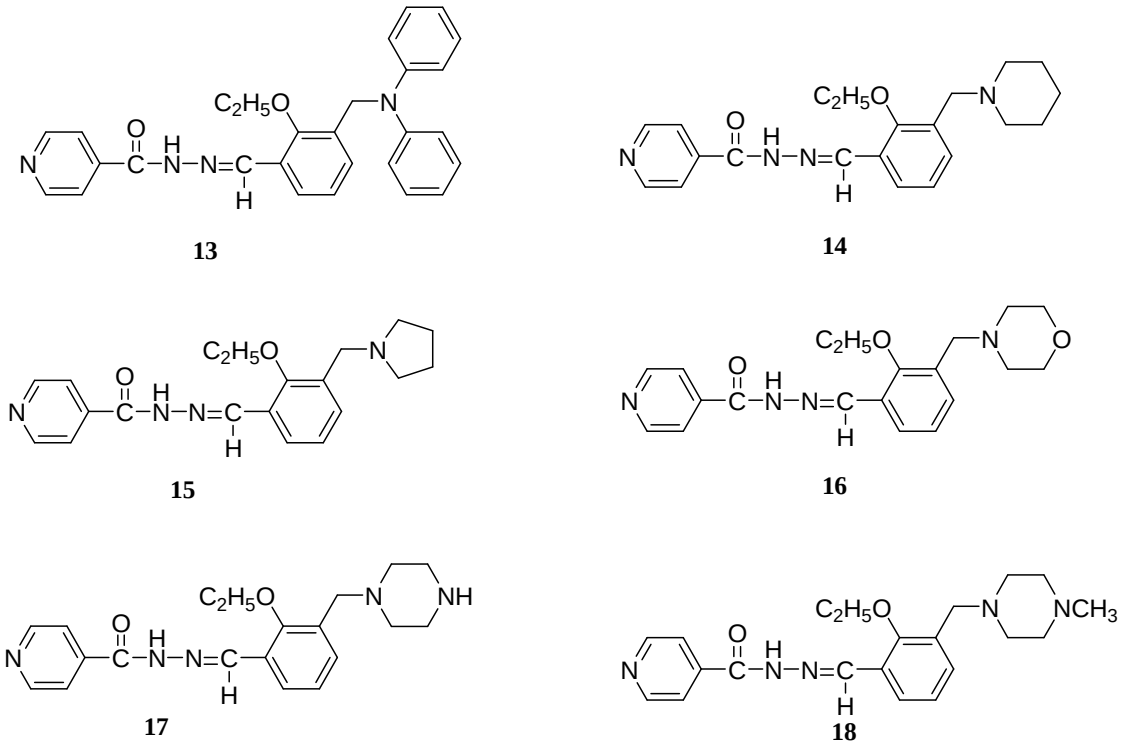


Figure 7: The biologically active Schiff-base derivatives **13-18**.[202]

Table 1: Zone of inhibition (mm) of the Schiff-base derivatives **13-18**

Compound	Concentration of the compound(µg/ml)	*B. substilis*	*S. aureus*	*E.coli*	*P. aeruginosa*	*C. albicans*	*C. gabrata*
13	100	26	31	22	32	21	22
14	100	23	25	20	25	16	16
15	100	22	26	20	27	18	19
16	100	27	32	22	31	21	21
17	100	27	34	23	33	24	23
18	100	26	33	22	32	22	20
Amoxicillin	50	25	30	21	30	-	-

The biological activity exhibited by the isoniazid Schiff-base derivatives **13-18** led us to pursue the synthesis of isoniazid derivatives of the 5,6-dihydroxylated benzo[*b*]furans discussed in Chapter 3.

6.2 RESULTS AND DISCUSSION

6.2.1 SYNTHESIS

The compounds that were used in this study, isoniazid **8** and the 5,6-dihydroxylated benzo[*b*]furans **19–27** (the synthesis of which was described in Chapter 3) are depicted in Figure 8.

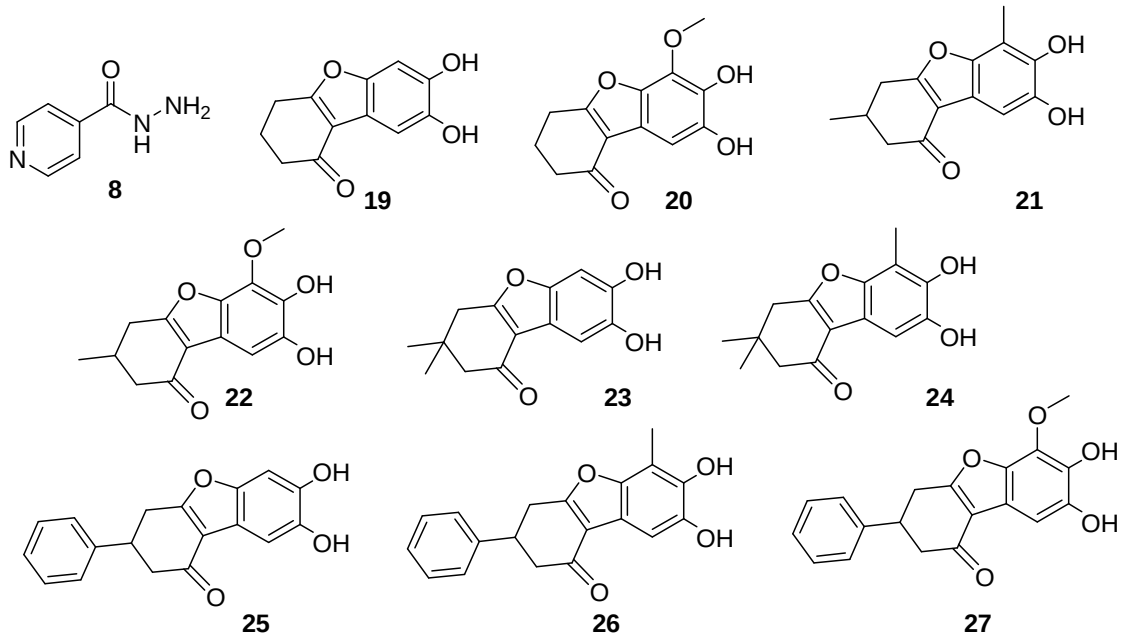
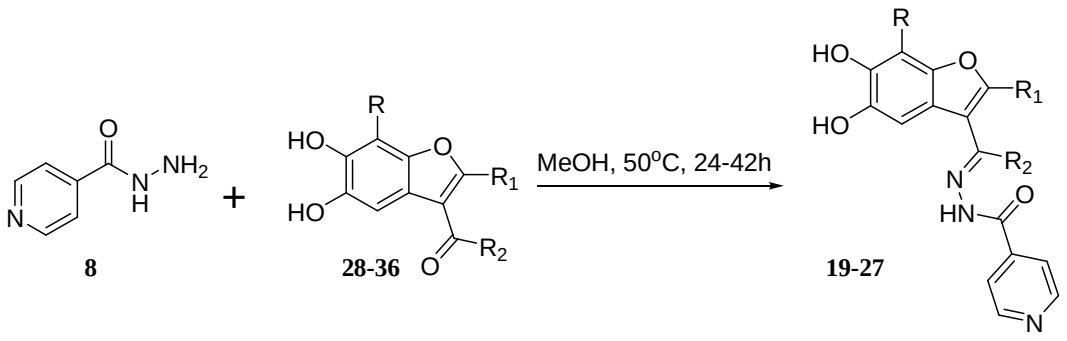


Figure 8. Isoniazid **5** and the 5,6-dihydroxylated benzo[*b*]furans **19-27** used in this study.

The synthesis of the isoniazid derivatives was conducted by reacting one equivalent of isoniazid **8** with one equivalent of each of the 5,6-dihydroxylated benzo[*b*]furan (**16-23**) and stirring in methanol under reflux in the presence of glacial acetic acid for 24 hours as shown in Scheme 1 (Method C).



Scheme 1: Synthesis of isoniazid derivatives of 5,6-dihydroxylated benzo[*b*]furans.

Acetic acid was added in order to make the hydroxyl group of the hemiamine intermediate a good leaving group. This intermediate is depicted in the mechanism of the reaction below. The second reason was to make the alpha carbon to be more electrophilic and susceptible to the lone pair of the nitrogen group. The reaction was monitored by TLC and the disappearance of the 5,6-dihydroxylated benzo[*b*]furan was followed. The reaction afforded an imine that migrated further up the TLC plate in comparison to the starting material. The resulting product was purified by flash chromatography. In some cases the product required further purification by either washing with EtOAc or recrystallizing from a mixture of MeOH and EtOAc. The yields of the reactions are shown in Table 1, and the structures of the products **19-27** are shown in Figure 9.

Table 1. The isoniazid derivatives **19-27** synthesised in methanol at 50°C.

Entry	benzo[*b*]furan	Reaction time [h]	Product (% Yield)
1	**28**	24	**19** (65)
2	**29**	24	**20** (63)
3	**30**	44	**21** (70)
4	**31**	24	**22** (76)
5	**32**	24	**23** (72)
6	**33**	42	**24** (70)
7	**34**	24	**25** (70)
8	**35**	42	**26** (74)
9	**36**	24	**27** (74)

Method C – isoniazid (1.0 mmol, 1 eq), 5,6-dihydroxylated benzo[*b*]furan (1.0 mmol), methanol (10.0 mL), stirring time at 50°C = 24-44 h.

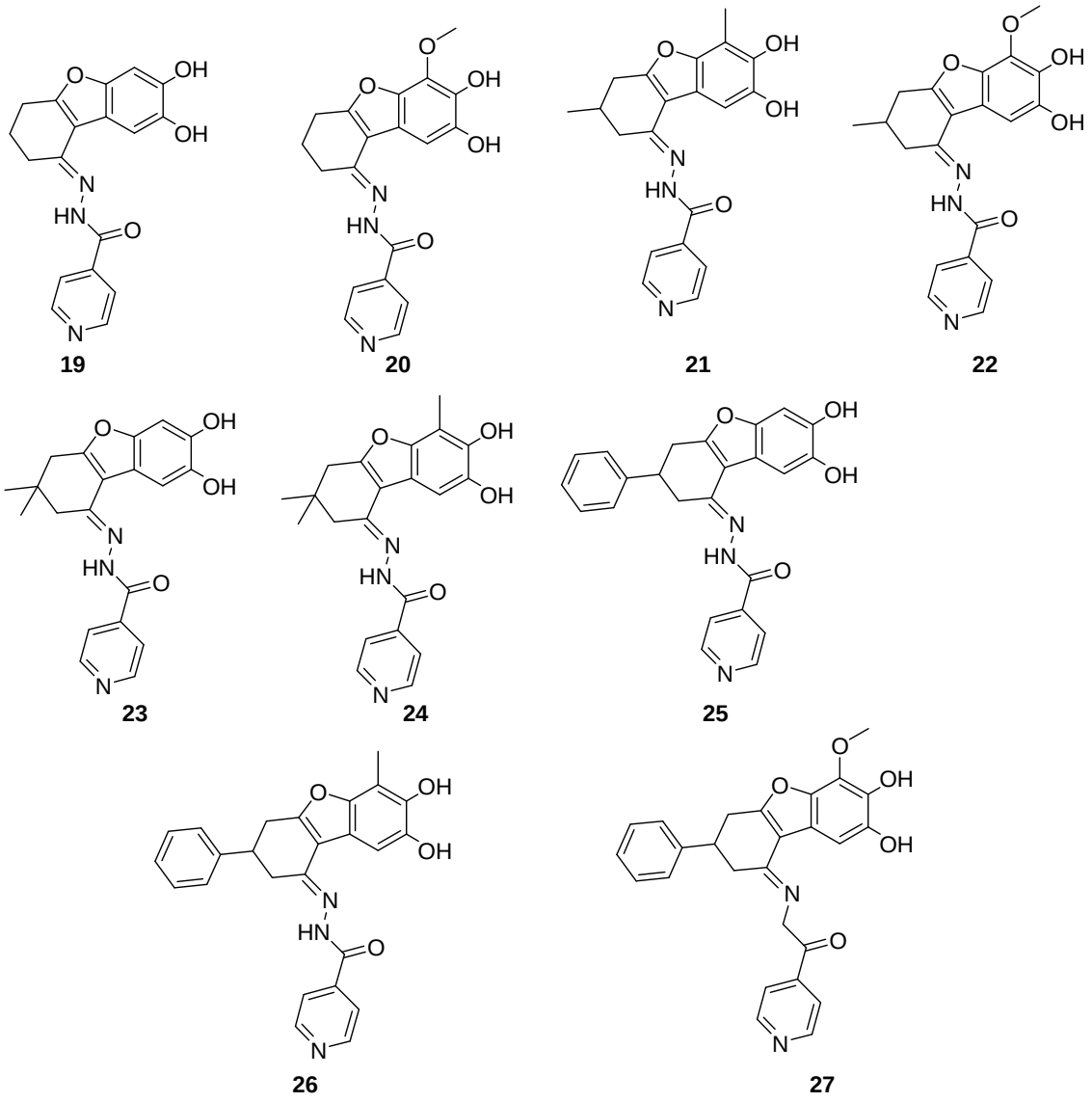
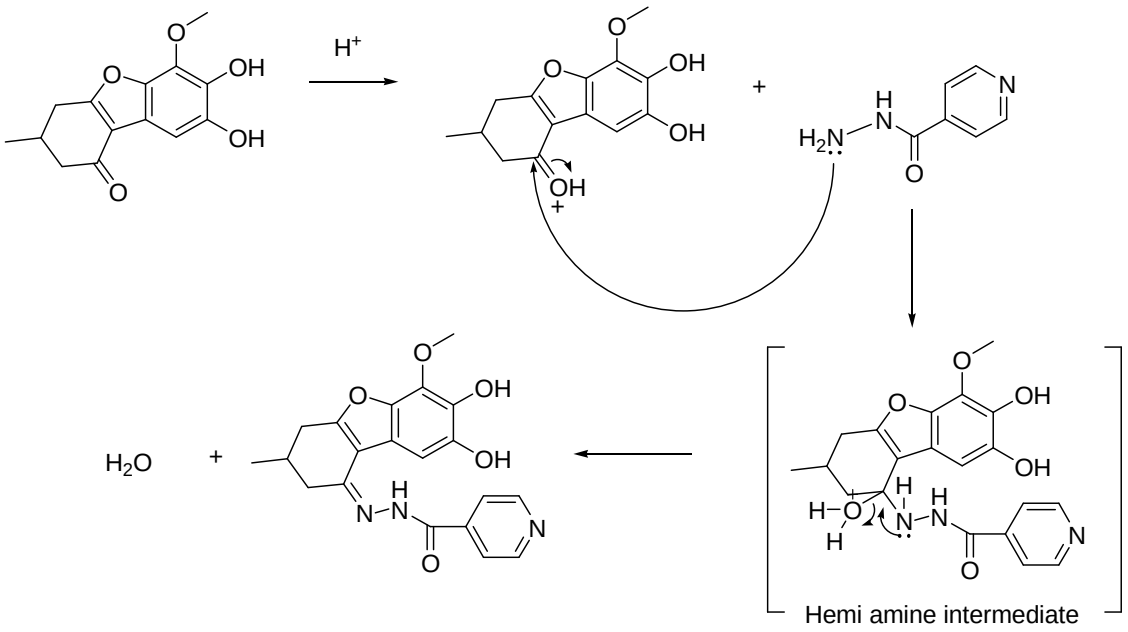


Figure 9: The structures of the isoniazid derivatives **19**-**27**.

6.2.2 MECHANISM OF FORMATION OF SCHIFF-BASE PRODUCTS

The Schiff-base product was obtained from the condensation reaction between a carbonyl group and and the hydrazine amino group via an intermediate hemiaminal, generating water as a by-product as shown in Scheme 2.



Scheme 2: Mechanism for synthesis of isoniazid derivatives of 5,6-dihydroxylated benzo[*b*]furans.

The isoniazid derivatives were successfully synthesised using the Schiff-base reaction in 63-76% yield. The highest yield that was obtained was 76% for **22** (Entry 4, Table 1) and the lowest was 63% for **20** (Entry 2, Table 1).

6.3 THIOSEMICARBAZIDE DERIVATIVES

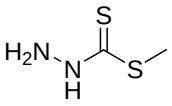
Thiosemicarbazide **2** is a versatile intermediate which is mostly used for preparing various heterocyclic derivatives such as **37**, **38**, and **39** among others (Figure 10).[203-206]



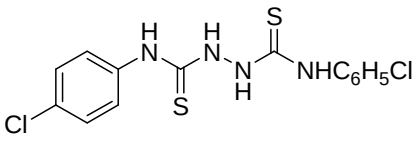
2 **37** **38** **39**

Figure 10: Thiosemicarbizide **2** and heterocyclic derivatives **28-30** prepared from it.

Thiazole, which contains both sulfur and nitrogen, is a component of the vitamin, thiamine B_1. Thiosemicarbazide **2** has displayed a wide range of biological activities such as anticancer, antiHIV, antitubercular, antiviral, antitumor, antiprotozoal, anticonvulsant, antidepressant, antimalarial, antifungal, pesticidal and antibacterial activity, antimicrobial, analgesic, sodium channel blockers and antiallergic agents.[207-210] It has also found application in drug development for the treatment of central nervous system disorders. [210] Methyl hydrazine carbodithioate **31** and hydrazine-carbothionamide analogue **32**, which are both thiosemicarbazide derivatives, possess antibacterial activity against Gram positive and Gram negative bacteria (Figure 11). Compound **32** has activity that is as potent as that of the standard, that was used. Therefore we thought it will be useful to incorporate thiosemicarbazide into the 5,6-dihydroxybenzo[*b*]furan.



31



32

Figure 11: The hydrazine derivatives that **31** and **32** exhibited antibacterial activity.

6.4 RESULTS AND DISCUSSION

6.4.1. Synthesis

The thiosemicarbazide **2** and the 5,6-dihydroxylated benzo[*b*]furans **19**, **20**, **22**, **25-27**, **33** and **34** (the synthesis of which was described in Chapter 3) used in this study are depicted in Figure 12 below.

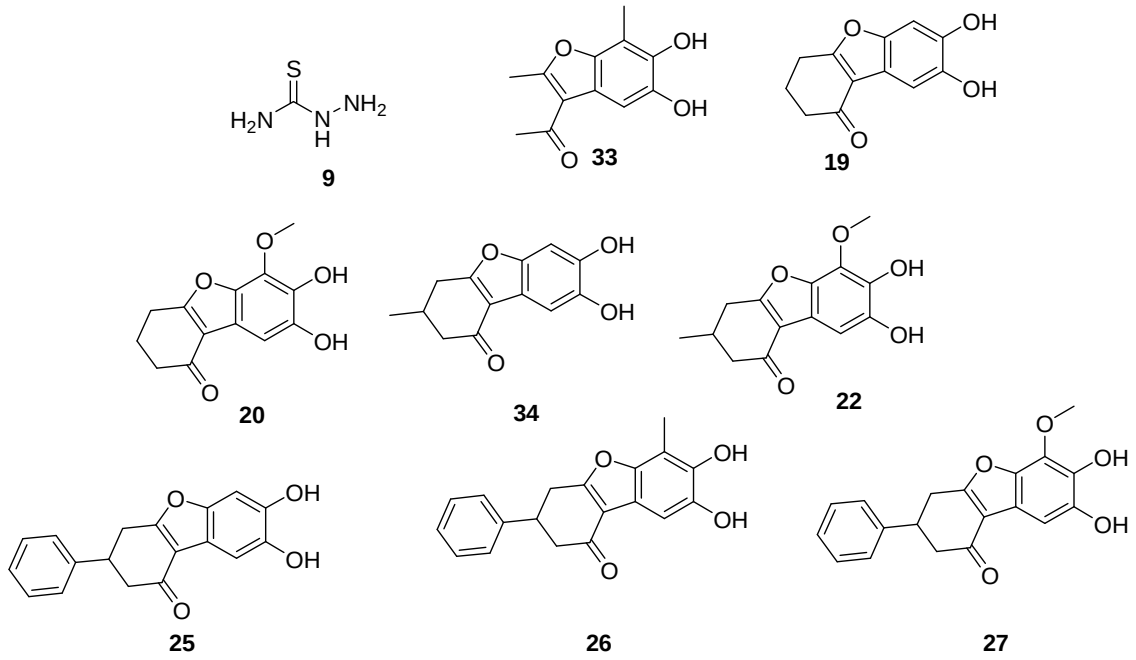
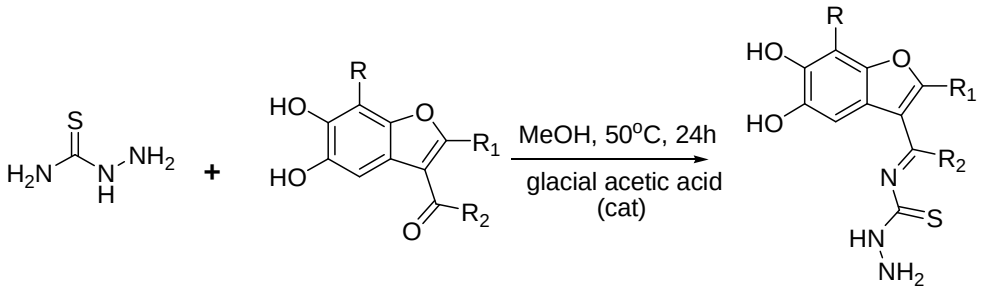


Figure 12: The thiosemicarbazide **2** and the 5,6-dihydroxylated benzo[*b*]furans **19**, **20**, **22**, **25-27**, **33** and **34** used in this study.

The synthesis of the thiosemicarbazide derivatives entailed reacting one equivalent of each of the 5,6-dihydroxylated benzo[*b*]furans **19**, **20**, **22**, **25-27**, **33** and **34** with one equivalent of the thiosemicarbazide **9** at 50ºC in methanol for 24 hours (Method C) as shown in Scheme 2. This was achieved in the presence of a catalytic amount of glacial acetic acid. The products were purified by flash chromatography.



6 **19,20,22,25-27,33, 34** **35-42**

Scheme 2: General synthesis of the thiosemicarbazide derivatives **35-42**.

The synthesis of the thiosemicarbazide derivatives was accomplished by employing the Schiff-base reaction and afforded the products in 66-80% yield. The highest yield that was obtained was 80% for **38** (Entry 4, Table 4) and the lowest was 66% for **35** (Entry 1, Table 4). The presence of a signal for a primary amine (NH_2) at about 4.50 ppm and a secondary amine (NH) at 8.60 ppm in the ^1H NMR spectrum, provided evidence for the structure of the compounds (Figure 12).

Table 4: The thiosemicarbazide derivatives **35-42** synthesised at 50°C in methanol for 48 h.

Entry	benzo[*b*]furans	Reaction time (h)	Product (%Yield)
1	**33**	**24**	**35** (66)
2	**19**	**24**	**36** (68)
3	**20**	**24**	**37** (76)
4	**34**	**48**	**38** (80)
5	**22**	**48**	**39** (78)
6	**25**	**48**	**40** (73)
7	**26**	**48**	**41** (72)
8	**27**	**48**	**42** (70)

Method C – thiosemicarbazide (1.0 mmol, 1 eq), 5,6-dihydroxylated benzo[*b*]furan (1.0 mmol), methanol (10.0 mL), stirring time at 50°C = 24 -48 h.

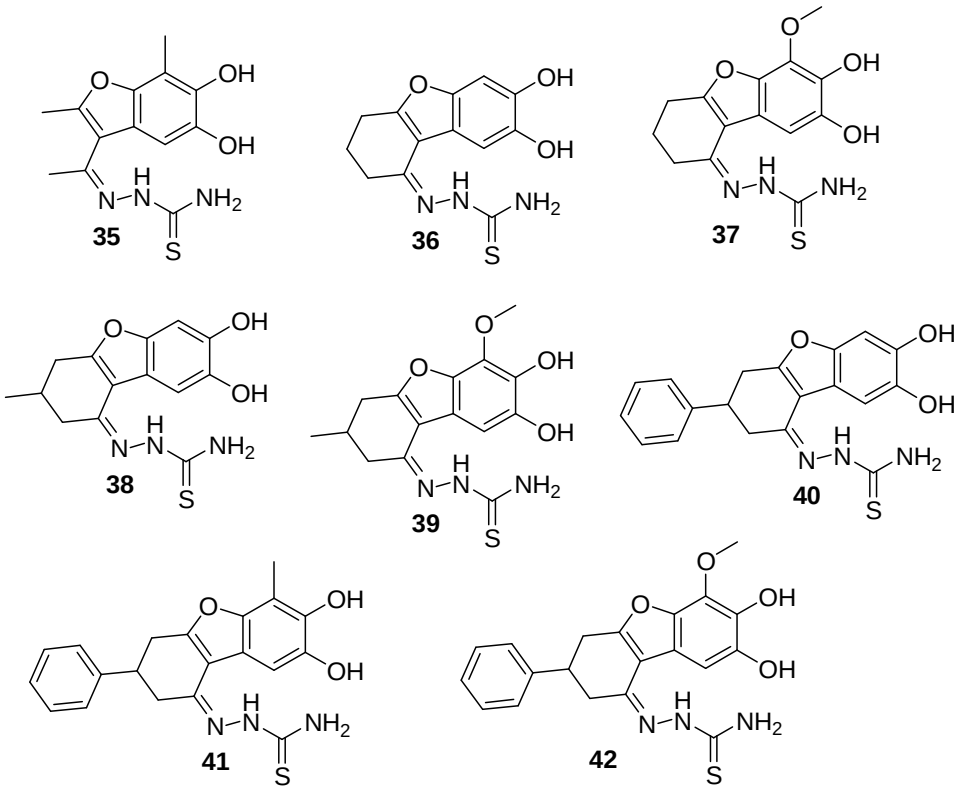


Figure 12: The structures of the synthesized thiosemicarbazide derivatives **35-42**.

6.5 CONCLUSION

This part of the project was inspired by the idea of multi-target drug discovery. The focus is on potential drug candidates that can modulate several targets which could improve efficacy and safety when compared to single target agents. Our goal was to react benzofurans with the well-known first-line anti-TB drug, isoniazid, as well as thiosemicarbazide through a Schiff-base reaction. The purpose of this derivatisation was to improve the biological activity against cancer and bacteria. All the synthesised compounds (**9** isoniazid derivatives **19-27** and 8 thiosemicarbazide derivatives **35-42)** are novel with potential increased potency when compared with our 5,6-dihydroxybenzo[*b*]furans. The results of the biological screening of the compounds are discussed in Chapter 7.

BIOLOGICAL EVALUATION

7.0 BIOLOGICAL EVALUATION

The synthesized compounds, as described in Chapters 3 to 6, were screened for their ability to inhibit cell growth of renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer cell lines. The cell lines were obtained from the National Cancer Institute (NCI) in a collaborative research program between the South African Council for Scientific and Industrial Research (CSIR) and the NCI.

The synthesized compounds were initially screened against four different bacterial strains i.e. *M. smegmatis* MC^2155, methicillin-sensitive *Staphylococcus aureus* (MSSA) 32710, *Bacillus substilis* 168 and *E. coli* DH10B and then against *M. tb*. The minimum inhibitory concentrations (MIC) was recorded. *M. smegmatis* was used as a model for further screening against *M. tb*. All the strains were from the International Centre for Public Health (ICPH), New Jersey, USA. This work was performed over a three month period during my PhD through a Fogarty Fellowship from the Aurum Institute that was awarded to myself, Tozama Qwebani-Ogunleye. The anticancer screening was done at the CSIR.

7.1 EVALUATION OF THE ANTICANCER ACTIVITY

We will first discuss the anticancer activity of the screened compounds. Screening was conducted against renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer cell lines using the Sulforhodamine B (SRB) assay to determine the growth

inhibitory effects of the compounds. These cell lines have been used routinely at the U.S. National Cancer Institute for screening for new anticancer agents and were derived from tumors that have different sensitivities to chemotherapeutic drugs. Etoposide, a well-known anticancer agent, was used as a positive control. It is known to be an inhibitor of topoisomerase, particularly topoisomerase II, and aids in DNA unwinding which causes the DNA strands to break. The results of a five dose screening were reported in three parameters: 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI) and the lethal concentration that kills 50% of cells (LC_{50}). The 5,6-dihydroxylated benzo[*b*] furans were the first compounds to be screened and are shown in Figure 2 below.

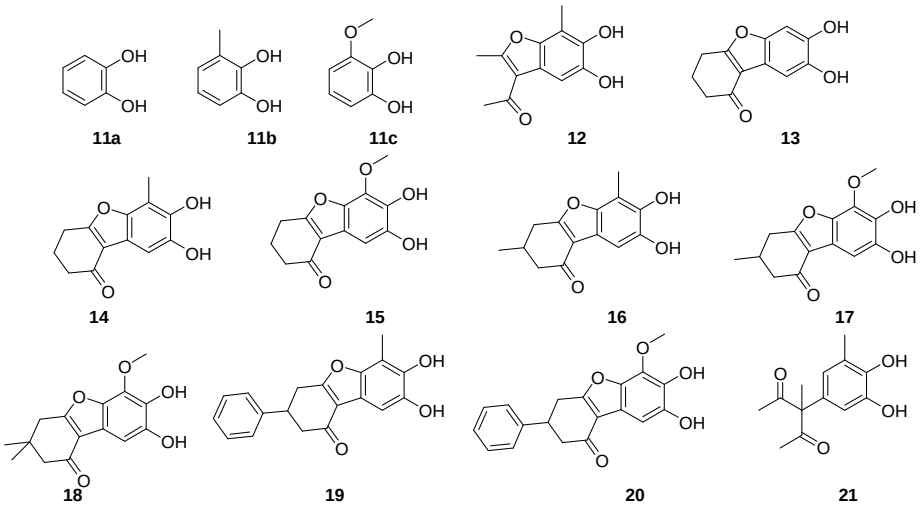


Figure 2: 5,6-Dihydroxylated benzo[*b*] furans screened for anticancer activity.

The screening results are shown in Table 1 and it is evident that several compounds exhibited potent cytostatic effects.

Table 1: *In vitro* anticancer screening of the compounds against renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer cells expressed as GI_{50}, TGI and LC_{50} values (µM).

Entry	Compound	Renal (TK10)			Melanoma (UACC62)			Breast (MCF7)			Cervical (HeLa)		
		GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
1	**11a**	68.02	>100	>100	45.00	>100	>100	52.41	>100	>100	[a]	[a]	[a]
2	**11b**	38.14	75.72	>100	**15.18**	46.22	77.26	**27.34**	64.31	>100	[a]	[a]	[a]
3	**11c**	51.63	96.98	>100	**23.64**	50.26	76.87	**27.55**	60.68	93.81	[a]	[a]	[a]
4	**12**	41.05	69.94	98.82	32.74	60.84	88.94	35.10	66.12	97.14	60.70	>100	>100
5	**13**	65.00	>100	>100	37.73	68.65	99.57	35.53	64.19	92.85	49.09	>100	>100
6	**14**	**24.25**	54.74	85.23	**23.63**	51.06	78.49	**25.97**	61.87	97.78	44.55	>100	>100
7	**15**	52.47	>100	>100	**6.04**	33.15	>100	**15.85**	54.74	93.63	54.46	>100	>100
8	**16**	**16.56**	55.32	94.09	**6.50**	33.85	74.35	**25.55**	68.82	>100	**25.93**	>100	>100
9	**17**	**26.54**	51.65	76.75	**0.78**	**18.32**	60.32	**8.79**	48.79	95.11	**26.68**	69.67	>100
10	**18**	**18.69**	46.14	73.59	**6.79**	**28.93**	66.27	**28.44**	60.90	93.35	34.37	62.15	89.93
11	**19**	**18.60**	48.25	77.90	**9.76**	>100	>100	**17.96**	60.79	>100	47.28	>100	>100
12	**20**	**9.73**	47.15	85.35	**0.77**	**23.60**	64.58	**9.30**	46.93	86.93	**22.32**	73.19	>100
13	**21**	86.99	>100	>100	51.29	>100	>100	68.03	>100	>100	[a]	[a]	[a]
14	Etoposide	**7.19**	49.74	>100	**0.89**	52.71	>100	**0.56**	>100	>100	**3.56**	40.18	87.54

Inactive, I: GI_{50} or TGI > 100 µM; weak activity, w: > 30 µM GI_{50} or TGI < 100 µM; moderate activity, m: < 30 µM GI_{50} or TGI > 10 µM; potent activity, p: GI_{50} or TGI < 10 µM. [a] Not screened.

The GI_{50} values of the catechols **11a–c** and selected benzo[*b*]furans were compared to that of etoposide, a well-known anticancer drug. Screening against the TK10 cell line showed that only compound **20** had potent activity (GI_{50} = 9.73 µM, Entry 12), which was not as good as that of etoposide (GI_{50} = 7.19 µM, Entry 14). Most of the compounds exhibited potent growth inhibitory activity against the UACC62 cell line. The potent activity exhibited by 1**7** (GI_{50} = 0.78 µM, Entry 9) and **20** (GI_{50} = 0.77 µM, Entry 12) was slightly better activity than that of etoposide (GI_{50} = 0.89 µM, Entry 14). Moreover, the same two compounds, 1**7** (GI_{50} = 8.79 µM, Entry 9) and **20** (GI_{50} = 9.30 µM, Entry 12), also exhibited potent growth inhibitory activity against the MCF7 cell line but this was not as good as that of etoposide (GI_{50} = 0.56 µM, Entry 14).

Most compounds exhibited weak TGI activity and three were inactive against the TK10 cell line. The activities of 1**8**, 1**9** and **20** (Entries 10–12, respectively) were slightly better (TGI = 46.14–48.25 µM) than that of etoposide (TGI = 49.74 µM, Entry 14). The compounds also exhibited moderate to weak activity against the UACC62 cell line with most compounds, 1**4**, 1**5**, 1**6**, 1**7,** 1**8** and **20**, exhibiting better activity (TGI = 18.32–51.06 µM) than that of etoposide (TGI = 52.71 µM, Entry 14). The best activity was observed for 1**7** (TGI = 18.32 µM, Entry 9) which was almost threefold better than that of etoposide. The test compounds exhibited weak activity against the MCF7 cell line, but the cytostatic effects of these compounds were better than that of etoposide which was inactive (TGI > 100 µM, Entry 14).

The LC_{50} values of the compounds were also compared to that of etoposide to get an idea of the cytotoxic effects of these compounds against the different cell lines. Screening against the TK10 cell line showed that most of the compounds were more lethal than etoposide (LC_{50} > 100 µM) with 1**8** (LC_{50} = 73.59 µM, Entry 10) being the most lethal.

Again, most of the compounds were more lethal than etoposide (LC_{50} > 100 µM) against the UACC62 cell line and 1**7** (LC_{50} = 60.32 µM, Entry 9) was the most lethal. Apart from 1**8**, none of the test compounds were lethal for HeLa cells, indicating a degree of selectivity between cell lines.

The compounds also exhibited more lethal cytotoxic effects against the MCF7 cell line than that of etoposide (LC_{50} > 100 µM, Entry 14) and **20** (LC_{50} = 86.93 µM, Entry 12) was the most lethal.

Apart from 1**7**, none of the test compounds were lethal against HeLa cells indicating a degree of selectivity between cell lines. The catechols **11a**–**c** and the catechol derivative **21** (Entry 13) did not exhibit any significant anticancer activity. From the results it can be seen that the GI_{50} concentrations of benzo[*b*]furan 1**2** (Entry 4) are almost half of those of **21** (Entry 13) against the renal (TK10), melanoma (UACC62) and breast (MCF7) cancer cell lines. It may thus be concluded that the presence of the furan ring enhances anticancer activity.

The benzo[*b*]furans were most effective against the melanoma (UACC62) cell line. Only one benzo[*b*]furan, **20**, exhibited growth inhibitory activity against all three cancer cell lines which may be attributed to the presence of the methoxy group on the benzene ring. When the methoxy group was replaced with a methyl group, as in 1**9**, growth activity against the renal (TK10) and breast (MCF7) cell lines was diminished. The benzo[*b*]furan 1**7** exhibited activity against melanoma **(**UACC62) and breast (MCF7) cell lines. The replacement of the methoxy-group with that of a methyl, as in 1**6**, also resulted in diminished activity and in this case against the breast (MCF7) cancer cell line. When comparing the structure of 1**9** to that of 1**7** it appears that the

replacement of the methyl group with that of a phenyl specifically affords growth inhibitory activity against the renal (TK10) cancer cell line.

It was concluded that the phenyl and methoxy-groups are essential for activity against all three cancer cell lines. We observed that the benzo[*b*]furans having the methoxy catechol were more biologically active and the activity increased with size as well as with hydrophobicity as shown in Figure 3. The benzo[*b*]furans having a methoxy catechol and a phenyl group showed enhanced activity when compared to the ones without the phenyl group.

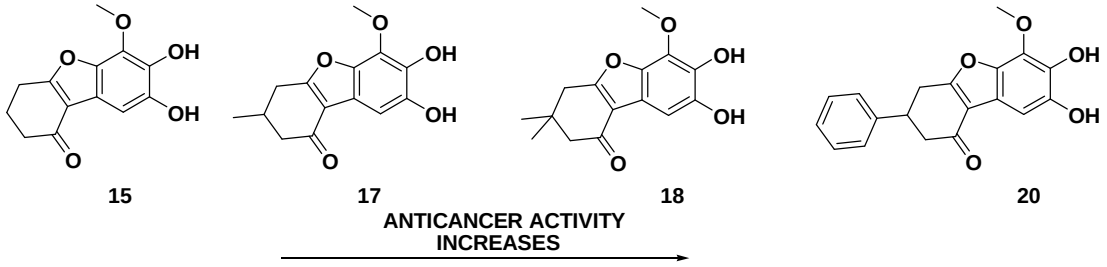


Figure 3: Evaluation of the anticancer activity of benzo[*b*]furan derivatives.

The benzofuro[3,2-*c*]chromen-6-one derivatives were the second class of compounds screened for anticancer activity and are shown in Figure 4 below.

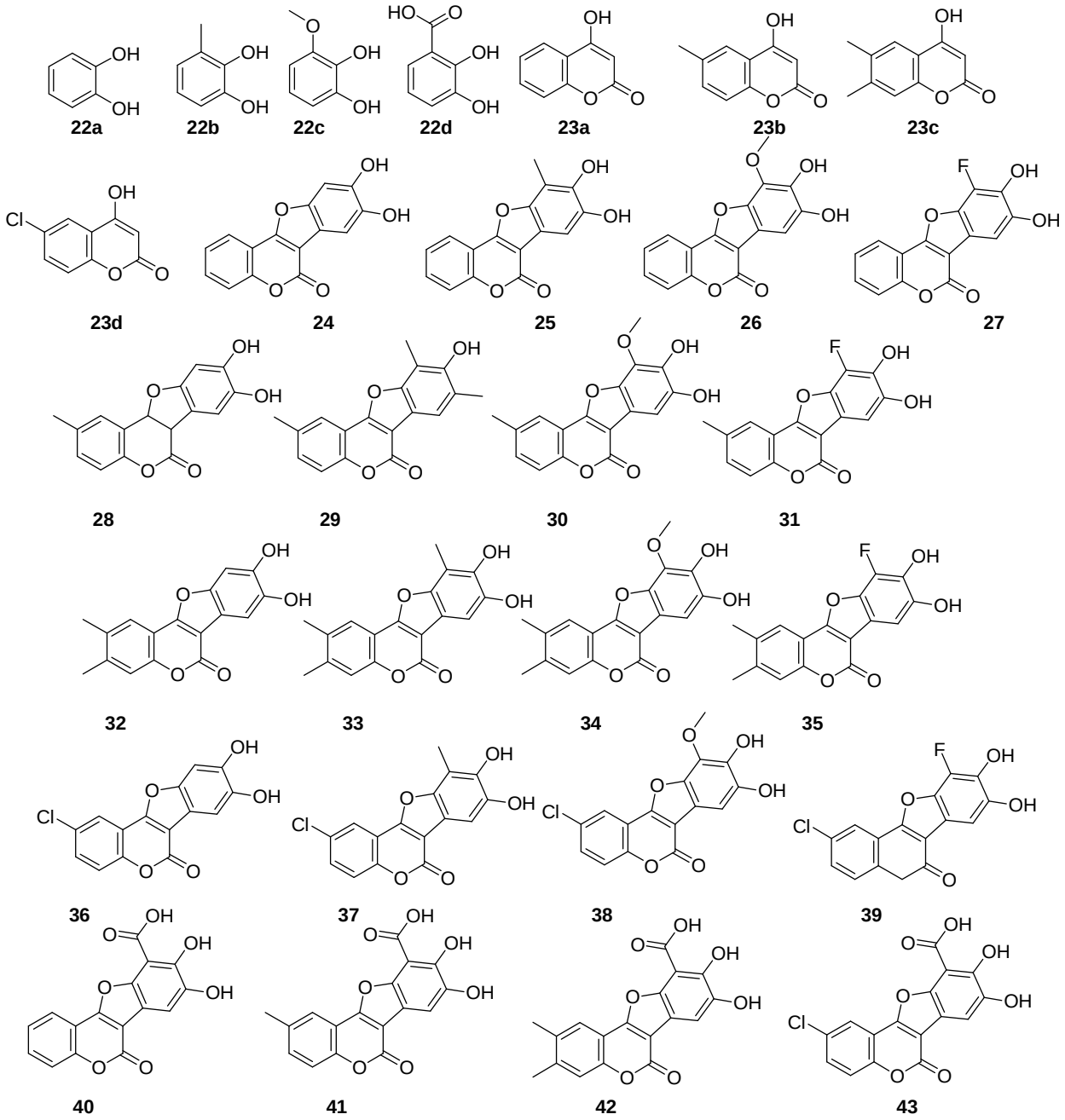


Figure 4: Benzofuro[3,2-*c*]chromen-6-one derivatives screened for anticancer activity.

The screening results of the catechols **22a-d**, coumarins **23a-d** and benzofuro[3,2-*c*]chromen-6-ones **24-41** were again compared to that obtained for etoposide.

It was evident that screening against the TK10 cell line showed that only compound **30** had potent activity against both melanoma (GI_{50} = 5.32 µM, Entry 14, Table 2) and breast cancer

(GI_{50} = 7.96 µM, Entry 14, Table 2). Most of the compounds exhibited moderate to weak activity. All the results are tabulated in Table 2 below.

Table 2. *In vitro* anticancer screening of the compounds against renal (TK10), melanoma (UACC62) and breast (MCF7) cancer cells expressed as GI_{50}, TGI and LC_{50} values (µM).

		Renal (TK10)			Melanoma (UACC62)			Breast (MCF7)		
Entry	**Cpd**	**GI_{50}**	**TGI**	**LC_{50}**	**GI_{50}**	**TGI**	**LC_{50}**	**GI_{50}**	**TGI**	**LC_{50}**
1	**22a**	68.02	>100	>100	45.00	>100	>100	52.41	>100	>100
2	**22b**	38.14	75.75	>100	**15.18**	46.22	77.26	**27.34**	64.31	>100
3	**22c**	51.63	96.98	>100	**23.64**	50.26	76.87	**27.55**	60.68	93.81
4	**22d**	84.68	>100	>100	35.24	78.43	>100	57.55	>100	>100
5	**22e**	a	a	a	a	a	a	a	a	a
5	**23a**	>100	>100	>100	83.60	>100	>100	95.34	>100	>100
6	**23b**	>100	>100	>100	84.66	>100	>100	84.40	>100	>100
7	**23c**	>100	>100	>100	80.48	>100	>100	82.26	>100	>100
8	**23d**	N/A	>100	>100	88.72	>100	>100	83.31	>100	>100
9	**24**	**28.73**	88.41	>100	33.26	61.88	90.56	39.40	77.31	>100
10	**25**	>100	>100	>100	55.57	>100	>100	54.92	>100	>100
11	**26**	>100	>100	>100	67.63	>100	>100	87.31	>100	>100
12	**27**	>100	>100	>100	67.57	>100	>100	53.06	>100	>100
13	**30**	51.96	>100	>100	**11.16**	50.94	90.71	54.70	>100	>100
14	**29**	48.81	>100	>100	**5.35**	**9.82**	89.94	**7.96**	78.16	>100
15	**31**	88.59	>100	>100	49.90	>100	>100	65.76	>100	>100
16	**32**	>100	>100	>100	>100	>100	>100	69.67	>100	>100
17	**34**	53.26	>100	>100	38.28	8.01	>100	45.97	86.34	>100
18	**35**	>100	>100	>100	67.63	>100	>100	87.31	>100	>100
19	**36**	**26.81**	66.41	>100	**18.12**	46.70	75.28	**29.51**	95.42	>100
20	**37**	>100	>100	>100	91.84	>100	>100	77.73	>100	>100
21	**39**	53.38	>100	>100	36.29	68.57	>100	47.54	89.75	>100
22	**40**	58.14	>100	>100	39.66	73.59	>100	63.14	>100	>100
23	**41**	>100	>100	>100	83.70	>100	>100	70.34	>100	>100
24	Etoposide	**7.19**	49.74	>100	**0.89**	52.71	>100	**0.56**	>100	>100

Inactive, GI_{50} or TGI > 100 mM; weak activity, > 30 mM GI_{50} or TGI < 100 mM; moderate activity: < 30 mM GI_{50} or TGI > 10 mM; potent activity, GI_{50} or TGI < 10 mM. [a] Not screened.

Compounds **24** (GI_{50} = 28.73 µM, Entry 9, Table 2) and **36** (GI_{50} = 26.81 µM, Entry 19, Table 2) showed moderate activity against the renal (TK10) cancer cell line.

Methyl-catechol **22b** (GI_{50} = 15.18 µM, Entry 2, Table 2) and methoxy-catechol **22c** (GI_{50} = 23.64 µM, Entry 3, Table 2) exhibited moderate activity against the melanoma (UACC62) cancer cell line. Compounds **30** (GI_{50} = 11.16 µM, Entry 13, Table 2) and **36** (GI_{50} = 18.12 µM, Entry 19, Table 2) also showed moderate activity against the melanoma cancer (UACC62) cell line. Compound **29** was the only compound that exhibited potent activity (GI_{50} = 5.35 µM, Entry 14, Table 2) against melanoma (UACC62) cancer cell line. This compound also exhibited potent total growth inhibition (TGI = 9.82 µM, Entry 14, Table 2)

Moderate activity was observed for methyl catechol **22b** (GI_{50} = 27.34 µM, Entry 2, Table 2), methoxy catechol **22c** (GI_{50} = 27.55 µM, Entry 3, Table 2) and compound **36** (GI_{50} = 29.51 µM, Entry 19, Table 2) against the breast (MCF7) cancer cell line while compound **30** (GI_{50} = 7.96 µM, Entry 14, Table 2) was the only compound to exhibit potent activity.

Compound **30** is the only compound that has exhibited potent activity against both melanoma (UACC62) and breast (MCF7) cancer cell lines.

The last class of compounds screened were the naphthoquinone derivatives as shown in Figure 5 below.

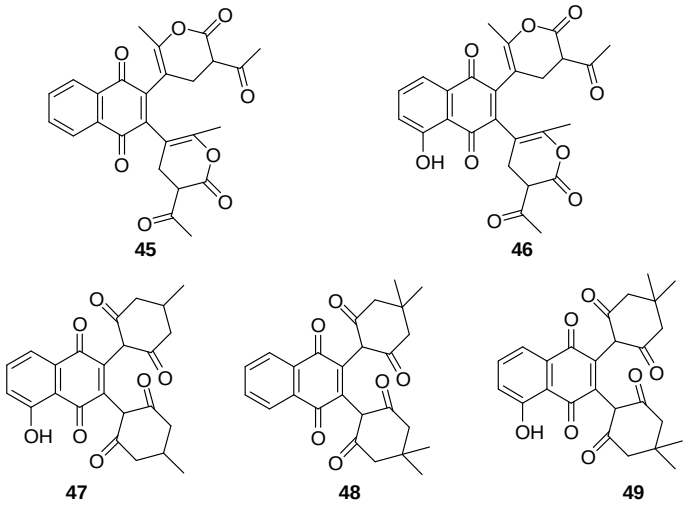


Figure 5: Naphthoquinone derivatives screened for anticancer activity.

The screening results of the naphthoquinone derivatives are in Table 3 below.

Table 3. In vitro anticancer screening of the compounds against renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer cells expressed as GI_{50}, TGI and LC_{50} values (µM).

Entry	Compound	renal (TK10)			melanoma (UACC62)			breast (MCF7)		
		GI_{50}	**TGI**	**LC_{50}**	**GI_{50}**	**TGI**	**LC_{50}**	**GI_{50}**	**TGI**	**LC_{50}**
1	**45**	49.57	>100	>100	32.96	63.16	93.36	44.02	80.96	>100
2	**46**	>100	>100	>100	**27.31**	56.00	84.69	52.88	>100	>100
3	**47**	**3.75**	**6.70**	**9.66**	**1.43**	**4.73**	**8.04**	**4.09**	**8.28**	98.71
4	**48**	>100	>100	>100	**28.02**	55.85	83.69	50.25	>100	>100
5	**49**	32.52	55.35	78.18	**6.28**	**27.31**	64.71	31.94	64.00	96.07
6	Etoposide	**7.19**	49.74	>100	**0.89**	52.71	>100	**0.56**	>100	>100

Inactive, GI_{50} or TGI > 100 mM; weak activity, > 30 mM GI_{50} or TGI < 100 mM; moderate activity: < 30 mM GI_{50} or TGI > 10 mM; potent activity, GI_{50} or TGI < 10 mM.

Compound **47** showed excellent activity against all three cancer cell lines.

7.2 EVALUATION OF THE ANTIBACTERIAL ACTIVITY

As previously mentioned in Section 7.1, it has been reported in the literature that benzofurans exhibit antibacterial activity.[108b] The benzo[*b*]furans synthesized in the present study were therefore screened against four different bacterial strains i.e. *M. smegmatis* MC^2155, methicillin-sensitive *Staphylococcus aureus* (MSSA) 32710, *Bacillus substilis* 168 and *E. coli* DH10B. The minimum inhibitory concentrations (MIC) were recorded. *M. smegmatis* was used as a model for further screening against *M.tb*. If good growth inhibitory activity was obtained against *M. smegmatis* then screening would be conducted against *M.tb*. Although this model is not completely precise we had to adopt it due to the time constraints as *M.tb* is a slow growing bacterium. Kanamycin and Rifampicin, which are well known antibiotics, were used as positive controls. The results are shown in Table 4 below and compound structures are shown in Figure 6.

Table 4. MIC values (µg/mL) of the 5,6-dihydroxylated benzo[*b*]furans screened against four bacterial strains.

Entry	Compound	*M. smegmatis*	*S. aureus*	*B. subtilis*	*E. coli*
1	**11a**	**100**	**>100**	**>20**	**>100**
2	**11b**	**100**	**>100**	**>20**	**50**
3	**11c**	**25**	**>100**	**>20**	**>100**
4	**12**	**>100**	**100**	**>100**	**>100**
5	**13**	**>100**	**>100**	**>100**	**>100**
6	**14**	>100	>100	>100	100
7	**15**	>100	>100	>100	100
8	**16**	>100	>100	>100	>100
9	**17**	>100	>100	>100	>100
10	**18**	>100	>100	>100	>100
11	**19**	>100	>100	>100	>100
12	**20**	>100	>100	>100	>100
13	**21**	>100	>100	>100	100
14	**Kanamycin**	**1.56**	-	-	-
15	**Rifampicin**	_	**<0.78**	**<0.78**	**<0.78**

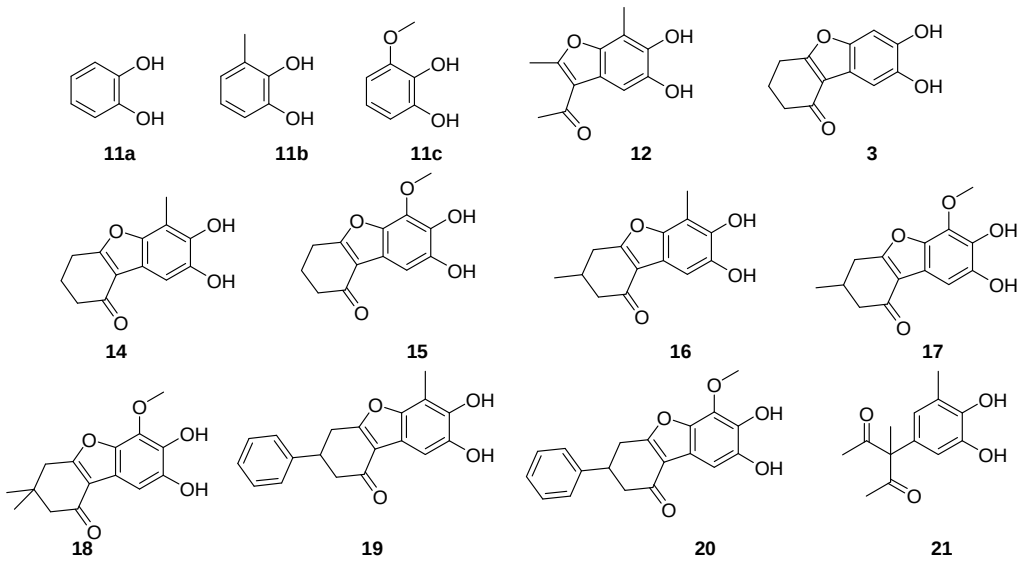


Figure 6: Benzo[*b*]furan derivatives screened for antibacterial activity.

From the results shown in Table 4 it is evident that the benzo[*b*]furans were not active against the four bacterial strains since no growth inhibition was observed. Since none of the compounds exhibited potent activity against *M. smegmatis* they were not screened against *M.tb*. We then proceeded to evaluate the antibacterial activity of benzofuro[3,2-*c*]chromen-6-ones against the same bacterial strains. The structures are shown in Figure 7 below.

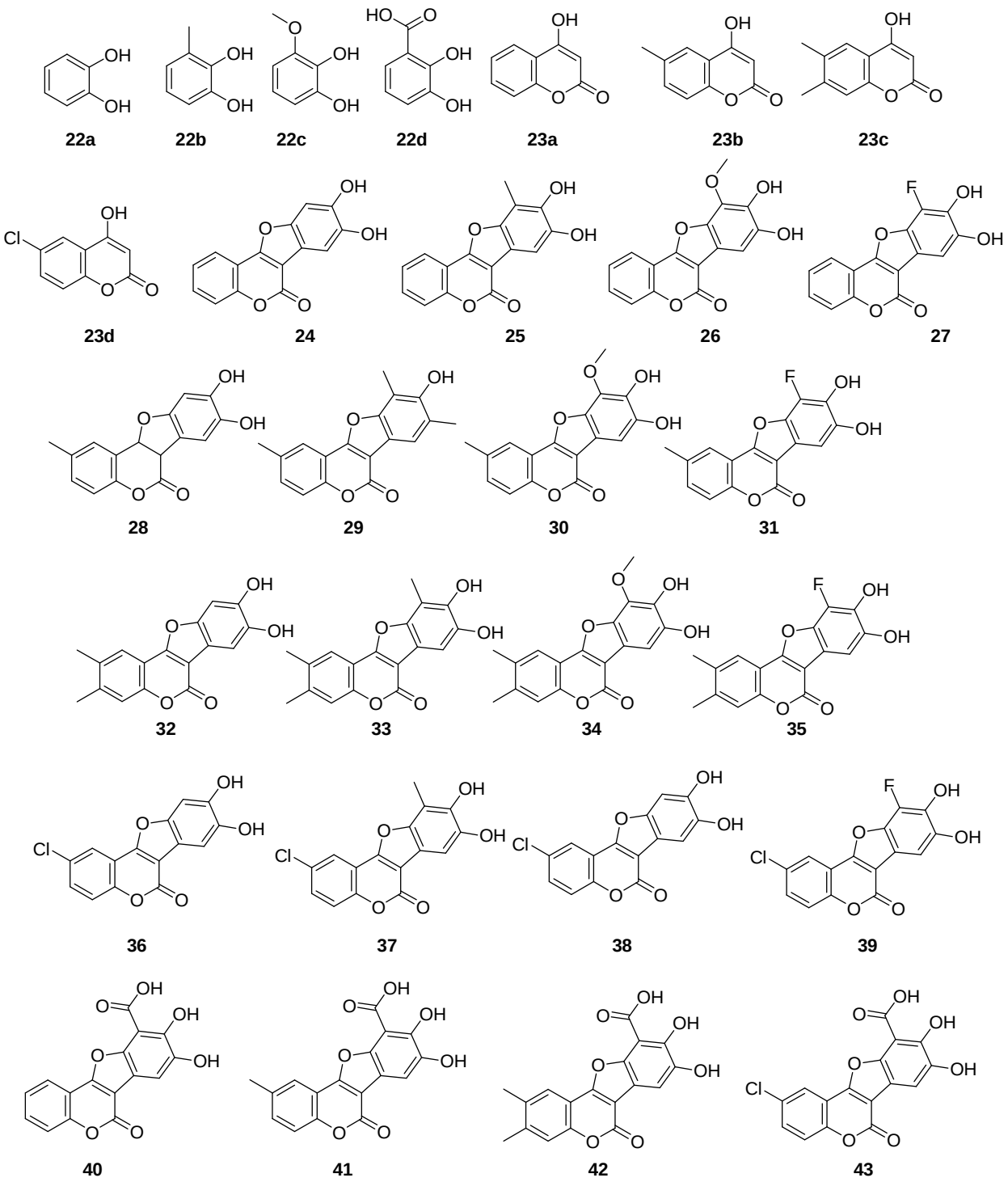


Figure 7: Benzofuro[3,2-*c*]chromen-6-one derivatives screened for antibacterial activity.

Table 5. *In vitro* antibacterial screening results of the catechols, coumarins and benzofuro[3,2-*c*]chromen-6-one derivatives against *M. smegmatis*, *S. aureus*, *B. subtilis* and *E. coli* expressed as MIC values (µg/mL).

Entry	Compound	*M. smegmatis* (MC^{2}155)	*S. aureus* (32710)	*B. subtilis* (168)	*E. coli* (DH10B)
1	**22a**	100	>100	>20	>100
2	**22b**	100	>100	>20	50
3	**22c**	**25**	>100	>20	>100
4	**22d**	50	>100	>20	>100
5	**23a**	50	>100	>20	>100
6	**23b**	25	>100	>20	>100
7	**23c**	50	>100	>20	>100
8	**23d**	100	>100	>20	>100
9	**24**	>100	>100	**< 20**	>100
10	**25**	>100	>100	>20	>100
11	**26**	>100	>100	>20	>100
12	**27**	>100	>100	>20	>100
13	**28**	>100	>100	>20	>100
14	**29**	>100	>100	>20	>100
15	**30**	>100	>100	>20	>100
16	**31**	>100	>100	>20	>100
17	**32**	>100	>100	>20	>100
18	**33**	>100	>100	>20	>100
19	**34**	>100	>100	>20	>100
20	**35**	>100	>100	>20	>100
21	**36**	>100	>100	>20	>100
22	**37**	>100	**6.25**	>20	>100
23	**38**	>100	>100	>20	>100
24	**39**	>100	>100	>20	>100
25	**Kanamycin**	**1.56**	-	-	-
26	**Rifampicin**	-	**<0.78**	**<0.78**	**<0.78**

Compound **37** (MIC = 6.25 µg/mL, Entry 37, Table 5) showed some activity against *S. aureus*. However, since none of the derivatives were potent against *M. smegmatis* it was decided not to screen benzofuro[3,2-*c*]chromen-6-one derivatives against *M.tb*.

We then proceeded to screen the naphthoquinone derivatives (Figure 8) against all four bacterial strains (Table 6). Compound **48** exhibited potent antibacterial activity (MIC = 6.25 µg/mL, Entry 4, Table 6) against *M. smegmatis* and we decided to proceed and screen it against three susceptible *M.tb* strains.

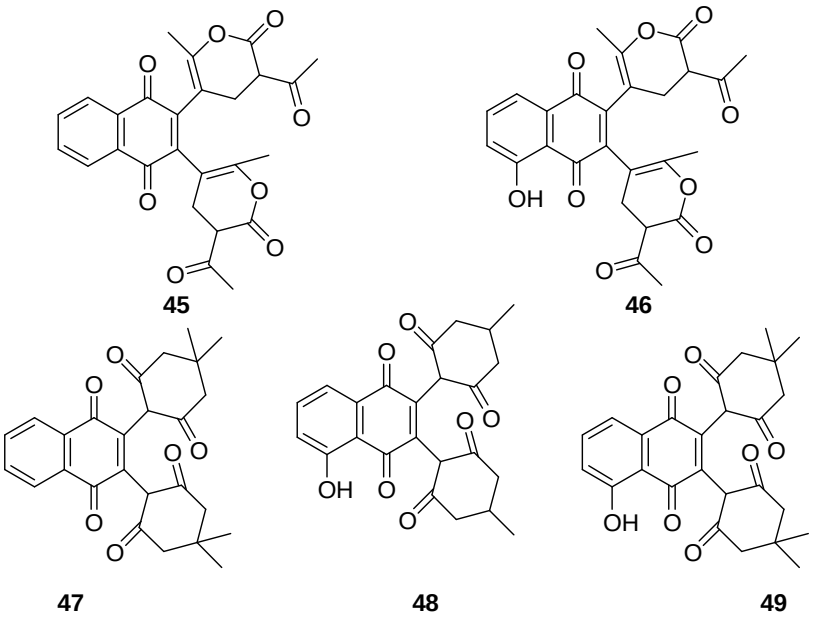


Figure 8: Naphthoquinone derivatives screened for antibacterial activity.

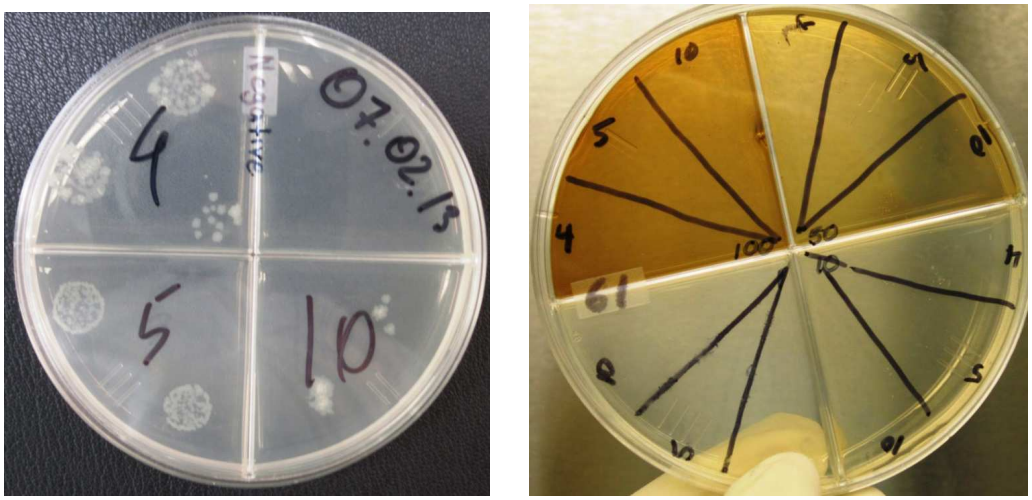
Table 6. MIC values (µg/mL) of compounds screened against four bacterial strains.

Entry	Compound	*Mycobacterium smegmatis*	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*
1	**45**	>100	>100	>20	>100
2	**46**	>100	>100	>20	>100
3	**47**	>100	>100	>20	>100
4	**48**	**6.25**	>100	>20	>100
5	**49**	>100	>100	>20	>100

Compound **48** was left in *M. smegmatis* for a number of days in order to determine if it was acting as a bactericidal or bacteriostatic agent. On the tenth day the bacterium started to slowly grow and this confirmed that this compound was acting as a bacteriostatic agent.

7.2.2 SCREENING OF COMPOUND 48 AGAINST THREE SUSCEPTIBLE *M.tb* STRAINS

Compound **48** was screened against three *M.tb* strains. Strain 4 is a MDR unique 001 strain with D516Y mutation in rpoB gene. Strain 5 is a MDR LL strain with S531L mutation in rpoB gene. Strain 10 is the drug susceptible strain called BE. Figure 9 shows two plates; the first one (Plate A) is the control which has three *M. tb* strains. The second one (Plate B) has three *M. tb* strains with compound **48** at different concentrations.



A **B**

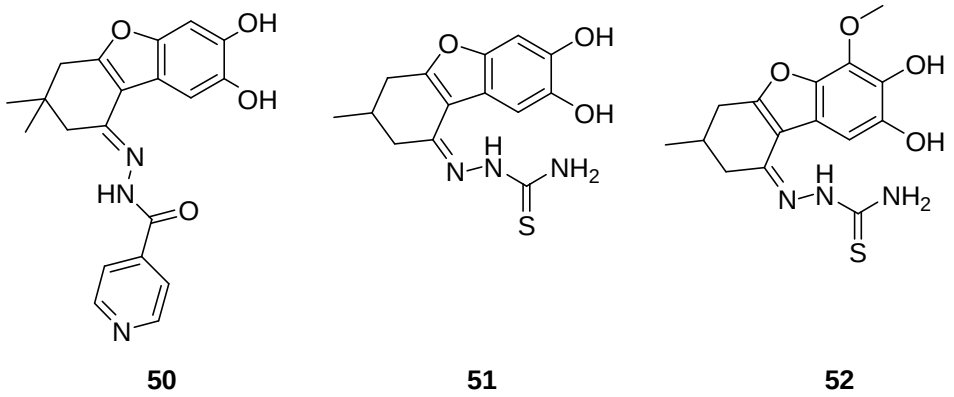
Figure 9: Plate A, the control and 3 *M.tb* strains. Plate B, 3 *M.tb* strains with compound **48** at different concentrations.

The growth of the bacteria took three weeks at 37ºC in 7H11 media. As depicted above compound **48** was active against resistant and susceptible clinical *M.tb* strains at 10, 50 and 100

mM as shown in Figure 9. This is evident as the bacteria were unable to grow at those concentrations.

7.2.3 ISONIAZID AND THIOSEMICARBAZIDE DERIVATIVES

In this section we will discuss the derivatized compounds that were screened for both anticancer and antibacterial activity. Because of time constraints we were unable to screen all the compounds. The compounds that were screened are shown in Figure 10 below.



50 **51** **52**

Figure 10: Benzo[*b*]furan isoniazid derivative **50** and thiosemicarbazide derivatives, **51** and **52**, screened for antibacterial and anticancer activity.

7.2.3.1 Evaluation of the anticancer activity

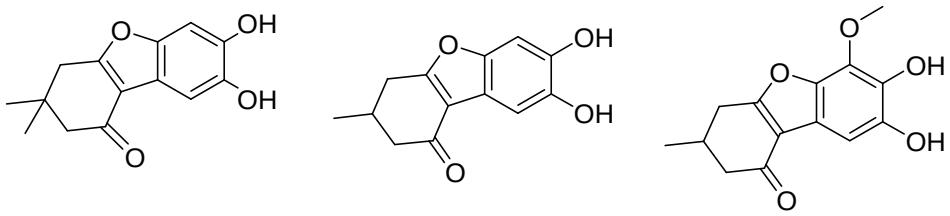
The results of the cancer screening are shown in Table 7 below.

Table 7: *In vitro* anticancer screening of compounds **17**, **50**, **51**, **52**, **53** and **54** against renal (TK10), melanoma (UACC62) and breast (MCF7) cancer cell lines expressed as GI_{50}, TGI and LC_{50} values (µM).

Entry	Cpd	Renal (TK10)			Melanoma (UACC62)			Breast (MCF7)		
		GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
1	**17**	26.54	51.65	76.75	**0.78**	18.32	60.32	**8.79**	48.79	95.11
2	**50**	37.25	84.01	>100	**17.12**	60.11	>100	56.87	>100	>100
3	**51**	45.56	82.44	>100	**4.38**	**8.32**	>100	33.22	78.55	>100
4	**52**	30.72	61.21	91.69	**0.65**	**4.32**	94.85	**9.27**	61.61	>100
5	**53**	19.41	47.00	74.59	29.56	59.48	89.39	44.55	80.91	>100
6	**54**	26.69	56.49	83.29	40.41	77.13	>100	52.63	98.60	>100
7	**Etoposide**	**7.19**	49.74	>100	**0.89**	52.71	>100	**0.56**	>100	>100

Cpd = compound; Inactive, I: GI_{50} or TGI > 100 µM; weak activity, w: > 30µM GI_{50} or TGI < 100µM; moderate activity, m: < 30µM GI_{50} or TGI > 10µM; potent activity, p: GI_{50} or TGI < 10µM.

The results were compared to its precursor **53**, **54** and **17 (**Figure 11) as well as to that of etoposide.



53 **54** **17**

Figure 11: Structures of the precursors, benzo[*b*]furans **53**, **54** and **17**.

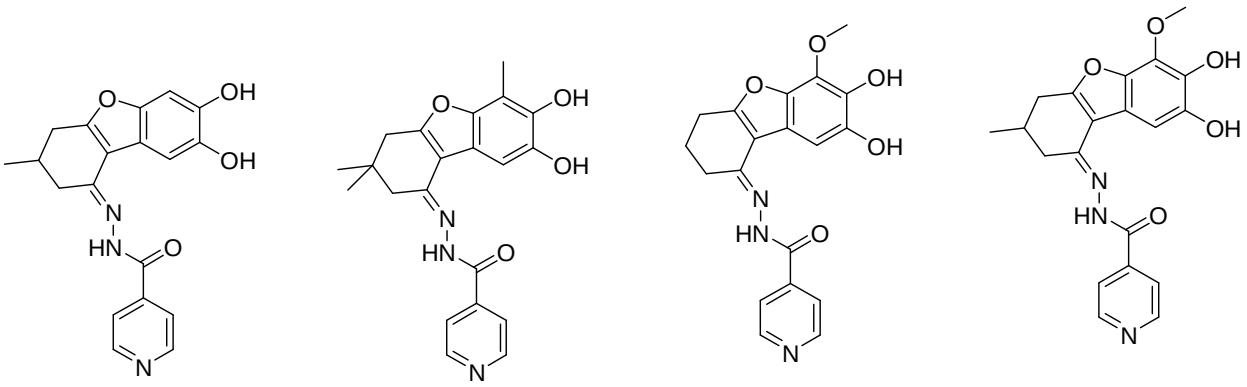
Both compounds **52** (GI_{50}, = 0.65 µM) and **17** (GI_{50} = 0.78 µM) exhibited potent activity against both melanoma and breast cancer cell lines. The precursor **17** was less potent than that of the derivative **52** against the melanoma cancer cell line. However, it was observed that the GI_{50} of the precursor **17** (GI_{50} = 8.79 µM) was better than that of the derivative **52** (GI_{50} = 9.27 µM) against the breast cancer cell line. These findings imply that the synthesized benzo[*b*]furan **17** is more biologically active and that derivitisation did not improve the biological activity against the breast cancer cell line.

Benzo[*b*]furans **53** and benzo[*b*]furans **54** exhibited moderate to weak activity as shown in Table 7 above. The GI_{50} values were compared to that of anticancer drug, etoposide. Compound **50** exhibited moderate to weak activity against the three cancer cell lines.

It was observed that **51** exhibited potent activity against the melanoma cell line (GI_{50} = 4.38 µM) but it was not as good as that of etoposide (GI_{50} = 0.89 µM, Entry 5, Table 7). The activity of **52** (GI_{50}, = 0.65 µM) against the melanoma cell line was better than that of etoposide (GI_{50} = 0.89 µM, Entry 5, Table 7). The activity of etoposide (GI_{50} = 0.56 µM, Entry 5, Table 7) was better than **52** (GI_{50} = 9.27 µM) against the breast cancer cell line. The difference between **51** and **52** is the methoxy group which enhanced the activity of compound **52** against all three cancer cell lines.

7.2.3.2 Evaluation of the antibacterial activity

7.2.3.2.1 Isoniazid derivatives

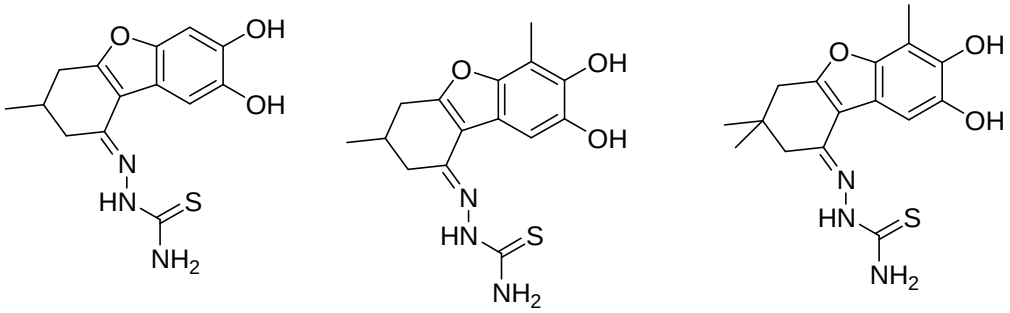


55 **56** **57** **58**

Figure 12: Structures of the selected isoniazid derivatives screened against *M. smegmatis.*

The selected isoniazid derivatives that were screened were inactive against the four bacterial strains since no growth inhibition was observed. Since none of the compounds exhibited potent activity against *M. smegmatis* the derivatives were not screened against *M.tb*.

7.2.3.2.2 Thiosemicarbazide derivatives



51 **59** **60**

Figure 13: Structures of the selected thiosemicarbazide derivatives screened against *M. smegmatis*.

The selected thiosemicarbizide derivatives were also like isoniazid derivatives inactive against the four bacterial strains since no growth inhibition was observed. Since none of the compounds exhibited potent activity against *M. smegmatis* the derivatives were not screened against *M.tb*.

7.3 CONCLUSION

It was possible to establish a structure-activity relationship for the 5,6-dihydroxylated benzo[*b*]furans that were screened against four cancer cell lines. It was observed that the phenyl and methoxy-groups in the 5,6-dihydroxylated benzo[*b*]furans are essential for activity against renal, melanoma breast and cervical cancer cell lines. Compounds; **15**, **17**, **18** and **20** were the most biologically active of the benzo[*b*]furans (Figure 11).

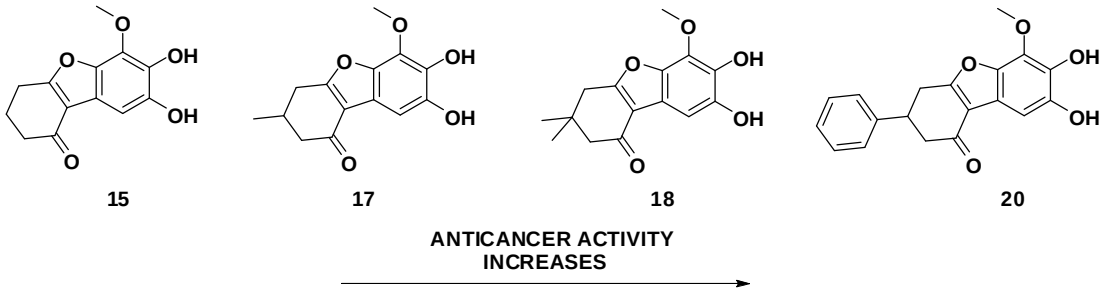


Figure 12: Structures of the biologically active benzo[*b*]furans.

The methoxy catechol derivatives were more biologically active and the activity increases as the size of the 1,3-diketone increases as shown in Figure 11 above. The 5,6-dihydroxylated benzo[*b*]furans having both a methoxy catechol and a phenyl group showed enhanced activity when compared to the ones without the phenyl group.

The benzo[3,2-c]chromen-6-ones did not exhibit a similar activity to that of the 5,6-dihydroxylated benzo[*b*]furans. Only benzofuro[3,2-*c*]chromen-6-one **29** exhibited potent activity against the melanoma cancer cell line (GI_{50} = 5.35 µM). Interestingly, compound **29** also has a methoxy group (Figure 13). Only compound **37** exhibited antibacterial activity, it has potent activity against *S. aureus* (MIC = 6.25 µg/mL).

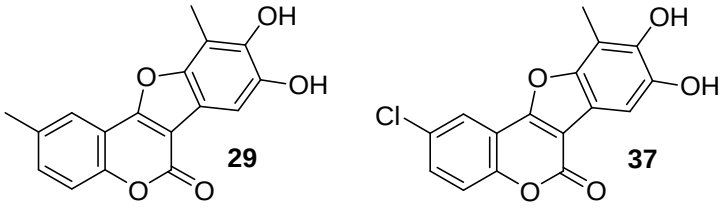
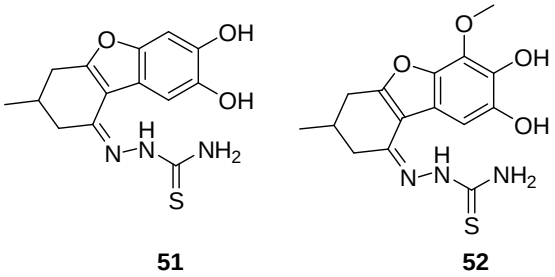


Figure 13: Benzofuro[3,2-*c*]chromen-6-one **29** exhibiting potent anticancer activity and **37** exhibiting potent antibacterial activity.

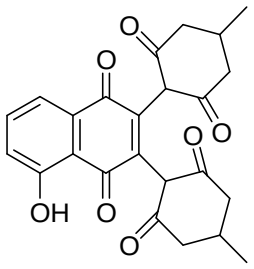
Compound **51** exhibited potent activity (GI_{50} = 4.38 µM) against the melanoma cell line (Figure 14). Compound **52** exhibits potent activity against two cell-lines, melanoma (GI_{50} = 0.65 µM) and breast cancer (GI_{50} = 9.27 µM), and its activity was better than that of etoposide (GI_{50} = 0.89 µM) against the melanoma cell-line.



51 **52**

Figure 14: Thiosemicarbazide derivatives exhibiting potent anticancer activity.

The methoxy group enhanced the activity of compound **52**. The isoniazid derivatives were inactive against the three cancer cell lines since no growth inhibition was observed, and the thiosemicarbizide derivatives did not show any significant increase in anticancer activity when compared to their precursors, the 5,6-dihydroxylated benzo[*b*]furans. For the antibacterial evaluation compound **48** (Figure 15) is the only compound that exhibited significant biological activity (MIC = 6.25 µg/mL) against *M. smegmatis* (MC2155).



48

Figure 15: The juglone derivative exhibiting anti-TB activity.

Compound **48** is active against both susceptible and resistant clinical *M.tb* strains.

CONCLUSION

8.0 CONCLUSION

8.1 BIOCATALYTIC SYNTHESIS

A new and broadly applicable synthetic sequence that is concise and cost effective has been successfully developed for the synthesis of benzo[*b*]furans **1**, methyl substituted 1,3-diketones catechol derivatives **2**, benzofuro[3,2-c]chromen-6-ones **3**, naphthoquinone menadione analogues **4** and C-C Bis coupled naphthoquinones **5** , **6** (Figure 1).

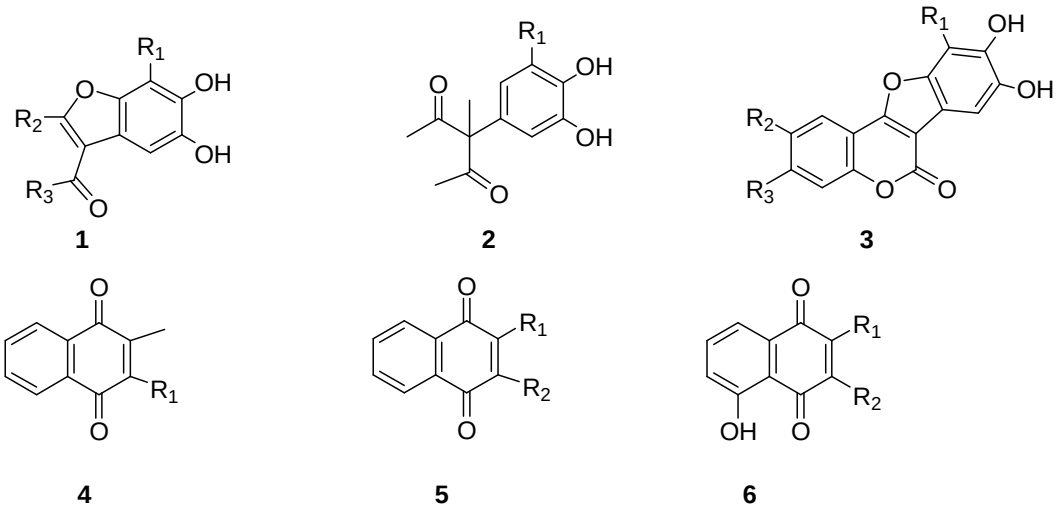


Figure 1: Structures synthesized compounds

The biocatalytic synthesis that entailed reacting a catechol or naphthohydroquinone with an equivalent of the 1,3-dicarbonyl, was based on the use of an enzyme, a laccase, which is marketed as Suberase®. The methodology utilised was environmentally friendly with high atom economy in that it was performed in an aqueous solvent at room temperature with atmospheric oxygen as a co-reactant, and generated water as the only by-product. No mediator compounds were required for these reactions. This broadly applicable biocatalytic method permitted the synthesis of a total of seventy compounds in this study, of which fifty one were novel.

We were able to synthesise fluorinated and acid derivatives of benzofuro[3,2-c]chromen-6-one **3**,which has not been reported in literature previously. We also demonstrated that the enzyme Suberase® can be utilised in cross coupling of enolates to unactivated sp2-hybridized electrophiles without the use of a transition-metal catalyst (compound **2**).

8.2 SYNTHESIS OF ISONIAZID AND THIOSEMICARBIZIDE DERIVATIVES

Our interest was in synthesising derivatives of the first-line anti-TB drug, isoniazid, as well as thiosemicarbazide derivatives from the previously synthesised 5,6-dihydroxylated benzo[*b*]furans **1**, with the aim to improve the biological activity against both cancer and bacteria. Schiff base methodology was utilised to give nine novel isoniazid derivatives and eight novel thiosemicarbizide derivatives.

8.3 BIOLOGICAL EVALUATION

Anti-cancer activity: The synthesised compounds were evaluated as anti-cancer compounds against cancer cell panels comprised of renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer lines. A number of the benzo[b]furans **1**, (compounds **7**, **8**, **9** and **10,** Figure 2) had a comparable potency to that of the anticancer drug etoposide on the melanoma cell line (etoposide (GI_{50} = 0.89 µM), compound **7** (GI_{50} = 6.04 µM), **8** (GI_{50} = 0.78 µM), **9** (GI_{50} = 6.79 µM) and **10** (GI_{50} = 0.77)).

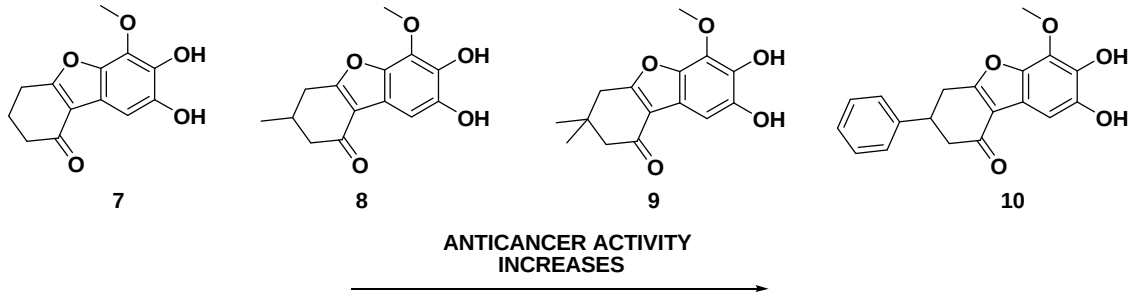


Figure 2: Structures of the benzo[*b*]furans with anti-cancer activity.

The methoxy catechol derivatives of the benzo[b]furans were more biologically active on melanoma cancer cell line and the activity increased as the size of the 1,3-diketone as increased. This observed increase in activity of these analogues was attributed to an increased lipophilic character. The benzo[3,2-c]chromen-6-ones **3** did not generally exhibit a similar anti-cancer activity to that of the 5,6-dihydroxybenzo[*b*]furans, and. only benzofuro[3,2-*c*]chromen-6-one **11** exhibited potent activity against the melanoma cell-line (GI_{50} = 5.35 µM) (Figure 3).

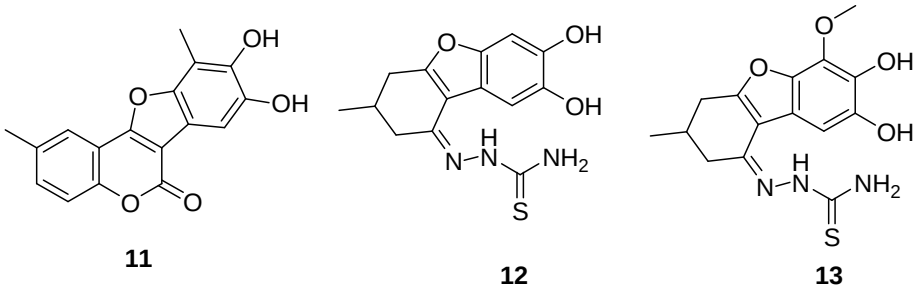
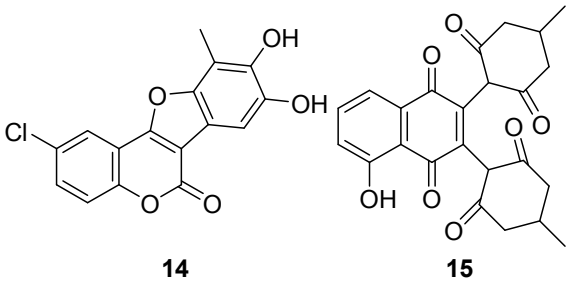


Figure 3: A benzofuro[3,2-*c*]chromen-6-one **11** and two thiosemicarbazide derivatives **12, 13** exhibiting potent anticancer activity**..**

A thiosemicarbizide analogue **12** exhibited potent activity (GI_{50} = 4.38 µM) against the melanoma cell line, while **13** exhibited potent activity against both melanoma (GI_{50} = 0.65 µM) and breast cancer (GI_{50} = 9.27 µM), the former being similar in potency to etoposide

(GI_{50} = 0.89 µM) against the same melanoma cell-line. However, the thiosemicarbizide derivatives did not show superior anticancer activity when compared to their chemical precursors, the 5,6-dihydroxyl benzo[b]furans. It is evident among the thiosemicarbizide derivatives that the methoxy group enhanced the activity of compound **13**.

Antibacterial activity: A selected number of compounds were evaluated for antibacterial properties against a range of bacteria which included the Gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, the Gram negative *Escherichia coli*, and the acid fast bacteria *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis.* Compound **14** (Figure 4) exhibited antibacterial activity, against *S. aureus* (*MIC* = 6.25 µg/mL). Only compound **15** exhibited significant biological activity (*MIC* = 6.25 µg/mL) against *M. smegmatis* (MC2155), but was active against both drug susceptible and drug resistant clinical *M.tb* strains.



14 **15**

Figure 4: A benzofuro[3,2-*c*]chromen-6-one **14** that exhibited potent antibacterial activity and a juglone derivative **15** that exhibited anti-TB activity .

8.3 CLOSING COMMENTS

In conclusion, this research has thoroughly explored the application of a laccase, one that is commercially available and accessible to chemists, for the synthesis of a broad range of benzofurans and structurally related compounds. These compounds were demonstrated to have biological activities, specifically relating to anti-cancer and anti-microbial properties, with potencies that are comparable with existing prescription medicines. The biocatalytic synthetic technique used here permitted the synthesis of many novel compounds, opening the way to access synthetic spaces that have not been previously accessed by non-biocatalytic methods.

The future work should include further studies of benzo[b]furans (**7-11**) and thiosemicarbizide (**12-13**) for their application in anticancer therapy. This encompasses further synthesis of **7-11**, **12,13** and evaluation of their anticancer activity in addition to structural modification of hits in order to determine whether the anticancer activity can be optimised. The results also warrant further derivatisation of the promising compounds **14** and **15** in order to enhance their antibacterial properties. The influence of other laccases on the synthetic profile and reaction yield should be explored, including those laccases with higher redox potentials or with differing active site configurations.

EXPERIMENTAL AND REFERENCES

9.1 EXPERIMENTAL

9.1 SUMMARY OF THE EXPERIMENTAL

9.1.1 General

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Mercury 400 MHz spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instrument at 100 MHz. Chemical shifts are reported in parts per million (ppm) relative to the solvent peaks and coupling constants are given in Hertz (Hz). A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. The PDA detector was used for all purity determinations (Maxplot 200–500 nm). All chemicals for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) while ultra-pure solvents were purchased from Honeywell (Burdick & Jackson, Muskegon, USA). Ultra-pure water was generated using a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France). Reactions were monitored by thin layer chromatography (TLC) on aluminium-backed Merck silica gel 60 F254 plates. Gravity column chromatography was performed using Merck Silica Gel 60 (70–230 mesh). Melting points were determined using a Glassco melting point apparatus and are uncorrected. All other chemicals were reagent grade materials. The 1,3-dicarbonyls were purchased from Sigma–Aldrich, South Africa. Suberase® (10757.8 PCU/mL) is a fungal laccase from *M. thermophila* produced by submerged fermentation of a genetically modified *Aspergillus oryzae*strain. The enzymatic preparation is supplied as a brown liquid which is completely miscible with water. Suberase® was obtained from Novozymes in South Africa.

9.1.2 METHODS FOR THE SYNTHESIS OF THE 5,6-DIHYDROXYLATED BENZO[*B*]FURANS

METHOD A

The laccase (Suberase®, 2.0 mL) was added to a mixture of the catechol (2.0 mmol), 1,3-dicarbonyl (2.0 mmol) and phosphate buffer (20.0 mL, 0.10 M, pH 7.15) in a 250-mL round-bottom flask stirred under air at rt. More laccase (2.0 mL) was added after 2, 18 and 20 h. The mixture was vigorously stirred under air until the substrates were consumed as judged by TLC. After stirring the reaction mixture was acidified with 32% HCl to pH 4.0. The mixture was extracted with EtOAc and washed with water (20.0 mL). The organic phases were then combined and the solvent evaporated. The residue, a powder, was purified by washing with EtOAc or recrystallizing from a combination of MeOH and EtOAc.

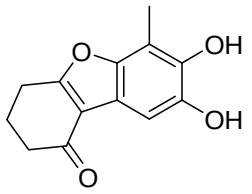
METHOD B

Same as Method B except that more laccase (2.0 mL) was added after 4, 24 and 28 h.

METHOD C

The laccase (Suberase®, 1.5 mL) was added to a mixture of the catechol (0.60 mmol), 1,3-dicarbonyl (2.40 mmol), phosphate buffer (4.0 mL, 0.10 M, pH 7.15) and DMF (2.0 mL) in a test tube stirred under air at rt. More laccase (1.5 mL) was added after 2 h and then again after 4 h. The mixture was vigorously stirred under air until the substrates had been consumed as judged by TLC. After stirring the reaction mixture was transferred to a separating funnel and the mixture extracted with EtOAc and washed with water (20.0 mL). The organic phases were combined, the solvent evaporated and the residue (a powder) purified by flash chromatography.

Synthesis of 7,8-Dihydroxy-6-methyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 4



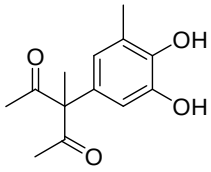
Method A

Stirring time 24 h.should really say how much you started with.for all of the experimental. The product was recrystallized from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (288 mg, 62%) (Found: M–H$^+$, 231.0683. $C_{13}H_{11}O_4$ requires M–H, 231.0657). UPLC 99.7%. R*f* 0.48 (EtOAc/hexane, 1:1). mp 250°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.14 (2H, m, CH_2), 2.26 (3H, s, CH_3), 2.45 (2H, t *J* 6.1 Hz, CH_2), 2.98 (2H, t *J* 6.1 Hz, CH_2), 7.11 (s, 1H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0, 22.2, 23.3, 37.9, 102.4, 107.9, 113.5, 115.9, 142.2, 143.4, 147.6, 169.6, 194.6.

Method B

Stirring time 44 h. The product was washed with EtOAc to afford a brown powder (311.0 mg, 67%).

Synthesis of 3-(3,4-Dihydroxy-5-methylphenyl)-3-methylpentane-2,4-dione 16



Method A

Stirring time 24 h. The product was washed with EtOAc to afford a dark-brown powder (1.60 g, 80%) (Found: M–H^+, 235.0926. $C_{13}H_{14}O_4$ requires M–H, 235.0970. UPLC 98.0%. R*f* 0.48 (EtOAc/hexane, 1:1). mp 82–85°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.60 (3H, s, CH_3), 2.03 (6H, s, CH_3), 2.08 (3H, s, CH_3), 6.43 (1H, d *J* 2.0 Hz, ArH), 6.46 (1H, d*J* 2.4 Hz, ArH), 8.36 (1H, br s, OH), 9.29 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.2, 19.0, 27.2, 30.7, 68.8, 112.4, 120.1, 124.7, 127.7, 143.0, 144.8, 207.8.

9.1.3 METHODS FOR THE SYNTHESIS OF Benzofuro[3,2-*c*]chromen-6-one

METHOD A

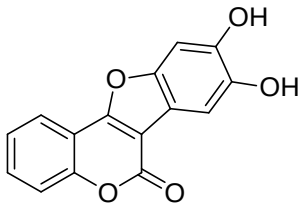
The laccase (Suberase®, 2.0 mL) was added to a mixture of the catechol (2.0 mmol), coumarin (2.0 mmol) and phosphate buffer (20.0 mL, 0.10 M, pH 7.15) in a 250 mL round bottom flask stirred under air at rt. More laccase (2.0 mL) was added after 2, 18 and 20 h. The mixture was vigorously stirred under air until the substrates were consumed as judged by TLC. The mixture was extracted with EtOAc and washed two times with water (20.0 mL). The organic phases were then combined and the solvent evaporated. The residue, a powder, was purified by washing with EtOAc, flash chromatography or recrystallization from a combination of MeOH and EtOAc. Products were characterized by ^{1}H NMR, ^{13}C NMR, and MS.

METHOD B

The laccase (Suberase®, 1.0 mL) was added to a mixture of the catechol (1.0 mmol), coumarin (1.0 mmol) and phosphate buffer (8.0 mL, 0.10 M, pH 7.15) in a 250 mL round bottom flask stirred under air at 40°C. More laccase (1.0 mL) was added after 2, 18 and 20 h. The mixture was

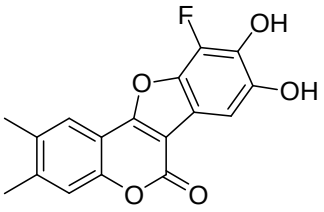
vigorously stirred under air until the substrates were consumed as judged by TLC. The solvent mixture was left for a day to allow the product to precipitate to the bottom and filtered. The residue, a powder, was purified by washing with EtOAc. Products were characterized by ^{1}H NMR, ^{13}C NMR, and MS.

Synthesis of 8,9-Dihydroxy-6H-benzofuro[3,2-c]chromen-6-one 17



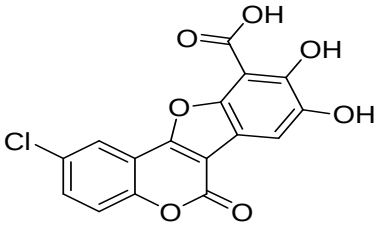
Method A. Stirring time = 24 h. Purification by a wash with EtOAc and the flash chromatography (silica: EtOAc/hexane, 1:5, 1:4, 1:3, 1:2 and EtOAc) to afford a light-brown solid (0.4600 g, 86.0%). (M-H^{+} Found: 267.0216. $C_{15}H_7O_5$ requires M-H^{+}, 267.0293). UPLC 99.8 %. R_f 0.54 (EtOAc/Hex, 1:2). mp 300^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.24 (1H, s, ArH). 7.31 (1H, s, ArH), 7.48 (1H, t, *J* 8.0 Hz, ArH), 7.58 (1H, d, *J* 8.0 Hz, ArH), 7.66 (1H, ddd, *J* 1.6, 8.0, 15.6 Hz, ArH), 8.00 (1H, dd, *J* 1.6, 8.0 Hz, ArH; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 98.9, 104.9, 105.5, 112.4, 113.8, 117.0, 121.1, 124.8, 131.2, 144.7, 146.6, 149.4, 152.3, 157.4, 157.7.

Synthesis of 10-Fluoro-8,9-dihydroxy-2,3-dimethyl-6H-benzofuro[3,2-c]chromen-6-one 30



Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a brown solid (0.500 g, 76.0%). ($M+H^+$ Found: 314.0588. $C_{17}H_{11}O_5F$ requires $M+H^+$, 314.0591). UPLC 93%. R_f 0.58 (EtOAc/hexane, 1:1). mp = 174 ºC; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.26 (3H, s, CH_3), 2.30 (3H, s, CH_3), 5.46 (1H, s, ArH), 7.15 (1H, s, ArH), 7.56 (1H, s, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 18.7, 19.6, 62.5, 63.4, 68.8, 71.4, 72.3, 73.7, 89.9, 113.6, 116.7, 123.0, 132.1, 142.1, 152.0, 162.3, 166.2.

Synthesis of 2-chloro-8,9-dihydroxy-6-oxo-6H-benzofuro[3,2-c]chromene-10-carboxylic acid 34



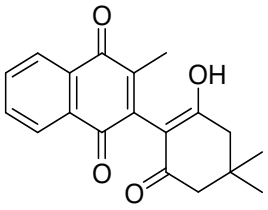
Method B. Stirring time = 24 h. Purification by a wash with EtOAc to afford a yellow solid (0.527 g, 76.0%). ($M+H^+$ Found: 346.9887 $C_{16}H_8O_7Cl$ requires $M+H^+$, 346.9959). UPLC 98.1%. R_f 0.31 (EtOAc/Hex, 2:1). mp 152ºC; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 7.13 (1H, s, H-Ar), 7.52 (1H, d, *J* 8.8 Hz, H-Ar), 7.60 (1H, dd, *J* 2.4, 8.8 Hz, H-Ar), 7.96 (1H, d, *J* 2.4 Hz, H-Ar), ^{13}C NMR (100 MHz, DMSO-d_6): δ = 98.1, 107.6, 109.0, 111.9, 113.7, 118.3, 120.1, 128.1, 130.1, 147.5, 147.9, 150.5, 154.6, 155.6, 168.4. 15

9.1.4 SYNTHESIS OF 1,4-NAPHTHOQUINONE DERIVATIVES

METHOD

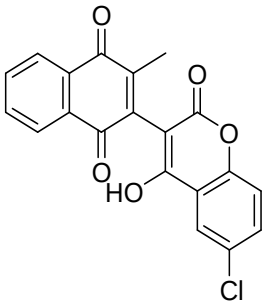
The laccase (Suberase1, 1.0 mL) was added to a mixture of the catechol (1.0 mmol), coumarin (1.0 mmol) and phosphate buffer (8.0 mL, 0.10 M, pH 7.15) in a test tube stirred under air at 40°C. More laccase (1.0 mL) was added after 2, 18 and 20 h. The mixture was vigorously stirred under air until the substrates were consumed as judged by TLC. The mixture was extracted with EtOAc and washed two times with water (20.0 mL). The organic phases were then combined and the solvent evaporated. The residue, a powder, was purified by flash chromatography. Products were characterized by ^{1}H NMR, ^{13}C NMR, and MS.

Synthesis of 2-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-enyl)-3-methylnaphthalene-1,4-dione 41



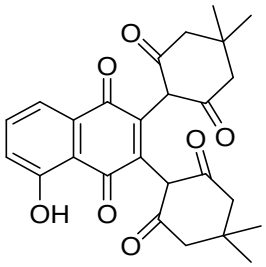
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (0.296g 88% yield), (M-H^{+} Found: 309.1012, $C_{22}H_{13}O_2$ requires M-H^{+}, 309.0916). UPLC 99.6%. R_f 0.52. (EtOAc/Hex, 1:2). mp 230ºC ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.09 (3H, s, CH_3), 1.16 (3H, s, CH_3), 1.90 (3H, s, CH_3),2.36 (4H, s, 2 x CH_2), 7.85 (2H, dd, *J* 3.4, 5.4 Hz, ArH), 7.96 (1H, dd, *J* 3.4, 5.4 Hz, ArH), 8.04 (1H, dd, *J* 3.4, 5.4 Hz, ArH), 10.8 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.9, 27.7, 28.0, 31.9, 108.9, 125.8, 125.8, 131.6, 132.0, 133.7, 133.9, 141.6, 145.4, 182.5, 184.6.

Synthesis of 2-(6-chloro-4-hydroxy-2-oxo-2H-chromen-3-yl)-3-methylnaphthalene-1,4-dione 46



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (M-H$^+$ Found: 365.0299, $C_{20}H_{10}ClO_5$ requires M-H$^+$, 365.0300). UPLC 90.1%. R_f 0.40 (EtOAc/Hex, 1:2). mp 260-261ºC ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.12 (3H, s, CH_3), 3.90 (1H, br s, OH), 6.98 (1H, s, ArH), 7.55 (1H, dd, *J* 7.50, 15.6 Hz, ArH), 7.64 (1H, m, ArH), 7.86 (2H, dd, *J* 3.60, 4.80 Hz, ArH), 7.97 (1H, t, *J* 4.50 Hz, ArH), 8.02 (1H, t, *J* 4.50 Hz, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 14.2, 95.9, 116.6, 116.7, 122.8, 122.9, 124.8, 125.4, 125.8, 130.2, 131.8, 133.0, 133.1, 133.3, 143.2, 146.2, 154.0, 161.8, 171.5, 183.1, 189.2.

Synthesis of 5-hydroxy-2,3-bis(4,4-dimethyl-2,6-dioxocyclohexyl)naphthalene-1,4-dione 50



Stirring time = 48h. The product was washed with EtOAc to afford light brown solid (0.292g 48% yield), (M–H$^+$ Found: 449.1595 $C_{26}H_{25}O_7$ requires M$^-$H, 449.1600). UPLC 91.2%. R_f = 0.51 (EtoAc) Mp = 170 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) 0.99 (3H, s, CH_3), 1.07 (3H, s, CH_3), 2.13 (2H, s, 2 X CH), 2.15 (2H, s, CH_2), 2.26 (2H,s,CH_2), 7.33 (1H, dd, *J* 1.0, 8.7 Hz, H-Ar), 7.50 (1H, dd, *J* 1.20, 7.60 Hz, H-Ar), 7.75 (1H, t, *J* 8.0 Hz, H-Ar), 12.0 (1H, s, OH); ^{13}C NMR (100 MHz,

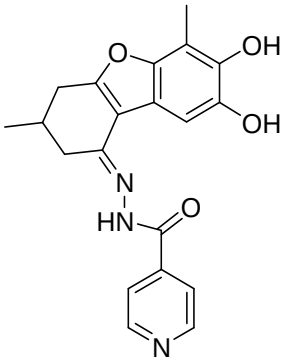
DMSO-d_6): δ 28.8, 29.3, 31.8, 31.6, 109.9, 125.8, 128.6, 129.3, 132.3, 134.6, 135.3, 139.8, 146.6, 183.6

9.1.5 SYNTHESIS OF ISONIAZID DERIVATIVES

METHOD

The synthesis of the isoniazid derivatives was conducted by reacting one equivalent of isoniazid with one equivalent of the 5,6-dihydroxylated benzo[*b*]furan and stir in methanol at 60ºC for 24-44 hours. The reaction was followed by TLC, the disappearance of the 5,6-dihydroxylated benzo[*b*]furan was monitored. The resulting product was purified by flash chromatography. In some cases the product required further purification by either washing with EtOAc or recrystallizing from a mixture of MeOH and EtOAc.

Synthesis of isoniazid derivative 56



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). mp 240°C. ($M+H^+$ Found: 365.1511, $C_{20}H_{20}N_3O_4$ requires $M+H^+$, 365.1566) R_f = 0.35 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.14 (3H, d, *J* 6.4 Hz, CH_3), 2.26 (3H, s, CH_3), 2.29 (1H, dd, *J* 4.7, 12.4 Hz, CH_2), 2.43 (2H, dd *J* 3.4, 12.8 Hz, CH_2), 2.68 (1H, m, CH), 3.03 (1H, dd *J* 4.7, 12.4 Hz, CH), 7.11 (1H, s, ArH), 7.72 (2H, dd, *J* 1.6, 4.4

Hz, ArH), 8.40 (1H, br s, OH), 8.70 (2H, d, *J* 6.0 Hz, ArH),9.31 (1H, br s, OH), 10.01 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 2.0, 23.1, 37.3, 60.3, 99.8, 114.8, 115.5, 120.9, 133.3, 136.1, 140.2, 144.9, 150.1, 163.8, 169.8, 194.4.

9.1.6 SYNTHESIS OF THIOSEMICARBIZIDE DERIVATIVES

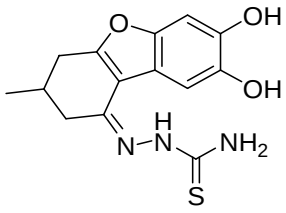
Method A

The benzofuran (**1.0** mmol) was added to a solution of the thiosemicarbazide (1.0 mmol) in methanol (10 ml). The resulting mixture was stirred at 40ºC for 48 h. The reaction mixture was extracted with EtOAc and washed with water (20.0 mL). The product was left to precipitate when the reaction vessel was left standing at room temperature. The residue, a powder, was purified by washing with EtOAc or recrystallizing from a mixture of MeOH and EtOAc.

Method B

This method is the same as Method A except that the resulting mixture was refluxed for 48 h in the presence of a catalytic amount of glacial acetic acid. After completion of the reaction, the reaction mixture was first extracted with EtOAc and then the organic phases were combined, the solvent evaporated and the residue (a powder) purified by flash chromatography.

Synthesis of thiosemicarbizide derivative 65



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). ($M+H^+$ Found: 306.0904, $C_{14}H_{16}N_3O_3S$ requires $M-H^+$, 306.0912). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.12 (3H, d, J 6.40 Hz, CH_3), 2.31 (1H, dd, J 4.6, 12.8 Hz, CH), 2.44 (2H, dd, J 3.6, 12.8 Hz, CH_2), 2.70 (1H, m, CH), 3.03 (1H, dd J 4.6, 12.8 Hz, CH), 4.49 (1H, s, NH), 6.97 (1H, s, ArH), 7.21 (1H, s, ArH) and 8.60 (2H, br s, OH), 9.05 (2H, s, NH_2), ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.6, 30.2, 30.6, 30.9, 45.6, 98.4, 105.3, 114.3, 115.2, 143.6, 144.3, 148.1, 169.2, 193.8.

9.1.7ANTICANCER EVALUATION

The growth inhibitory effects of the compounds were tested in a 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using the Sulforhodamine B (SRB) assay. The SRB assay was developed by Skehan and colleagues to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye, sulforhodamine B (Acid Red 52), to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilised for measurement. The SRB assay is performed at CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for this screen.

MATERIALS AND METHOD

The human cell lines TK10, UACC62 and MCF7 were obtained from the NCI in a collaborative research program between the CSIR and the NCI. cell lines were routinely maintained as a

monolayer cell culture at 37°C, 5% CO_2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50 µg/mL gentamicin. For the screening experiment the cells (3–19 passages) were inoculated in a 96-well microtitre plate at plating densities of 7–10 000 cells/well and were incubated for 24 h. After 24 h one plate was fixed with TCA to represent a measurement of the cell population for each cell line at the time of drug addition (T0). The other plates with cells were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce five concentrations (0.01–100 µM). Cells without drug addition served as control. The blank contains complete medium without cells. Etoposide was used as a reference standard. The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at a wavelength of 540 nm using a multiwell spectrophotometer. Optical density measurements were used to calculate the net percentage cell growth. The optical density of the test well after a 48 h period of exposure to test drug is T_i, the optical density at time zero is T_0, and the control optical density is C. Percentage cell growth is calculated as:

.

$$\frac{[(T_i - T_0)}{(C - T_0)]} \times 100 \text{ for concentrations at which } T_i \geq T_0$$

$$[(T_i - T_0) \times 100 \text{ for concentrations at which } T_i < T_0$$

The results of a five dose screening were reported as TGI (total growth inhibition). The TGI is the concentration of test drug where $100 \times (T - T_0)/(C - T_0) = 0$. The TGI signifies a cytostatic effect. The biological activities were separated into four categories: inactive (GI_{50} or TGI > 100 µM), weak activity (30 µM < GI_{50} or TGI < 100 µM), moderate activity (10 µM < GI_{50} or TGI < 30 µM) and potent activity (GI_{50} or TGI < 10 µM). For each tested compound, three response parameters, GI_{50} (50% growth inhibition and signifies the growth inhibitory power of the test agent), TGI (which is the drug concentration resulting in total growth inhibition and signifies the cytostatic effect of the test agent) and LC_{50} (50% lethal concentration and signifies the cytotoxic effect of the test agent), were calculated for each cell line.

9.1.8 ANTIBACTERIAL SCREENING

Mycobacterium smegmatis - (MC^2 155)

The MICs of the compounds against the four different bacteria were determined in 96 well microtiter plate. A 10.00 mg/mL stock solution of each compound was prepared by dissolving it in 1 mL of DMSO. The stock solution was kept in a refrigerator at +4 ^{0}C. Kanamycin was used as a positive control. The negative control was a well with **7H9 media** and bacterial dilutions. The second negative control was wells with only the media in order to check for contamination. A 1:2 serial dilution of the tested compound was prepared. The initial concentration was 100 µg/ml of the stock solution in well 1 and a final concentration of 0.78 µg/mL in the well. A bacterial suspension was prepared by scraping a small amount of fresh bacteria from a plate onto a wet cotton stick. 7H9 media was used to wet the cotton wool. The bacterium was dispensed by rubbing the wet cotton wool with the bacterium against the walls of a tube with 2.5 mL of 7H9 media. The

tube was vortexed and the OD measured at 600 nm. The standard for OD is 0.3 = 5 x 10^8 CFU/mL, Serial dilutions of the bacterial suspension were made up to the required concentrations(for our experiment: C_1 = 1x10^2/well = 2 x10^3 CFU/mL and C_2 = 1x10^3/well = 2 x10^6 CFU/mL). A volume of 100 µL of the required concentrations of the bacteria was dispensed into each well to have a total volume of 200 µL per well. The concentrations of the bacteria in the wells were determined using a microtitre plate reader. The 96 well plates were then placed in a plastic bag which was incubated at 37°C. The concentrations of the bacteria were read daily for 4-5 days.

7H9 Medium Preparation (7H9 + 0.2% glycerol + 0.05% Tween 80 + ADC)

Middlebrook 7H9 broth base (4.7 g) was added to a 2 L flask to which water (900 mL) was added. Tween 80 (0.5 g) was added to a 100 mL beaker to which broth (10 mL) was added. The mixture was dissolved using moderate heat while mixing. The contents from the 100 mL beaker were then transferred back into the 2 L flask. To ensure that all the contents were transferred to the 2 L flask, the beaker was rinsed several times with broth. Glycerol (2 mL) was then added to the 2 L flask. This was then mixed and 180 mL dispensed into each of five 500 mL screw cap bottles. The latter was then autoclaved for 10 min at 121^o C, allowed to cool and then stored at 2-8^o C. ADC (20 mL) was added to each 180 mL broth before use.

Staphylococcus aureus - 32710 (*MSSA*)

Same procedure but instead of 7H9 media, LB media was used. LB was bought from the chemical store in UMDNJ.

Escherichia coli - DH10B strain

Same procedure but instead of 7H9, LB media was used. LB was bought from the chemical store in UMDNJ., Rifampicin was used Instead of Kanamycin.

Bacillus subtilis - strain no 168

A 96 wells Microtitre plates was used to determine the MICs for bacteria. A 10.00 mg/mL solution of each compound was prepared by dissolving the powder in a required volume of DMSO. For example 10.12 mg of the compound should be dissolved in 1.012 mL of DMSO. Keep stock solution in the refrigerator at +4 °C. Rifampicin was used as a positive control. The negative control was wells with **LB media** and bacteria dilutions. The second negative control is wells with only the media in order to check for contamination. The solutions were diluted to 20 µg/ml. The bacteria suspension was prepared by scraping small amount of fresh bacteria from a plate with a wet cotton stick. LB media was used to make the cotton wet. The bacteria were dispensed by rubbing the wet cotton wool with bacteria against the walls of a tube with 2.5 mL of LB media. The tube was vortexed and then measured the OD at 600 nm. Standard for OD is 0.3 = 5 x 10^8 CFU/mL. Make serial dilutions of the bacterial suspension to the required concentration. (for our experiment C_1 = 1x10^2/well = 2 x10^3 CFU/mL and C_2 = 1x10^3/well = 2 x10^6 CFU/mL). We ispensed(a word?) 100 µL of the required concentrations of the bacteria into each well to have a total volume of 200 µL per well. The negative control was wells with media and bacteria dilutions. The positive control should was with media and kanamycin. We read the concentration of the bacteria in a microtitre plate reader. We put the 96 well plates in a plastic bag and incubate at 37°C. We ead the concentration of the bacteria every day.

Mycobacterium smegmatis - (MC^2 155)

A 96 well microtiter plates was used to determine the MICs for bacteria. A 10.00 mg/mL solution of each compound was prepared by dissolving the powder in a required volume of DMSO. For example 10.12 mg of the compound should be dissolved in 1.012 mL of DMSO. Keep stock solution in the refrigerator at $+4^0$C. Kanamycin was used as a positive control. The negative control was wells with **7H9 media** and bacteria dilutions. The second negative control was wells with only the media in order to check for contamination. A 1:2 serial dilution of the tested compound was prepared. Initial concentration of 100 µg/ml in well 1 and final concentration of 0.78 µg/ml. The bacteria suspension was prepared by scraping small amount of fresh bacteria from a plate with a wet cotton stick. 7H9 media was used to make the cotton wet. The bacteria were dispensed by rubbing the wet cotton wool with bacteria against the walls of a tube with 2.5 mL of 7H9 media. The tube was vortexed and then the OD 600 nm measured . Standard for OD was 0.3 = 5 x 10^8 CFU/mL. Where necessary serial dilutions of the bacterial suspension where made to the required concentration For readings (for our experiment $C_1 = 1x10^2$/well = 2 x10^3 CFU/mL and $C_2 = 1x10^3$/well = 2 x10^6 CFU/mL). Dispensed volumes (100 µL) of the required concentrations of the bacteria into each well had a total volume of 200 µL per well. The negative control comprised of wells with media and bacteria dilutions. The positive control comprised of wells with media and kanamycin. The concentration of the bacteria was determined using a microtiter plate reader at time zero and then daily. The 96 well plates were incubated at 37°C in a plastic bag.

9.2 FULL EXPERIMENTAL

9.2 General

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Mercury 400 MHz spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instrument at 100 MHz. Chemical shifts are reported in parts per million (ppm) relative to the solvent peaks and coupling constants are given in Hertz (Hz).

A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. The PDA detector was used for all purity determinations (Maxplot 200–500 nm). All chemicals for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) while ultra-pure solvents were purchased from Honeywell (Burdick & Jackson, Muskegon, USA). Ultra-pure water was generated using a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France). Reactions were monitored by thin layer chromatography (TLC) on aluminium-backed Merck silica gel 60 F254 plates. Gravity column chromatography was performed using Merck Silica Gel 60 (70–230 mesh).Melting points were determined using a Glassco melting point apparatus and are uncorrected. All other chemicals were reagent grade materials. The 1,3-dicarbonyls were purchased from Sigma–Aldrich, South Africa. Suberase® (10757.8 PCU/mL) is a fungal laccase from *M. thermophila* produced by submerged fermentation of a genetically modified *Aspergillus oryzae*strain. The enzymatic preparation is supplied as a brown liquid which is completely miscible with water. Suberase® was obtained from Novozymes in South Africa.

Methods for the synthesis of the 5,6-dihydroxylated benzo[*b*]furans

Method A

The laccase (Suberase®, 2.0 mL) was added to a mixture of the catechol (2.0 mmol), 1,3-dicarbonyl (2.0 mmol) and phosphate buffer (20.0 mL, 0.10 M, pH 7.15) in a 250-mL round-bottom flask stirred under air at rt. More laccase (2.0 mL) was added after 2, 18 and 20 h. The mixture was vigorously stirred under air until the substrates were consumed as judged by TLC. After stirring the reaction mixture was acidified with 32% HCl to pH 4.0. The mixture was extracted with EtOAc and washed with water (20.0 mL). The organic phases were then combined and the solvent evaporated. The residue, a powder, was purified by washing with EtOAc or recrystallizing from a combination of MeOH and EtOAc.

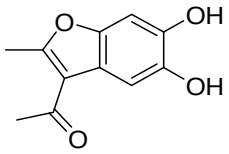
Method B

Same as Method B except that more laccase (2.0 mL) was added after 4, 24 and 28 h.

Method C

The laccase (Suberase®, 1.5 mL) was added to a mixture of the catechol (0.60 mmol), 1,3-dicarbonyl (2.40 mmol), phosphate buffer (4.0 mL, 0.10 M, pH 7.15) and DMF (2.0 mL) in a test tube stirred under air at rt. More laccase (1.5 mL) was added after 2 h and then again after 4 h. The mixture was vigorously stirred under air until the substrates had been consumed as judged by TLC. After stirring the reaction mixture was transferred to a separating funnel and the mixture extracted with EtOAc and washed with water (20.0 mL). The organic phases were combined, the solvent evaporated and the residue (a powder) purified by flash chromatography.

1-(5,6-Dihydroxy-2-methyl-1-benzofuran-3-yl)ethanone 1[121, 122]



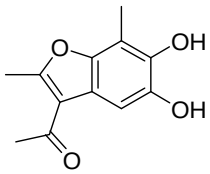
Method A

Stirring time 24 h. The product was recrystallized from 5% MeOH in EtOAc solution at 35°C to afford a dark-brown powder (198 mg, 48%) (Found: M–H^+, 205.0518. $C_{11}H_9O_4$ requires M–H, 205.0501). UPLC 95.5%. R*f* 0.40 (EtOAc/hexane, 1:1). mp 200–203°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.51 (3H, s, CH_3), 2.68 (3H, s, CH_3), 6.91 (1H, s, ArH), 7.33 (1H, s, ArH), 8.94 (1H, br s, OH), 9.06 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.3, 30.8, 97.8, 106.4, 117.2, 117.2, 143.6, 144.2, 147.0, 160.7, 193.9.

Method B

Stirring time 44 h. The product was washed with EtOAc to afford a black powder (202.0 mg, 49%).

1-(5,6-Dihydroxy-2,7-dimethyl-1-benzofuran-3-yl)ethanone 2[121,123]

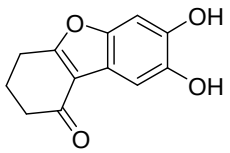


Method A

Stirring time 24 h. The product was washed with EtOAc to afford a red-brown powder (220 mg, 50%) (Found: M–H^+, 219.0615. $C_{12}H_{11}O_4$ requires M–H, 219.0657). UPLC 97.3%. R*f* 0.46

(EtOAc/hexane, 1:1). mp 234°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.19 (3H, s, CH_3), 2.45 (3H, s, CH_3), 2.64 (3H, s, CH_3), 7.17 (1H, s, ArH), 8.38 (1H, br s, OH), 9.25 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 8.9, 15.4, 30.7, 103.2, 107.1, 116.2, 117.4, 141.8, 143.1, 146.5, 160.5, 193.9.

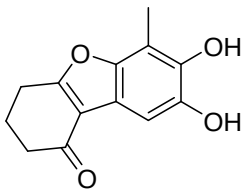
7,8-Dihydroxy-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 3[124, 125]



Method A

Stirring time 24 h. The product was recrystallized from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (284 mg, 65%) (Found: M–H$^+$, 217.0458. $C_{12}H_9O_4$ requires M–H, 217.0501). UPLC 96.2%. R*f* 0.48 (EtOAc/hexane, 1:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.21 (2H, m, CH_2), 2.47 (2H, t *J* 6.4 Hz, CH_2) 2.96 (2H, t, *J* 6.4 Hz, CH_2), 7.00 (1H, s, ArH), 7.22 (1H, s, ArH), 9.18 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.2, 23.2, 37.3, 98.4, 105.4, 114.4, 115.6, 143.7, 144.4, 147.9, 169.7, 194.4.

7,8-Dihydroxy-6-methyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 4[124]



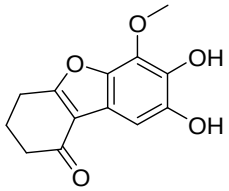
Method A

Stirring time 24 h. The product was recrystallized from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (288 mg, 62%) (Found: M–H$^+$, 231.0683. $C_{13}H_{11}O_4$ requires M–H, 231.0657). UPLC 99.7%. R*f* 0.48 (EtOAc/hexane, 1:1). mp 250°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.14 (2H, m, CH_2), 2.26 (3H, s, CH_3), 2.45 (2H, t, *J* 6.1 Hz, CH_2), 2.98 (2H, t *J* 6.1 Hz, CH_2), 7.11 (s, 1H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0, 22.2, 23.3, 37.9, 102.4, 107.9, 113.5, 115.9, 142.2, 143.4, 147.6, 169.6, 194.6.

Method B

Stirring time 44 h. The product was washed with EtOAc to afford a brown powder (311.0 mg, 67%).

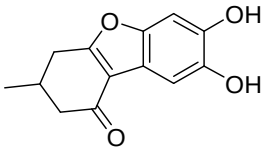
7,8-Dihydroxy-6-methoxy-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 5[124, 126]



Method A

Stirring time 24 h. The product was recrystallized from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (348 mg, 70%) (Found: M–H$^+$, 247.0557. $C_{13}H_{11}O_5$ requires M–H, 247.0606). UPLC 97.0%. R*f* 0.38 (EtOAc/hexane, 2:1). mp 232°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.14 (2H, m, CH_2), 2.46 (2H, t, *J* 6.4 Hz, CH_2), 3.00 (2H, t, *J* 6.4 Hz, CH_2), 3.94 (3H, s, CH_3), 7.00 (1H, s, ArH), 8.71 (1H, br s, OH), 9.24 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ22.1, 23.2, 37.4, 60.4, 99.9, 114.8, 115.6, 133.3, 136.2, 140.3, 145.0, 169.9, 194.5.

7,8-Dihydroxy-3-methyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 6[124, 127-129,131]



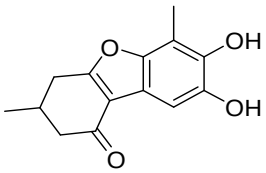
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a brown powder (274 mg, 59%) (Found: M–H$^+$, 231.0610. $C_{13}H_{11}O_4$ requires M–H, 231.0657). UPLC 95.3%. R*f* 0.38 (EtOAc/hexane, 1:1). mp 263°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.11 (3H, d, *J* 6.0 Hz, CH_3), 2.30 (1H, dd, *J* 4.6, 12.8 Hz, CH), 2.43 (2H, dd, *J* 3.6, 12.8 Hz, CH_2), 2.68 (1H, m, CH), 3.01 (1H, dd, *J* 4.6, 12.8 Hz, CH), 6.98 (1H, s, ArH), 7.21 (1H, s, ArH) and 9.15 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.7, 30.3, 40.0, 45.6, 98.5, 105.3, 114.3, 115.3, 143.7, 144.4, 148.2, 169.4 and 194.0.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 0.5:9.5, 1:9, 2.5:7.5, 1:1) to afford a brown powder (84 mg, 30%).

7,8-Dihydroxy-3,6-dimethyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 7[124, 129, 131]



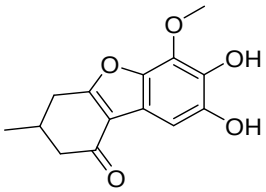
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a light-brown powder (384 mg, 78%) (Found: M–H$^+$, 245.0817. $C_{14}H_{13}O_4$ requires M–H, 245.0814). UPLC 96.0%. R*f* 0.74 (EtOAc/hexane, 1:1). mp 165–168°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.11 (3H, d *J* 6.0 Hz, CH_3), 2.25 (3H, s, CH_3), 2.29 (1H, dd, *J* 4.7, 12.4 Hz, CH_2), 2.43 (2H, dd, *J*3.4, 12.8 Hz, CH_2), 2.68 (1H, m, CH), 3.03 (1H, dd, *J* 4.7, 12.4 Hz, CH), 7.11 (1H, s, ArH), 8.47 (1H, br s, OH), 9.38 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0, 20.7, 30.3, 31.0, 45.6, 102.3, 107.9, 113.4, 115.5, 142.1, 143.3, 147.8, 169.2, 194.1.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 0.5:9.5, 1:9, 1:1; EtOAc) to afford a light-brown powder (209.0 mg, 71%).

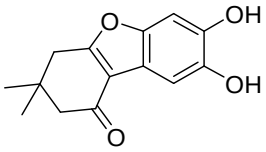
7,8-Dihydroxy-6-methoxy-3-methyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 8[124,126]



Method A

Stirring time 24 h. The product was washed with EtOAc to afford a light-brown powder (194 mg, 37%) (Found: M–H$^+$, 261.0843. $C_{14}H_{13}O_4$ requires M–H, 261.0763). UPLC 99.6%. R*f* 0.50 (EtOAc/hexane, 1:1). mp 179–182°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.12 (3H, d *J* 6.4 Hz, CH_3), 2.32 (1H, dd, *J* 4.0, 12.4 Hz, CH) 2.44 (2H, dd, *J* 3.4, 13.2 Hz, CH_2), 2.72 (1H, m, CH), 3.06 (1H, dd, *J* 5.0, 12.4 Hz, CH), 3.94 (3H, s, CH_3), 6.97 (1H, s, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.7, 30.3, 31.0, 45.7, 60.4, 99.8, 114.8, 115.3, 133.4, 136.3, 140.6, 145.1, 169.6, 194.1.

7,8-Dihydroxy-3,3-dimethyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 9[126]



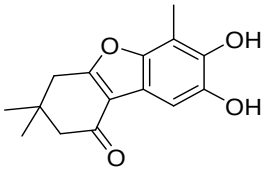
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a yellow powder (286 mg, 58%). (Found: M–H$^+$, 245.0867. $C_{14}H_{14}O_4$ requires M–H, 245.0814). UPLC 99.8%. R*f* 0.64 (EtOAc/hexane, 1:1). mp 278–280°C [lit. 280°C]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.09 (6H, s, 2× CH_3), 2.38 (2H, s, CH_2), 2.88 (2H, s, CH_2), 6.98 (1H, s, ArH), 7.20 (1H, s, ArH), 9.15 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.0 (2× CH_3), 34.9, 36.7, 51.5, 98.5, 105.3, 114.2, 114.4, 143.7, 144.3, 148.3, 168.6, 193.7.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 0.5:9.5, 1:9, 2.5:7.5, 1:1) to afford a yellow powder (118 mg, 40%).

7,8-Dihydroxy-3,3,6-trimethyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 10[124]



Method A

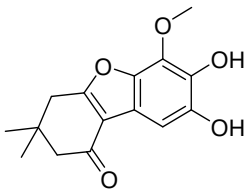
Stirring time 24 h. The product was recrystallized from a 5% MeOH in EtOAc solution at 35°C to afford a light-brown powder (510 mg, 98%). (Found: M–H$^+$ 259.0931. $C_{15}H_{15}O_4$ requires M–H,

259.0970). UPLC 96.6%. R*f* 0.45 (EtOAc/hexane, 1:2). mp 255°C [lit. 260–262°C]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.09 (6H, s, 2 × CH_3), 2.26 (3H, s, CH_3) 2.37 (2H, s, CH_2), 2.90 (2H, s, CH_2), 7.10 (1H, s, ArH), 8.41 (1H, br s, OH), 9.32 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0, 28.0, 34.9, 36.8, 51.5, 102.4, 107.9, 113.3, 114.7, 142.0, 143.3, 168.5, 193.8.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 5:95, 1:9, 1:1) to afford a light-brown powder (184 mg, 59%).

7,8-Dihydroxy-3,3,6-trimethyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 11[124]



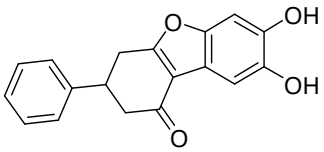
Method A

Stirring time 24 h. The product was recrystallized from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (403 mg, 73%). (Found: M–H$^+$, 275.0922. $C_{15}H_{15}O_5$ requires M–H, 275.0919). UPLC 94.5%. R*f* 0.44 (EtOAc/hexane, 1:1). mp 288–291°C [lit. 289–291°C]. ^{1}H NMR (400 MHz, MeOH-*d4*): δ 1.16 (6H, s, 2 × CH_3), 2.43 (2H, s, CH_2) 2.90 (2H, s, CH_2), 4.06 (3H, s, CH_3), 7.01 (1H, s, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.1, 35.0, 36.8, 51.2, 60.4, 99.8, 114.5, 114.7, 113.4, 136.2, 140.8, 145.1, 168.9, 193.9.

Method B

Stirring time 44 h. The product was washed with EtOAc to afford a brown powder (425 mg, 77%).

- **7,8-Dihydroxy-3-phenyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 12**



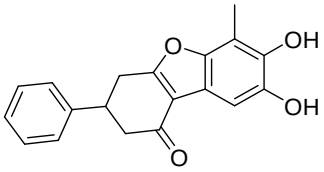
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a white solid (449 mg, 76%). (Found: M–H$^+$, 293.0821. $C_{18}H_{13}O_4$ requires M–H, 293.0814). UPLC 99.8%. R*f* 0.50 (EtOAc/hexane, 1:1). mp 240–243°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.58 (1H, dd *J* 3.2, 16.0 Hz, CH), 2.93 (1H, dd, *J* 12.4, 16.0 Hz, CH), 3.24 (2H, m, H-11), 3.66 (1H, m, CH), 7.01 (1H, s, ArH), 7.26 (1H, s, ArH), 7.35 (2H, t, *J* 7.2, 7.6 Hz, ArH), 7.42 (2H, d *J* 7.6 Hz, ArH), 9.09 (1H, s, CH), 9.13 (1H, s, CH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.7, 44.6, 62.5, 63.4, 68.8, 71.5, 72.3, 73.8, 98.5, 105.4, 114.2, 115.5, 126.9, 128.6, 143.1, 143.9, 144.5, 148.3, 169.0, 193.1.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 5:95, 1:9, 1:1; EtOAc) to afford a white solid (132 mg, 50%).

7,8-Dihydroxy-6-methyl-3-phenyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 13[124]

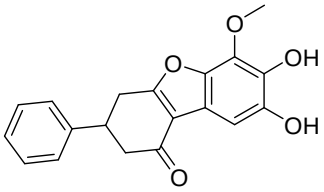


Method A

Stirring time 24 h. The product was recrystallized from 5% MeOH in EtOAc solution at 35°C to afford a light-brown powder (279 mg, 80%) (Found: M–H$^+$, 307.0977.1120. $C_{19}H_{15}O_4$ requires

M–H$^+$, 307.0970). UPLC 99.0%. R*f* 0.45 (EtOAc/hexane, 1:1). mp 250–253°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.28 (3H, s, CH_3), 2.59 (1H, dd *J* 4.0, 16.4 Hz, CH), 2.91 (1H, dd, *J* 12.2, 16.2 Hz, CH), 3.28 (1H, m, CH_2), 3.66 (1H, m, H-11 CH), 7.16 (1H, s, ArH), 7.26 (1H, t *J* 7.0 Hz, ArH), 7.35 (2H, t, *J* 7.6 Hz, ArH), 7.42 (2H, d, *J* 8.0 Hz, ArH), 8.45 (1H, br s, OH), 9.36 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.1, 30.7, 44.7, 102.4, 108.0, 113.4, 115.8, 126.9, 127.0, 127.1, 128.6, 142.3, 143.2, 143.4, 148.0, 168.8, 193.2.

7,8-Dihydroxy-6-methoxy-3-phenyl-3,4-dihydrodibenzo[*b,d*]furan-1(2*H*)-one 14



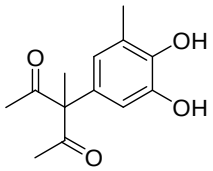
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a dull grey powder (279 mg, 43%). (Found: M–H$^+$, 323.0922. $C_{19}H_{15}O_5$ requires M–H, 323.0919). UPLC 96.8%. R*f* 0.38 (EtOAc/hexane, 1:2). mp 165–168°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.57 (1H, dd *J* 4.0, 16.0 Hz, CH), 2.93 (1H, dd, *J* 12.0 Hz, 12.4 Hz, CH), 3.26 (2H, m, CH_2), 3.67 (1H, m, CH), 3.95 (3H, s, CH_3), 7.03 (1H, s, ArH), 7.26 (1H, t, *J* 7.2 Hz, ArH), 7.35 (2H, t, *J* 7.2, 8.0 Hz, ArH), 7.42 (2H, d, *J* 8.0 Hz, ArH), 8.76 (1H, br s, OH) and 9.33 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.6, 44.6, 60.4, 99.8, 114.6, 115.5, 126.8, 127.0, 128.5, 133.4, 136.2, 140.6, 143.0, 145.0, 169.0, 193.0.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 5:95, 1:9, 1:1; EtOAc) to afford a black powder (58.0 mg, 15%).

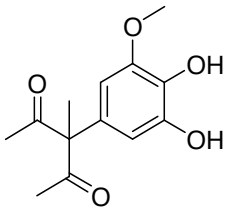
3-(3,4-Dihydroxy-5-methylphenyl)-3-methylpentane-2,4-dione 15



Method A

Stirring time 24 h. The product was washed with EtOAc to afford a dark-brown powder (1.60 g, 80%) (Found: M–H$^+$, 235.0926. $C_{13}H_{14}O_4$ requires M–H, 235.0970. UPLC 98.0%. R*f* 0.48 (EtOAc/hexane, 1:1). mp 82–85°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.60 (3H, s, CH_3), 2.03 (6H, s, CH_3), 2.08 (3H, s, CH_3), 6.43 (1H, d, *J* 2.0 Hz, ArH), 6.46 (1H, d, *J* 2.4 Hz, ArH), 8.36 (1H, br s, OH), 9.29 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.2, 19.0, 27.2, 30.7, 68.8, 112.4, 120.1, 124.7, 127.7, 143.0, 144.8, 207.8.

3-(3,4-Dihydroxy-5-methoxyphenyl)-3-methylpentane-2,4-dione 16



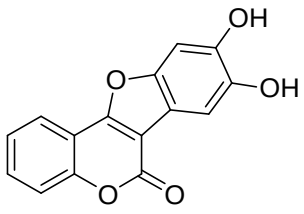
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a dark-brown powder (1.10 g, 55%) (Found: M–H$^+$, 251.0933. $C_{13}H_{14}O_5$ requires M–H, 251.0919. R*f* 0.48 (EtOAc/hexane, 1:1). mp 108–110°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.62 (3H, s, CH_3), 2.05 (6H, s, CH_3), 3.74 (3H, s, OMe), 6.27 (1H, s, ArH), 6.28 (1H, s, ArH), 8.44 (1H, br s, OH), 9.03 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.1, 27.3, 56.0, 69.0, 103.1, 108.7, 127.7, 133.9, 145.8, 148.5, 207.7

METHOD A

The laccase (Suberase®, 2.0 mL) was added to a mixture of the catechol (2.0 mmol), coumarin (2.0 mmol) and phosphate buffer (20.0 mL, 0.10 M, pH 7.15) in a 250 mL round bottom flask stirred under air at rt. More laccase (2.0 mL) was added after 2, 18 and 20 h. The mixture was vigorously stirred under air until the substrates were consumed as judged by TLC. The mixture was extracted with EtOAc and washed two times with water (20.0 mL). The organic phases were then combined and the solvent evaporated. The residue, a powder, was purified by washing with EtOAc, flash chromatography or recrystallization from a combination of MeOH and EtOAc. Products were characterized by ^{1}H NMR, ^{13}C NMR, and MS.

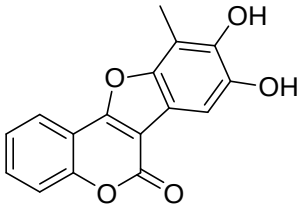
- **8,9-Dihydroxy-6H-benzofuro[3,2-c]chromen-6-one 17[154-158]**



Method A. Stirring time = 24 h. Purification by a wash with EtOAc and the flash chromatography (silica: EtOAc/hexane, 1:5, 1:4, 1:3, 1:2 and EtOAc) to afford a light-brown solid (0.4600 g, 86.0%). (M-H^{+} Found: 267.0216. $C_{15}H_7O_5$ requires M-H^{+}, 267.0293). UPLC 99.8 %. R_f 0.54 (EtOAc/Hex, 1:2). mp 300^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.24 (1H, s, ArH). 7.31 (1H,

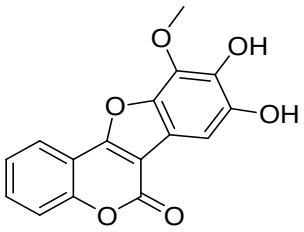
s, ArH), 7.48 (1H, t, *J* 8.0 Hz, ArH), 7.58 (1H, d, *J* 8.0 Hz, ArH), 7.66 (1H, ddd, *J* 1.6, 8.0, 15.6 Hz, ArH), 8.00 (1H, dd, *J*, 1.6, 8.0 Hz, ArH; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 98.9, 104.9, 105.5, 112.4, 113.8, 117.0, 121.1, 124.8, 131.2, 144.7, 146.6, 149.4, 152.3, 157.4, 157.7.

- **8,9-Dihydroxy-10-methyl-6H-benzofuro[3,2-c]chromen-6-one 18[156,157]**



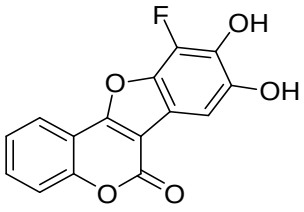
Method A. Stirring time = 24 h. Purification by washing with EtOAc and recrystallization from a mixture of methanol and hexane at 42ºC to afford a grey crystalline product (0.429 g, 76.0%). M+H$^+$ Found: 283.0638, $C_{16}H_{11}O_5$ requires M+H$^+$, 283.0606). UPLC 98.8%. R_f 0.38 (EtOAc/hexane, 1:2). mp 298ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.41 (3H, s, CH_3), 7.21 (1H, s, ArH), 7.46 (1H, t, *J* 7.6 Hz, ArH), 7.58 (1H, d, *J* 7.6 Hz, ArH), 7.66 (1H, t, *J* 8.0 Hz, ArH), 8.05 (1H, dd, *J* 1.6, 8.0 Hz, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 9.51, 102.3, 106.2, 108.9, 113.0, 117.5, 121.7, 125.4, 131.7, 144.8, 149.5, 152.8, 158.1.

- **8,9-Dihydroxy-10-methoxy-6H-benzofuro[3,2-c]chromen-6-one 19[156]**



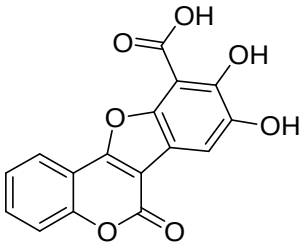
Method A. Stirring time = 24 h. Purification by washing with EtOAc and recrystallization from a mixture of methanol and hexane at 42ºC to afford a light-brown crystalline product (0.441 g, 74.0%). ($M+H^+$ Found: 299.0519, $C_{16}H_{11}O_6$ requires $M+H^+$, 299.0556). UPLC 95.1%. R_f 0.38 (EtOAc/hexane, 1:2). mp 234ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 4.10 (3H, s, OMe), 7.08 (1H, s, ArH), 7.49 (1H, t, *J* 7.80 Hz, ArH), 7.58 (1H, d, *J* 8.40 Hz, ArH), 7.69 (1H, t, *J* 8.4 Hz, ArH), 8.07 (1H, dd, *J* 1.4, 7.8 Hz, ArH), 9.10 (1H, s, ArH), 9.61 (1H, s, ArH).^{13}C NMR (100 MHz, DMSO-d_6): δ = 60.6, 99.3, 105.5, 112.3, 114.2, 117.0, 121.3, 124.9, 131.4, 133.6, 138.1, 141.7, 145.8, 152.4, 157.4, 157.9.

- **10-Fluoro-8,9-dihydroxy-6H-benzofuro[3,2-c]chromen-6-one 20**



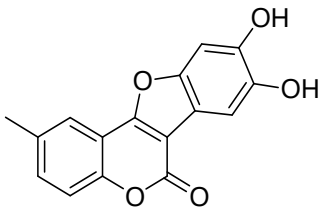
Method A. Stirring time = 24 h. Purification by a wash with EtOAc to afford a light brown solid (0.412 g, 72.0%). ($M+H^+$ Found: 287.0299, $C_{15}H_8O_5F$ requires $M+H^+$, 287.0356). UPLC 94.0%. R_f = 0.41 (EtOAc/hexane, 1:1). mp 142ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.58 (1H, s, ArH), 7.34 (1H, m, ArH), 7.64 (1H, ddd, *J* 1.6, 7.6, 9.6 Hz, ArH), 7.83 (1H, dd *J* 1.6, 7.6 Hz, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = ^{13}C NMR (100 MHz, DMSO-d_6): δ =59.6, 62.5, 68.3, 71.4, 72.3, 73.7, 85.1, 115.4, 121.2, 124.0, 124.2, 129.6, 154.5, 164.6, 174.4.

- **8,9-dihydroxy-6-oxo-6H-benzofuro[3,2-c]chromene-10-carboxylic acid 21**



Method B. Stirring time = 24 h. The product precipitated, was filtered and washed by EtOAc to afford a light-brown solid (0.486 g, 78.0%). ($M+H^+$ Found: 313.0306, $C_{16}H_9O_7$ requires $M+H^+$, 313.0348). UPLC 96.9%. R_f 0.38 (EtOAc/Hex, 2:1). mp 170^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.13 (1H, s, ArH), 7.41 (1H, t, *J* 7.20 Hz, ArH), 7.50 (1H, d, *J* 8.4 Hz, ArH), 7.60 (1H, t, *J* 8.40 Hz, ArH), 7.96 (1H, d, *J* 7.20 Hz, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): 98.4, 106.6, 111.7, 112.3, 116.4, 121.0, 124.3, 130.9, 147.3, 147.6, 152.1, 155.2, 127.2, 168.4.

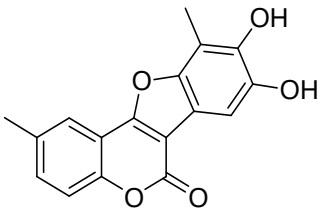
- **8,9-Dihydroxy-2-methyl-6H-benzofuro[3,2-c]chromen-6-one 22**



Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a brown solid (0.440 g, 78.0%). ($M+H^+$ Found: 283.0512, $C_{16}H_{11}O_5$ requires $M+H^+$, 283.0606). UPLC 88.4%. R_f 0.47 (EtOAc/hexane, 1:1). mp 283^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.41 (3H, s, CH_3), 7.21 (1H, s, ArH). 7.28 (1H, s, ArH), 7.42 (2H, d, *J* 1.2 Hz, ArH), 7.73 (1H, s, ArH), 9.61 (2H, s,

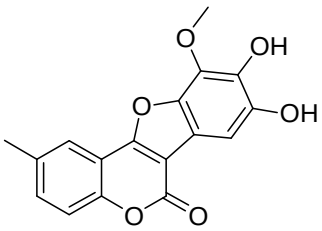
2 x OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 20.4, 98.9, 104.9, 105.3, 112.0, 113.9, 116.8, 120.6, 132.1, 134.3, 144.7, 146.5, 149.4, 150.5, 157.6, 157.7.

- **8,9-Dihydroxy-2,10-dimethyl-6H-benzofuro[3,2-c]chromen-6-one 23[154]**



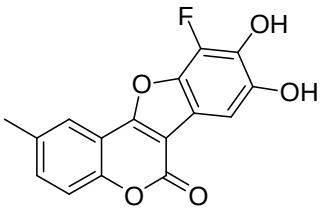
Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a brown solid (0.468 g, 79.0%). (M+H$^+$ Found: 297.0708, $C_{17}H_{13}O_5$ requires M+H$^+$, 297.0763). UPLC 80.1%. R_f 0.38 (EtOAc/hexane, 1:2). mp 234^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.41 (3H, s, CH_3), 2.46 (3H, s, CH_3) 7.20 (1H, s, ArH), 7.47 (1H, d, *J* 1.2 Hz, ArH), 7.85 (1H, d, *J* 0.8 Hz, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 8.9, 20.3, 101.9, 105.6, 108.5, 112.1, 113.0, 116.8, 120.6, 132.1, 134.3, 144.1, 149.0, 150.5, 157.6.

- **8,9-Dihydroxy-10-methoxy-2-methyl-6H-benzofuro[3,2-c]chromen-6-one 24[155]**



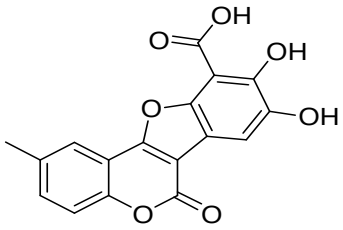
Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a grey solid (0.488 g, 78.0%). (M+H$^+$ Found: 313.0609, $C_{17}H_{13}O_6$ requires M+H$^+$, 313.0712). UPLC 99.2%. R_f 0.30 (EtOAc/hexane, 1:1). mp 260^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.45 (3H, s, CH_3), 4.09 (3H, s, OMe), 7.07 (1H, s, ArH). 7.47 (1H, s, ArH), 7.87 (1H, s, ArH), 9.09 (1H, s, OH), 9.59 (1H, s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 20.2, 20.3, 60.5, 60.7, 99.3, 105.4, 112.0, 114.3, 116.8, 120.7, 120.9, 133.6, 134.4, 138.0, 141.7, 145.8, 150.6, 157.5, 158.0.

- **10-Fluoro-8,9-dihydroxy-2-methyl-6H-benzofuro[3,2-c]chromen-6-one 25**



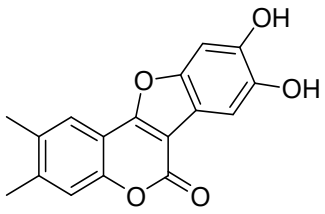
Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a light-brown solid (0.431 g, 72.0%). (M-H$^+$ Found:, $C_{16}H_8O_5F$ requires M-H$^+$, 299). UPLC 89 %. R_f 0.38 (MeOH/EtOAc, 1:10). mp 142^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.30 (3H, s, CH_3), 5.56 (1H, s, ArH). 7.25 (1H, d, *J* 8.4 Hz, ArH), 7.44 (1H, dd, *J* 1.8, 8.4 Hz, ArH), 7.61 (1H, d, *J* 1.2 Hz, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 20.8, 91.4, 115.9, 116.7, 123.2, 133.6, 133.9, 152.1, 162.4, 166.1.

8,9-dihydroxy-2-methyl-6-oxo-6H-benzofuro[3,2-c]chromene-10-carboxylic acid 26



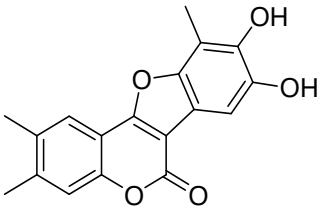
Method B. Stirring time = 24 h. Purification by a wash with EtOAc to afford a light yellow solid (0.493 g, 76.0%). (M-H^+ Found: 325.0254 $C_{17}H_9O_7$ requires M-H^+, 325.0348). UPLC 95.0%. R_f 0.41 (EtOAc:Hex, 2:1). mp = 180 ^oC; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.37 (3H, s, CH_3), 7.08 (1H, s, H-Ar), 7.45 (2H, m, H-Ar), 7.76 (1H, s, H-Ar); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 20.3, 97.6, 107.0, 112.1, 116.0, 120.5, 131.5, 133.5, 147.1, 147.4, 150.2, 153.0, 155.1, 156.9, 168.0.

8,9-Dihydroxy-2,3-dimethyl-6H-benzofuro[3,2-c]chromen-6-one 27



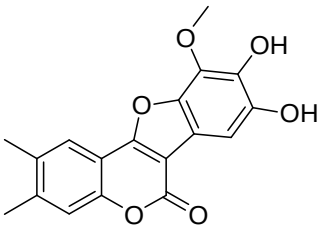
Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a brown solid (0.461 g, 78.0%). (M+H^+ Found: 297.0768 $C_{17}H_{13}O_5$ requires M+H^+, 297.0763). UPLC 99%. R_f 0.56 (EtOAc/hexane, 1:2). mp 272 ^oC; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.32 (3H, s, CH_3), 2.33 (3H, s, CH_3), 7.19 (1H, s, ArH), 7.27 (1H, s, ArH), 7.34 (1H, s, ArH), 7.71(1H, s, ArH), 9.49 (2H, s, 2 x OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 18.7, 19.7, 98.8, 104.5, 104.8, 109.8, 113.9, 117.3, 120.8, 133.5, 141.1, 144.5, 146.2, 150.8, 157.6, 158.1.

8,9-Dihydroxy-2,3,10-trimethyl-6H-benzofuro[3,2-c]chromen-6-one 28



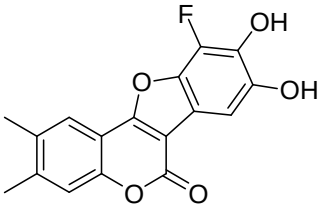
Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a brown solid (0.459 g, 74.0%). ($M+H^+$ Found: 311.0939 $C_{18}H_{15}O_5$ requires $M+H^+$, 311.0919). UPLC 92%. R_f 0.52 (EtOAc/hexane, 1:2). mp 280ºC; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.35 (6H, s, 2 × CH_3), 2.40 (3H, s, CH_3), 7.18 (1H, s, ArH). 7.38 (1H, s, ArH), 7.79 (1H, s, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 8.9, 18.6, 19.7, 101.8, 104.8, 108.5, 109.9, 113.1, 117.4, 120.8, 133.5, 141.0, 143.9, 144.0, 148.7, 150.8, 157.7, 158.0.

8,9-Dihydroxy-10-methoxy-2,3-dimethyl-6H-benzofuro[3,2-c]chromen-6-one 29



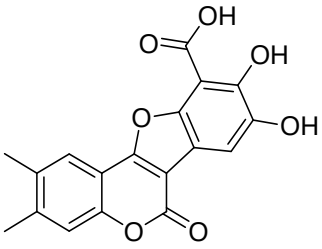
Method A. Stirring time = 24 h. Purification by wash with EtOAc to afford a brown solid (0.500 g, 76.0%). ($M-H^+$ Found: 325.0667. $C_{18}H_{13}O_6$ requires $M-H^+$, 325.0712). UPLC 99.0%. R_f 0.38 (EtOAc/hexane, 1:2). mp = 274 ºC; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.37 (6H, s, CH_3), 4.08 (3H, s, OMe), 7.06 (1H, s, ArH). 7.41 (1H, s, ArH), 7.85 (1H, s, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): ^{13}C NMR (100 MHz, DMSO-d_6): δ = 62.5, 63.0, 63.4, 68.8, 71.4, 72.3, 73.7, 93.0, 127.3, 128.4, 128.7, 128.8, 132.6, 185.0.

10-Fluoro-8,9-dihydroxy-2,3-dimethyl-6H-benzofuro[3,2-c]chromen-6-one 30



Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a brown solid (0.500 g, 76.0%). ($M+H^+$ Found: 314.0588. $C_{17}H_{11}O_5F$ requires $M+H^+$, 314.0591). UPLC 93%. R_f 0.58 (EtOAc/hexane, 1:1). mp = 174 ^oC; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.26 (3H, s, CH_3), 2.30 (3H, s, CH_3), 5.46 (1H, s, ArH), 7.15 (1H, s, ArH), 7.56 (1H, s, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 18.7, 19.6, 62.5, 63.4, 68.8, 71.4, 72.3, 73.7, 89.9, 113.6, 116.7, 123.0, 132.1, 142.1, 152.0, 162.3, 166.2.

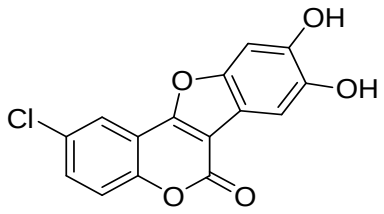
- **8,9-dihydroxy-2,3-dimethyl-6-oxo-6H-benzofuro[3,2-c]chromene-10-carboxylic acid 31**



Method B. Stirring time = 24 h. Purification by a wash with EtOAc to afford a light-grey solid (0.483 g, 74.0%). ($M+H^+$ Found: 341.0594. $C_{18}H_{12}O_7$ requires $M+H^+$, 341.0661). UPLC 82%. R_f 0.45 (EtOAc:Hex). Mp = 140 ^oC; ^1H NMR (400 MHz, DMSO-d_6): δ = 2.27 (3H, s, CH_3), 2.30

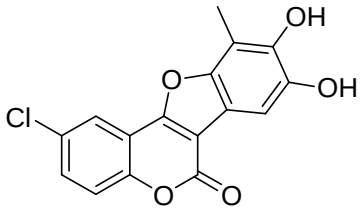
(3H, s, CH_3), 5.43 (1H, s, H-Ar), 7.13 (1H, s, H-Ar), 7.56 (1H, s, H-Ar); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 18.7, 19.7, 90.1, 90.2, 113.3, 116.8, 122.9, 123.0, 132.4, 142.4, 152.0 162.3, 165.8.

2-Chloro-8,9-dihydroxy-6H-benzofuro[3,2-c]chromen-6-one 32



Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a dark brown solid (0.442 g, 73.0%). (M+H$^+$ Found: 303.0002. $C_{15}H_8O_5Cl$ requires M+H$^+$, 303.0060). UPLC 88%. R_f 0.32 (EtOAc/hexane, 1:2). mp = 273^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 7.22 (H, s, ArH), 7.29 (1H, s, ArH), 7.61 (1H, d, *J* 9.0 Hz, ArH), 7.68 (1H, d, *J* 8.4 Hz, ArH), 8.02 (1H, s, ArH), 8.02 (2H, s, 2 × OH) ; ^{13}C NMR (600 MHz, DMSO-d_6): δ = 98.8, 98.9, 104.8, 106.3, 113.6, 113.8, 120.2, 120.3, 128.9, 144.9, 147.0, 149.6, 149.6, 150.8, 156.4, 157.0.

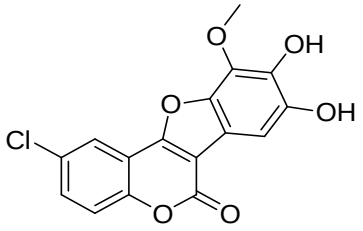
2-Chloro-8,9-dihydroxy-10-methyl-6H-benzofuro[3,2-c]chromen-6-one 33



Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a brown solid (0.494 g, 78.0%). (M+H$^+$ Found: 315.0018. $C_{16}H_8O_5Cl$ requires M+H$^+$, 315.0060). UPLC 99%. R_f 0.46 (EtOAc/hexane, 1:2). mp = 210^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.41 (3H, s, CH_3)

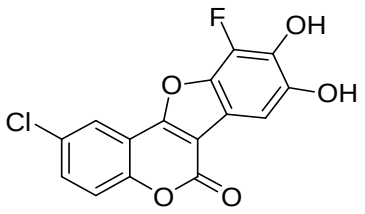
7.20 (1H, s, ArH), 7.61 (1H, d, *J*, 8.80 Hz, ArH), 7.68 (1H, dd, *J* 2.20, 8.80 Hz, ArH), 8.08 (1H, dd, s, ArH), 9.00 (1H, s, OH), 9.83 (1H, s, OH);^{13}C NMR (100 MHz, DMSO-d_6): δ = 62.5, 63.1, 63.4, 68.8, 71.4, 72.3, 73.9, 90.2, 109.6, 118.4, 119.3, 122.7, 127.5, 131.7, 152.4, 162.2, 167.0.

- **2-Chloro-8,9-dihydroxy-10-methoxy-6H-benzofuro[3,2-c]chromen-6-one 34**



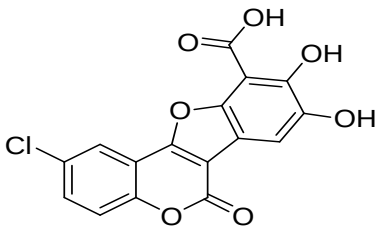
Method A. Stirring time = 24 h. Purification by washing with EtOAc to afford a dark-brown solid (0.494 g, 78.0%). (M-H$^+$ Found: 330.9915. $C_{16}H_8O_6Cl$ requires M-H$^+$, 331.0009). UPLC 98.6%. R_f 0.36 (EtOAc:Hex, 1:2). mp = 193-195 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 4.11 (3H, s, OMe) 7.01 (1H, s, ArH), 7.62 (1H, d, *J*, 9.00 Hz, ArH), 7.69 (1H, d, *J* 9.0 Hz, ArH), 8.13 (1H, d, *J*, 1.80 Hz, ArH);^{13}C NMR (600 MHz, DMSO-d_6): δ = 60.6, 60.7, 99.1, 106.3, 113.7, 114.0, 120.5, 120.5, 128.9, 133.6, 138.4, 141.9, 146.0, 150.9, 156.6, 157.1.

- **2-Chloro-10-fluoro-8,9-dihydroxy-6H-benzofuro[3,2-c]chromen-6-one 35**



Method A. Stirring time = 24 h. Purification by a washing with EtOAc to afford a light-brown solid (0.448 g, 70.0%). (M-H^+ Found: 313.0692 $C_{15}H_5O_4FCl$ requires M-H^+ 313.0643). UPLC 90.6%. R_f 0.36 (EtOAc/hexane, 1:1). mp = 192^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.57 (1H, s, ArH), 7.40 (1H, d, *J* 8.40 Hz, ArH), 7.66 (1H, dd, *J* 2.4, 8.4 Hz, ArH), 7.77 (1H, d, *J* 2.4 Hz, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 62.5, 63.4, 68.8, 71.4, 72.3, 73.7, 85.2, 117.6, 123.5, 125.2, 125.2, 125.7, 129.3, 153.0, 164.2, 173.0.

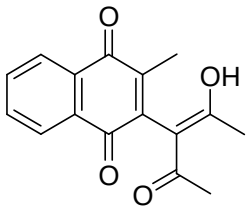
- **2-chloro-8,9-dihydroxy-6-oxo-6H-benzofuro[3,2-c]chromene-10-carboxylic acid 36**



Method B. Stirring time = 24 h. Purification by a wash with EtOAc to afford a yellow solid (0.527 g, 76.0%). (M+H^+ Found: 346.9887 $C_{16}H_8O_7Cl$ requires M+H^+, 346.9959). UPLC 98.1%. R_f 0.31 (EtOAc/Hex, 2:1). mp 152^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 7.13 (1H, s, H-Ar), 7.52 (1H, d, *J* 8.80 Hz, H-Ar), 7.60 (1H, dd, *J* 2.4, 8.80 Hz, H-Ar), 7.96 (1H, d, *J* 2.40 Hz, H-Ar), ^{13}C

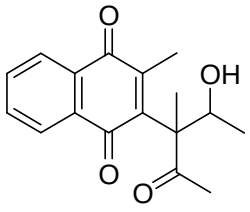
NMR (100 MHz, DMSO-d_6): δ = 98.1, 107.6, 109.0, 111.9, 113.7, 118.3, 120.1, 128.1, 130.1, 147.5, 147.9, 150.5, 154.6, 155.6, 168.4.

2-((E)-2-hydroxy-4-oxopent-2-en-3-yl)-3-methylnaphthalene-1,4-dione 37



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization into afford a light yellow solid (M-H$^+$ Found: 271.0872, $C_{16}H_{15}O_4$ requires M-H$^+$, 271.0970). UPLC 97.8%. Rf = 0.59 (EtOAc/Hex, 1:2). mp 170°C ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.14 (3H, s, CH_3), 2.22 (3H, s, CH_3), 2.44 (3H, *s*, CH_3), 7.47 (2H, *m*, ArH) 7.64 (1H, dd *J* 1.8, 7.8, ArH), 8.20 (1H, dd *J* 1.8, 7.8, ArH), 9.06 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 12.9, 14.0, 20.3, 42.4, 118.8, 121.0, 122.1, 124.9, 125.0, 125.1, 125.2, 125.8, 137.8, 147.5, 169.5, 205.0.

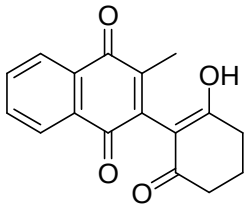
2-methyl-3-(3-methyl-2,4-dioxopentan-3yl)naphthalene-1,4-dione 38



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization into afford a light brown solid (M-H$^+$ Found: 283.2972, $C_{17}H_{15}O_4$ requires M-H$^+$, 283.2970). UPLC 90.1%. %. Rf = 0.55 (EtOAc/Hex, 1:2). mp 165°C δ = 2.10 (3H, s, CH_3), 2.21 (3H, s, CH_3), 2.91 (6H, *s*, 2 x

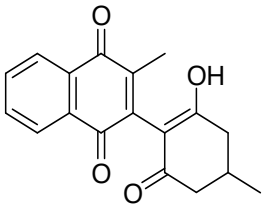
CH_3), 7.60 (1H, dd *J* 1.7, 7.8, ArH), 8.30 (1H, dd *J* 1.7, 7.8, ArH), 9.03 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.9, 17.1, 25.7, 25.8, 43.0, 79.2, 130.4, 130.5, 132.0, 132.2, 135.1, 135.3, 142.1, 147.2, 183.2, 183.3, 201.8

2-(2-hydroxy-6-oxocyclohex-1-enyl)-3-methylnaphthalene-1,4-dione 39



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid. (M-H$^+$ Found: 281.2299, $C_{17}H_{13}O_4$ requires M-H$^+$, 281.2289). UPLC 89.6%. R_f 0.52. (EtOAc/Hex, 1:2). mp 180ºC ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.42 (2H, m, CH_2), 1.90 (3H, s, CH_3),1.94 (3H, t, J CH_2), 2.92 (2H, t, CH_2), 7.72 (2H, dd, *J* 3.4, 5.4 Hz, ArH), 7.96 (1H, dd, *J* 3.4, 5.4 Hz, ArH), 11.8 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.5, 20.1, 31.3, 36.5, 107.1, 130.4, 130.6, 131.9, 134.9, 135.1, 140.0, 177.4, 183.1, 184.7, 194.5

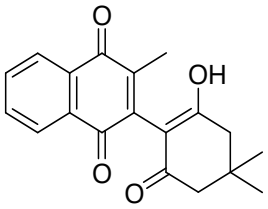
2-(2-hydroxy-4-methyl-6-oxocyclohex-1-enyl)-3-methylnaphthalene-1,4-dione 40



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid. (M-H$^+$ Found: 295.3188, $C_{18}H_{15}O_4$ requires M-H$^+$, 295.3180). UPLC 85.7%. R_f 0.50. (EtOAc/Hex, 1:2). mp 185ºC δ 1.10 (3H, d *J* 6.0 Hz, CH_3), 1.93 (3H, s, CH_3), 2.10 (1H, dd, *J* 4.7,

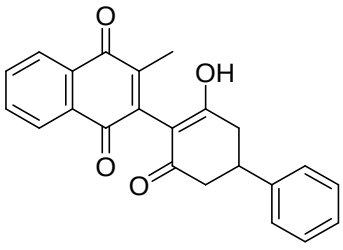
12.4 Hz, CH_2), 2.43 (1H, dd, *J* 3.4, 12.8 Hz, CH_2), 2.70 (1H, m, CH), 2.79 (1H, dd, *J* 3.4, 12.8 Hz, CH2), 3.03 (1H, dd, *J* 4.7, 12.4 Hz, CH2), 7.73 (2H, dd, *J* 3.4, 5.4 Hz, ArH), 8.01 (1H, dd, *J* 3.4, 5.4 Hz, ArH), 12.1 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.8, 20.9, 23.8, 41.3, 47.0, 106.9, 130.2, 130.5, 136.1, 136.9, 132.9, 133.2, 140.2, 147.9, 177.6, 183.1, 184.7, 194.5.

2-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-enyl)-3-methylnaphthalene-1,4-dione 41



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid. (M-H^+ Found: 309.1012, $C_{22}H_{13}O_2$ requires M-H^+, 309.0916). UPLC 99.6%. R_f 0.52. (EtOAc/Hex, 1:2). mp 230ºC ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.09 (3H, s, CH_3), 1.16 (3H, s, CH_3), 1.90 (3H, s, CH_3),2.36 (4H, s, 2 x CH_2), 7.85 (2H, dd, *J* 3.4, 5.4 Hz, ArH), 7.96 (1H, dd, *J* 3.4, 5.4 Hz, ArH), 8.04 (1H, dd, *J* 3.4, 5.4 Hz, ArH), 10.8 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.9, 27.7, 28.0, 31.9, 108.9, 125.8, 125.8, 131.6, 132.0, 133.7, 133.9, 141.6, 145.4, 182.5, 184.6.

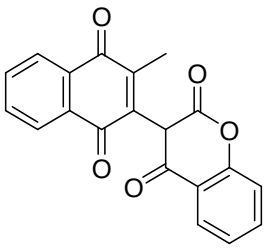
2-(2-hydroxy-6-oxo-4-phenylcyclohex-1-enyl)-3-methylnaphthalene-1,4-dione 42



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallisation to afford a yellow solid (M-H^+ Found: 357.1061, $C_{23}H_{17}O_4$ requires M-H^+, 357.1127). UPLC 99.4%. R_f 0.34

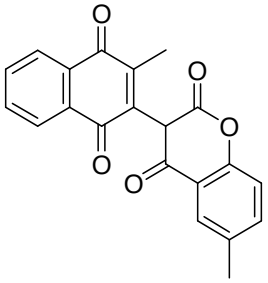
(EtOAc/Hex, 1:2). mp 239ºC ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.90 (3H, s, CH_3), 2.60 (2H, dd, CH_2), 2.80 (2H, dd, CH_2), 3.45 (1H, m, CH), 7.23 (1H, t, *J* 7.2 Hz, ArH), 7.33 (2H, t *J* 7.2 Hz, ArH), 7.40 (2H, dd, *J* 7.8, 21.6 Hz, ArH), 7.83 (2H, dd, *J* 3.3, 5.7 Hz, ArH), 7.95 (1H, d *J* 5.4 Hz, ArH), 8.01 (1H, dd, *J* 4.2, 9.0 Hz, ArH), 11.0 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.9, 38.2, 38.3, 109.7, 125.7, 125.8, 126.6, 126.7, 126.9, 127.0, 128.4, 128.5, 131.6, 131.9, 132.1, 133.6, 133.9, 143.4, 145.3, 182.3, 184.7.

2-(4-hydroxy-2-oxo-2H-chromen-3-yl)-3-methylnaphthalene-1,4-dione 43



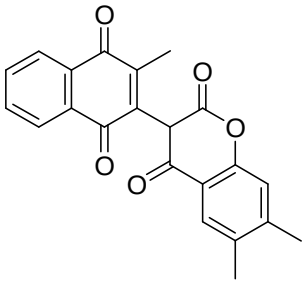
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid. UPLC 89.3%. R_f 0.42 (EtOAc/Hex, 1:2). mp 230ºC ($M+H^+$ Found: 333.0658, $C_{20}H_{13}O_5$ requires $M+H^+$, 333.0552). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.97 (3H, s, CH_3), 2.34 (3H, s, CH_3), 7.08 (1H,d, *J* 8.40 Hz, ArH), 7.27(1H, d, *J* 8.40 Hz, ArH), 7.61 (1H, s, ArH), 7.81 (2H, dd, *J* 3.20, 5.60 Hz, ArH), 7.95 (1H,dd, *J* 3.60, 5.60 Hz, ArH), 7.95 (1H, dd, *J* 3.6, 4.8 Hz, ArH), 8.02 (1H, dd, *J* 3.20, 5.60 Hz, ArH). δ = 13.9, 18.3, 95.2, 114.4, 120.2, 127.0, 136.5, 158.3, 159.5, 130.5, 130.9, 132.9, 133.0, 136.1, 136.4, 140.1, 160.2, 177.5, 183.1

2-(4-hydroxy-6-methyl-2-oxo-2H-chromen-3-yl)-3-methylnaphthalene-1,4-dione 44



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallisation to afford a brown solid. UPLC 90.1%. R_f 0.41 (EtOAc/Hex, 1:2). mp 234ºC, (M-H^+ Found: 345.1011, $C_{21}H_{13}O_5$ requires M-H^+, 345.1076). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.00 (3H, s, CH_3), 7.15 (2H,t, *J* 7.80 Hz, ArH), 7.44 (1H, t, *J* 7.20 Hz, ArH), 7.80 (2H, dd, J 3.6, 6.0 Hz, ArH), 7.84 (1H,d, *J* 7.80 Hz, ArH), 7.95 (1H, dd, *J* 3.6, 4.8 Hz, ArH), 8.01 (1H, dd, *J* 3.6, 5.1 Hz, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 14.2, 92.9, 115.6, 115.7, 121.8, 122.9, 124.8, 125.4, 125.8, 130.2, 131.8, 133.0, 133.1, 133.3, 143.2, 146.2, 154.0, 161.8, 171.5, 183.1, 185.2.

2-(4-hydroxy-6,7-dimethyl-2-oxo-2H-chromen-3-yl)-3-methylnaphthalene-1,4-dione 45

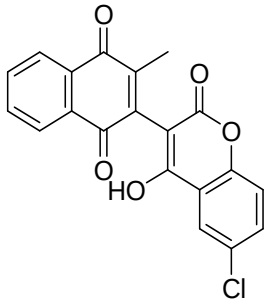


Stirring time = 24 h. Purification by a wash with EtOAc and recrystallisation to afford a brown solid (M-H^+ Found: 359.3620, $C_{22}H_{15}O_5$ requires M-H^+, 359.3619). UPLC 89.4%. R_f 0.34 (EtOAc/Hex, 1:2). mp 239ºC ^{1}H NMR (400 MHz, DMSO-d_6): ^{1}H NMR (400 MHz, DMSO-d_6): δ

= 1.97 (3H, s, CH_3), 2.34 (3H, s, CH_3), 2.39 (3H, s, CH_3), 6.70 (1H,d, s, ArH), 6.95(1H, s, ArH), 7.73 (1H, s, ArH), 8.01 (1H, dd, J 3.6, 4.8 Hz, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 14.4, 22.9, 93.9, 115.6, 115.7, 122.6, 122.9, 124.8, 125.4, 125.8, 130.2, 131.8, 133.0, 133.1, 133.3, 143.2, 146.2, 154.0, 161.8, 171.5, 183.1, 185.3.

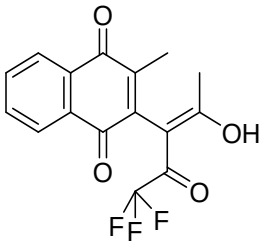
^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.9, 18.3, 95.2, 114.4, 120.2, 127.0, 136.5, 158.3, 159.5, 130.5, 130.9, 132.9, 133.0, 136.1, 136.4, 140.1, 160.2, 177.5, 183.1

2-(6-chloro-4-hydroxy-2-oxo-2H-chromen-3-yl)-3-methylnaphthalene-1,4-dione 46



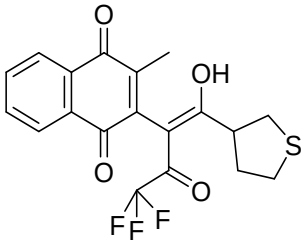
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (M-H$^+$ Found: 365.0299, $C_{20}H_{10}ClO_5$ requires M-H$^+$, 365.0300). UPLC 90.1%. R_f 0.40 (EtOAc/Hex, 1:2). mp 260-261ºC ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.12 (3H, s, CH_3), 3.90 (1H, br s, OH), 6.98 (1H, s, ArH), 7.55 (1H, dd, *J* 7.50, 15.6 Hz, ArH), 7.64 (1H, m, ArH), 7.86 (2H, dd, *J* 3.60, 4.80 Hz, ArH), 7.97 (1H, t, *J* 4.50 Hz, ArH), 8.02 (1H, t, *J* 4.50 Hz, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 14.2, 95.9, 116.6, 116.7, 122.8, 122.9, 124.8, 125.4, 125.8, 130.2, 131.8, 133.0, 133.1, 133.3, 143.2, 146.2, 154.0, 161.8, 171.5, 183.1, 189.2.

2-((Z)-1,1,1-trifluoro-4-hydroxy-2-oxopent-3-en-3-yl)-3-methylnaphthalene-1,4-dione 47



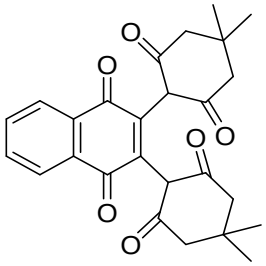
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (EtOAc/Hex, 1:2). mp 220ºC (M-H^+ Found: 345.1083, $C_{22}H_{17}O_4$ requires M-H^+, 345.1127). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.24 (3H, s, CH_3), 1.49 (3H, s, CH_3), 3.93 (1H, br s, OH), 7.54 (1H, dd, *J* 1.80, 7.20 Hz, ArH), 7.56 (1H, dd, *J* 1.80, 7.20 Hz, ArH), 7.64 (2H, m, ArH).

2-((E)-4,4,4-trifluoro-1-(tetrahydrothiophen-3-yl)-1-hydroxy-3-oxobut-1-en-2-yl)-3-methylnaphthalene-1,4-dione 48



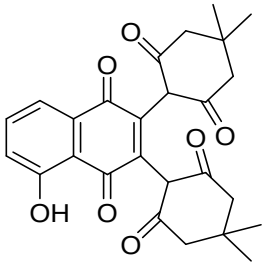
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (EtOAc/Hex, 1:2). mp 210-212°C (M-H^+ Found: 345.1071, $C_{22}H_{17}O_4$ requires M-H^+, 345.1127). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.49 (3H, s, CH_3), 3.93 (1H, br s, OH), 7.55 (2H, dd, *J* 1.80, 7.20 Hz, ArH), 7.62 (2H, dd, *J* 1.80, 7.20 Hz, ArH).

2,3-bis(4,4-dimethyl-2,6-dioxocyclohexyl) naphthalene-1,4-dione 49



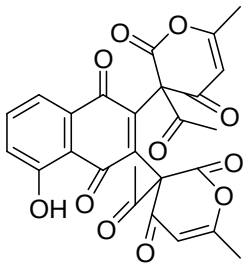
Stirring time 24h. The product was washed with EtOAc to afford dark brown solid (0.293g, 55% yield), ($M+H^+$ Found: 433.109 $C_{18}H_{15}O_4$ requires $M+H^+$, 433.126); R_f = 0.58 (1:10 MeOH:EtoAc). mp = 200 ^oC. ^1H NMR (400 MHz, DMSO-d_6) 0.99 (3H, s, CH_3), 1.08 (3H, s, CH_3), 2.11 (2H, s, 2 x CH), 2.13 (2H, s, CH_2), 2.24 (2H, s, CH_2), 7.85 (1H, dd, *J* 3.20, 5.60 Hz, H-Ar), 7.98 (1H, dd, *J* 3.20, 5.60 Hz, H-Ar). ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.8, 28.3, 30.8, 31.6, 109.9, 125.8, 128.6, 129.3, 132.3, 133.6, 134.3, 138.8, 144.6, 182.6

5-hydroxy-2,3-bis (4,4-dimethyl-2,6-dioxocyclohexyl) naphthalene-1,4-dione 50



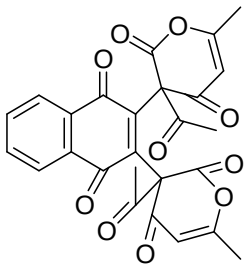
Stirring time = 48h. The product was washed with EtOAc to afford light brown solid (0.292g 48% yield), ($M–H^+$ Found: 449.1595 $C_{26}H_{25}O_7$ requires M^-H, 449.1600). UPLC 91.2%. R_f = 0.51 (EtoAc) Mp = 170 ^oC. ^1H NMR (400 MHz, DMSO-d_6) 0.99 (3H, s, CH_3), 1.07 (3H, s, CH_3), 2.13 (2H, s, 2 X CH), 2.15 (2H, s, CH_2), 2.26 (2H,s,CH_2), 7.33 (1H, dd, *J* 1.0, 8.7 Hz, H-Ar), 7.50 (1H, dd, *J* 1.20, 7.60 Hz, H-Ar), 7.75 (1H, t, *J* 8.0 Hz, H-Ar), 12.0 (1H, s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.8, 29.3, 31.8, 31.6, 109.9, 125.8, 128.6, 129.3, 132.3, 134.6, 135.3, 139.8, 146.6, 183.6

2,3-bis (5-acetyl-5,6-dihydro-2-methyl-6-oxo-4H-pyran-3-yl)-5-hydroxynaphthalene-1,4-dione 51



Stirring time = 24h. The product was purified by flash chromatography loaded with Et_2O and washed with hexane, (Silica: 1:10, 1:8, 1:4, 1:2, Et2O-hexane, Et_2O) to afford an orange solid (0.084g, 30% yield), ($M–H^+$ Found: 509.0500. $C_{28}H_{13}O_{10}$ requires M–H, 509.0509); R_f = 0.56 (1:20 MeOH: EtoAc). mp = 200 ^oC. 2.26 (6H, d, J 0.80 Hz, 2 x CH_3), 2.55 (6H, s, 2 x CH_3), 6.30 (1H, d, *J* 0.4 Hz, CH), 7.09 (1H, q, *J* 10.8 Hz, CH), 7.37 (1H, dd, *J* 1.2, 8.8 Hz, H-Ar), 7.52 (1H, dd, *J* 1.2, 7.20 Hz, H-Ar), 7.77 (1H, dd, *J* 7.6, 8.4 Hz, H-Ar), 11.8 (1H, s, OH).

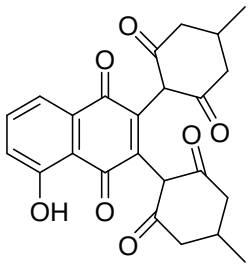
2,3-bis(5-acetyl-5,6-dihydro-2-methyl-6-oxo-4H-pyran-3-yl)naphthalene-1,4-dione 52



Stirring time =24h. Purification by flash chromatography loaded with Et_2O and washed with hexane, (Silica: 1:10, 1:8, 1:4, 1:2, Et_2O-hexane, Et_2O, then 5:1 Et_2O:Hex Preparative TLC plate) to afford an orange solid; R_f = 0.25 0.607 (1:1 EtoAc:MeOH). mp = 220 ^oC. ^1H NMR (400

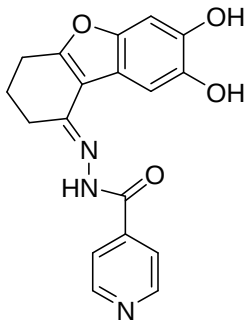
MHz, DMSO-d_6) 2.39 (6H, s, CH_3), 2.43 (3H, s, CH_3), 6.91 (1H, dd, *J* 1.60, 8.00 Hz, H-Ar), 7.32 (1H, dd, *J* 1.60, 8.00 Hz, H-Ar), 7.34 (1H, t, *J* 7.6 Hz, H-Ar), 13.7 (1H, s, OOH).

5-hydroxy-2,3-bis(4-methyl-2,6-dioxocyclohexyl)naphthalene-1,4-dione 53



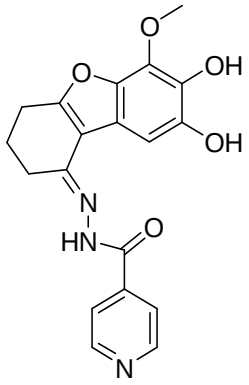
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). mp 220°C ($M\text{-}H^+$ Found: 421.1071, $C_{24}H_{21}O_7$ requires $M\text{-}H^+$, 345.10270). R_f = 0.25 (1:1 EtoAc: MeOH) ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.11 (3H, d, *J* 6.4 Hz, CH_3), 2.03 (1H, dd, *J* 5.8, 17.6 Hz, CH), 2.33 (1H, m, CH), 2.67 (1H, dd, *J* 2.0, 2.4 Hz, CH_2), 2.71 (1H, d, *J* 6.4 Hz, CH), 2.80 (1H, dd *J* 6.0 Hz, CH), 7.16 (1H, dd, *J* 1.6, 8.4 Hz, ArH), 7.47 (1H, dd *J* 1.2, 7.2 Hz, ArH), 7.57 (1H, t *J* 7.8 Hz, ArH), 13.8 (1H, s, OH).

- **Isoniazid derivative 54**



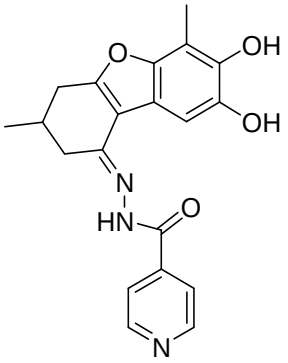
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.33 (2:1 EtoAc: MeOH) ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.20 (2H, s, CH_2), 2.73 (2H, d J 6.4 Hz, CH_2) 2.87 (2H, s J 6.4 Hz, CH_2), 6.93 (1H, s, ArH), 7.49 (1H, s, ArH), 7.80 (2H, s, ArH), 8.76 (2H, dd J 1.60, 4.40 Hz, ArH),8.98 (2H, br s, OH), 11.00 (1H, br s, NH);); ^{13}C NMR (100 MHz, DMSO-d_6): δ 36.2, 59.3, 98.8, 113.8, 115.5, 120.9, 133.3, 136.1, 140.2, 144.9, 150.1, 162.8, 169.8, 194.2.

- **Isoniazid derivative 55**



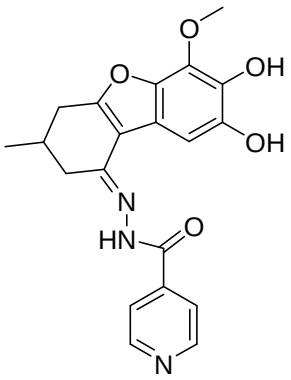
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH) . ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.15 (2H, m, CH_2), 2.46 (2H, t J 6.4 Hz, CH_2), 2.99 (2H, t J 6.4 Hz, CH_2), 3.94 (3H, s, CH_3), 7.00 (1H, s, ArH), 7.72 (2H, dd J 1.60, 4.40 Hz, ArH), 8.62 (1H, br s, OH), 8.70 (2H, d J 6.0 Hz, ArH), 9.17 (1H, br s, OH) 10.10 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.0, 23.1, 37.3, 60.3, 99.8, 114.8, 115.5, 120.9, 133.3, 136.1, 140.2, 144.9, 150.1, 163.8, 169.8, 194.4.

- **Isoniazid derivstive 56**



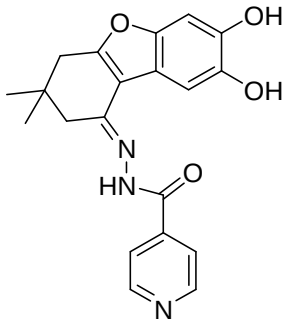
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). mp 240°C. ($M+H^+$ Found: 365.1511, $C_{20}H_{20}N_3O_4$ requires $M+H^+$, 365.1566) R_f = 0.35 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.14 (3H, d, J 6.4 Hz, CH_3), 2.26 (3H, s, CH_3), 2.29 (1H, dd, J 4.7, 12.4 Hz, CH_2), 2.43 (2H, dd J 3.4, 12.8 Hz, CH_2), 2.68 (1H, m, CH), 3.03 (1H, dd J 4.7, 12.4 Hz, CH), 7.11 (1H, s, ArH), 7.72 (2H, dd, J 1.6, 4.4 Hz, ArH), 8.40 (1H, br s, OH), 8.70 (2H, d, J 6.0 Hz, ArH),9.31 (1H, br s, OH), 10.01 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 2.0, 23.1, 37.3, 60.3, 99.8, 114.8, 115.5, 120.9, 133.3, 136.1, 140.2, 144.9, 150.1, 163.8, 169.8, 194.4.

- **Isoniazid derivative 57**



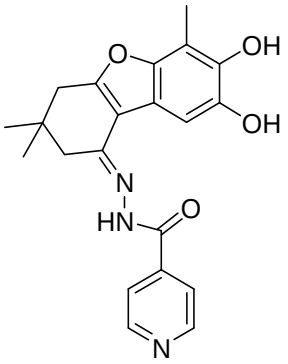
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). mp 235°C. R_f = 0.30 (2:1 EtoAc: MeOH).-^{1}H NMR (400 MHz, DMSO-d_6): δ 1.12 (3H, d J 6.8 Hz, CH_3), 2.32 (1H, dd J 12.0, 16.8 Hz, CH) 2.44 (2H, dd J 3.20, 16.8 Hz, CH_2), 2.72 (1H, m, CH), 3.06 (1H, dd J 5.0, 17.4 Hz, CH), 3.95 (3H, s, OCH_3), 6.99 (1H, s, ArH), 7.72 (2H, dd, J 1.80, 4.20 Hz, ArH), 8.8.62 (1H, br s, OH), 8.70 (2H, d J 61.60, 4.80 Hz, ArH),9.18 (1H, br s, OH), 10.10 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.6, 30.1, 30.9, 45.6, 60.3, 99.8, 114.7, 115.2, 120.9, 133.3, 136.1, 140.2, 140.5, 144.9, 150.2, 163.8, 169.4, 193.9.

- **Isoniazid derivative 58**



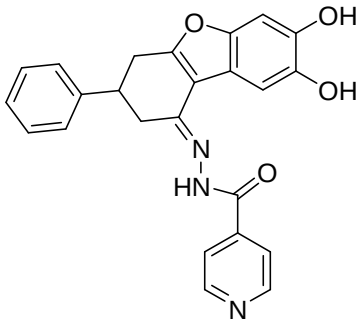
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.10 (6H, s, 2 × CH_3), 2.38 (2H, s, CH_2), 2.88 (2H, s, CH_2), 6.98 (1H, s, ArH), 7.21 (1H, s, ArH), 7.72 (2H, dd *J* 2.0, 4.4 Hz, ArH), 8.70 (2H, dd *J* 1.20, 4.4 Hz, ArH), 9.12 (2H, br s, OH), 10.1(1H, br s, NH). 13C?

- **Isoniazid derivative 59**



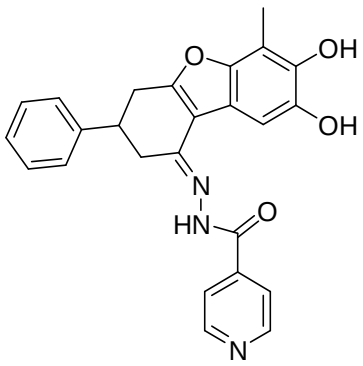
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). mp 240°C. ($M+H^+$ Found: 378.1493, $C_{21}H_{20}N_3O_4$ requires $M+H^+$, 379.1551) R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.09 (6H, s, 2× CH_3), 2.27 (3H, s, CH_3) 2.61 (2H, s, CH_2), 2.77 (2H, s, CH_2), 7.38 (1H, s, ArH), 7.80 (2H, d *J* 5.60 Hz, ArH),8.27 (1H, br s, OH), 8.76 (2H, dd, *J* 1.20, 4.40 Hz, ArH), 9.26 (1H, br s, OH), 11.00 (1H, br s, NH);

- **Isoniazid derivative 60**



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH)**.** ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.58 (1H, dd, *J* 3.2, 16.0 Hz, CH), 2.93 (1H, dd *J* 12.4, 16.0 Hz, CH), 3.24 (2H, m, CH_2), 3.66 (1H, m, CH), 7.01 (1H, s, ArH), 7.26 (1H, s, ArH), 7.35 (2H, t *J* 7.2, 7.6 Hz, ArH), 7.42 (2H, d *J* 7.6 Hz, ArH), 7.72 (2H, d *J* 6.0 Hz, ArH), 7.72 (2H, d *J* 6.40 Hz, ArH), 9.11 (1H, s, 2 x OH), 10.10 (1H, br s, NH); ^{13}C NMR 30.5, 44.5, 96.4, 105.3, 114.2, 115.4, 120.9, 126.9, 127.0, 140.2, 143.0, 143.7, 144.4, 148.2, 150.1, 163.8, 166.8, 192.9.

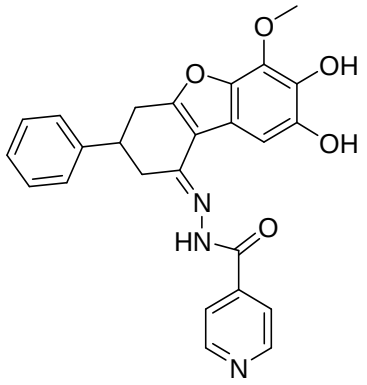
- **Isoniazid derivative 61**



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). ($M+H^+$ Found: 428.1546, $C_{25}H_{22}N_3O_4$ requires $M+H^+$, 428.1610) mp 236°C. R_f = 0.31 ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.28 (3H, s, CH_3), 2.60 (1H, dd *J* 4.0,

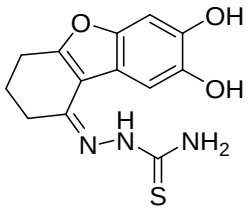
16.4 Hz, CH), 2.91 (1H, dd, *J* 12.2, 16.2 Hz, CH), 3.29 (1H, m, CH_2), 3.68 (1H, m, H-11 CH), 7.16 (1H, s, ArH), 7.26 (1H, t *J* 7.0 Hz, ArH), 7.35 (2H, t, *J* 7.6 Hz, ArH), 7.39 (2H, d *J* 7.6 Hz, ArH), 7.42 (2H, d, *J* 8.0 Hz, ArH), 7.86 (2H, d *J* 6.0 Hz, ArH), 8.68 (2H, d *J* 6.40 Hz, ArH).

- **Isoniazid derivative 62**



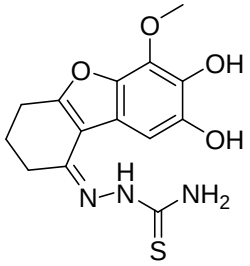
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). UPLC 99.5%. ($M\text{-}H^+$ Found: 442.1313, $C_{25}H_{20}N_3O_5$ requires $M\text{-}H^+$, 442.1403). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.68 (1H, dd, *J* 4.0, 16.0 Hz, CH), 2.93 (1H, dd *J* 12.0 Hz, 12.4 Hz, CH), 3.26 (2H, m, CH_2), 3.67 (1H, m, CH), 3.95 (3H, s, CH_3), 7.03 (1H, s, ArH), 7.26 (1H, t *J* 7.2 Hz, ArH), 7.35 (2H, t *J* 7.2, 8.0 Hz, ArH), 7.75 (2H, d *J* 3.60 Hz, ArH), 8.56 (1H, br s, OH), 8.72 (2H, d *J* 6.00 Hz, ArH), 9.10 (1H, br s, OH) 11.00 (1H, br s, NH); ^{13}C NMR 31.5, 44.5, 60.3, 96.5, 106.3, 114.2, 115.4, 121.9, 126.9, 127.0, 140.2, 143.0, 143.7, 144.4, 148.2, 150.1, 163.8, 166.8, 194.9.

Isoniazid derivative 63



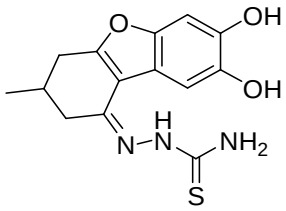
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ = 2.14 (2H, m, CH_2), 2.46 (2H, t, J 6.60 Hz, CH_2), 2.96 (2H, t, *J* 6.3 Hz, CH_2), 4.48 (1H, s, NH), 6.97 (1H, s, Ar-H), 7.22 (1H, s, Ar-H), 9.09 (2H, s, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.2, 29.2, 30.8, 97.4, 105.3, 113.3, 114.9, 143.6, 144.3, 148.1, 168.2, 191.9.

- **Thiosemicarbizide derivative 64**



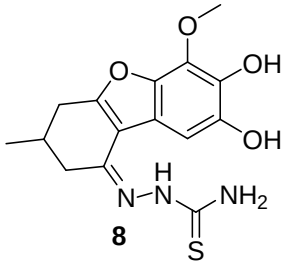
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). (M-H$^+$ Found: 320.0668, $C_{14}H_{14}O_4N_3$,S requires M-H$^+$, 320.0705). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.13 (2H, m, CH_2), 2.46 (2H, t, *J* 6.8 Hz, CH_2), 3.01 (2H, t *J* 6.4 Hz, CH_2), 3.94 (3H, s, OCH_3), 4.48 (1H, s, NH); 7.00 (1H, s, ArH), 7.16 (1H, br s, OH), 7.51 (1H, br s, OH) 8.60 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.2, 30.6, 30.8, 43.6, 97.4, 105.3, 114.3, 115.2, 143.6, 144.3, 148.1, 168.2, 192.8.

- **Thiosemicarbizide derivative 65**



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a light brown solid (MeOH/EtOAc 1:2). ($M+H^+$ Found: 306.0904, $C_{14}H_{16}N_3O_3S$ requires $M-H^+$, 306.0912). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.12 (3H, d, J 6.40 Hz, CH_3), 2.31 (1H, dd, J 4.6, 12.8 Hz, CH), 2.44 (2H, dd, J 3.6, 12.8 Hz, CH_2), 2.70 (1H, m, CH), 3.03 (1H, dd J 4.6, 12.8 Hz, CH), 4.49 (1H, s, NH), 6.97 (1H, s, ArH), 7.21 (1H, s, ArH) and 8.60 (2H, br s, OH), 9.05 (2H, s, NH_2), ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.6, 30.2, 30.6, 30.9, 45.6, 98.4, 105.3, 114.3, 115.2, 143.6, 144.3, 148.1, 169.2, 193.8.

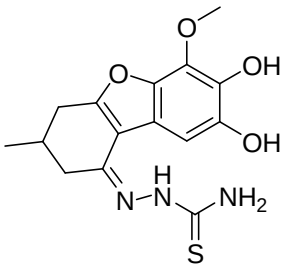
- **Thiosemicarbizide derivative 66**



8

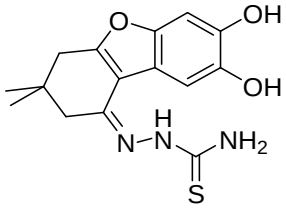
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a white solid (MeOH/EtOAc 1:2). ($M-H^+$ Found: 334.0829, $C_{15}H_{16}O_4N_3S$ requires $M-H^+$, 334.0862). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ = 1.13 (3H, d, J 6.40 Hz, CH_3), 2.32 (1H, dd, J 11.6, 16.8 Hz, CH), 2.46 (2H, dd, J 3.80, 16.8 Hz, CH_2), 3.07 (1H, dd, J 5.0, 17.4 Hz, CH), 3.29 (1H, s, NH), 3.95 (3H, s, OCH_3), 6.99 (1H, s, ArH), 8.61 (2H, s, NH_2), 9.18 (1H, s, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.3, 31.2, 33.1, 35.9, 46.6, 62.2, 98.4, 105.3, 114.6, 115.4, 143.6, 145.3, 148.1, 170.2, 193.8.

- **Thiosemicarbizide derivative 67**



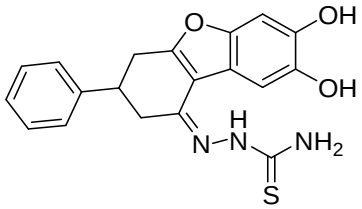
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a white solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.13 (3H, d *J* 6.4 Hz, CH_3), 2.32 (1H, dd *J* 12.4, 16.8 Hz, CH) 2.45 (2H, dd *J* 3.6, 16.8 Hz, CH_2), 2.73 (1H, dd, *J* 9.4, 17.0 Hz, CH), 3.07 (1H, m, CH), 3.94 (3H, s, CH_3), 4.48 (2H, s, NH_2), 6.99 (1H, s, OH), 7.17 (1H, s, OH), 8.60 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 19.0, 32.4, 32.6,36.3, 47.1, 56.5, 99.8, 102.0, 120.8, 126.0, 131.8, 137.8, 146.8, 147.0, 160.0, 181.4.

- **Thiosemicarbizide derivative 68**



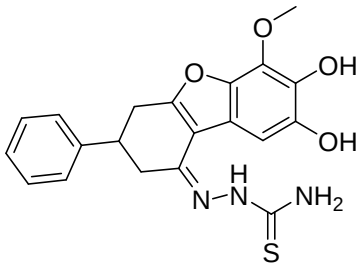
Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a dark brown solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ =1.04 (3H, s, CH_3), 2.33 (2H, s, CH_2), 2.83 (2H, s, CH_2), 4.42 (2H, s, NH_2), 6.92 (1H, s, H-Ar), 7.15 (1H, s, H-Ar), 8.54 (1H, s, NH), 9.00 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): ^{13}C NMR (100 MHz, DMSO-d_6): 25.7, 26.1, 36.4, 39.7, 46.9, 99.5, 103.1, 108.6, 119.9, 145.9, 146.7, 148.5, 148.8, 163.1, 181.4

- **Thiosermicarbizide derivative 69**



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.58 (1H, dd *J* 4.0, 16.0 Hz, CH), 2.93 (1H, dd *J* 12.2, 16.4 Hz, CH), 3.24 (2H, m, H-11), 3.70 (1H, m, CH), 7.01 (1H, s, ArH), 7.26 (1H, s, ArH), 7.35 (2H, t *J* 7.2, 7.6 Hz, ArH), 7.42 (2H, d *J* 7.6 Hz, ArH), 9.09 (1H, s, CH), 9.13 (1H, s, CH); ^{13}C NMR (100 MHz, DMSO-d_6): 34.4, 35.3, 45.6, 99.4, 102.0, 107.5, 119.8, 126.3, 126.4, 126.7, 128.9, 129.3, 141.4, 145.9, 146.7, 147.4, 148.0.

- **Thiosemicaribizide derivative 70**



Stirring time = 24 h. Purification by a wash with EtOAc and recrystallization to afford a brown solid (MeOH/EtOAc 1:2). (M+H$^+$ Found: 398.1158, $C_{20}H_{20}N_3O_4S$ requires M+H$^+$, 398.1175). mp 240°C. R_f = 0.30 (2:1 EtoAc: MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.16 (1H, t *J* 13.2 Hz, CH), 2.93 (1H, dd *J* 12.0 Hz, 12.4 Hz, CH), 3.26 (2H, m, CH_2), 3.67 (1H, m, CH), 3.95 (3H, s, CH_3), 7.03 (1H, s, ArH), 7.26 (1H, t *J* 7.2 Hz, ArH), 7.35 (2H, t *J* 7.2, 8.0 Hz,

ArH), 7.42 (2H, d *J* 8.0 Hz, ArH), 8.76 (1H, br s, OH) and 9.33 (1H, br s, OH). ^{13}C NMR (100 MHz, DMSO-d_6): 34.2, 34.4, 43.6, 56.5, 102.0, 120.8, 126.0, 126.3, 126.5, 126.7, 128.5, 128.6, 131.8, 137.7, 146.6, 148.6, 147.0, 160.0, 186.1.

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PUBLISHED RESEARCH MANUSCRIPT

Full Paper

One-Pot Laccase-Catalysed Synthesis of 5,6-Dihydroxylated Benzo[*b*]furans and Catechol Derivatives, and Their Anticancer Activity

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A commercial laccase, Suberase® from Novozymes, was used to catalyse the synthesis of 5,6-dihydroxylated benzo[*b*]furans and catechol derivatives. The yields were, in some cases, similar to or better than that obtained by other enzymatic, chemical or electrochemical syntheses. The synthesised derivatives were screened against renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer cell lines. GI_{50}, TGI and LC_{50} are reported for the first time. Anticancer screening showed that the cytostatic effects of the 5,6-dihydroxylated benzo[*b*]furans were most effective against the melanoma (UACC62) cancer cell line with several compounds exhibiting potent growth inhibitory activities (GI_{50} = 0.77–9.76 μM), of which two compounds had better activity than the anticancer agent etoposide (GI_{50} = 0.89 μM). One compound exhibited potent activity (GI_{50} = 9.73 μM) against the renal (TK10) cancer cell line and two exhibited potent activity (GI_{50} = 8.79 and 9.30 μM) against the breast (MCF7) cancer cell line. These results encourage further studies of the 5,6-dihydroxylated benzo[*b*]furans for their potential application in anticancer therapy.

Keywords: 1,3-Dicarbonyls / Anticancer / Benzo[*b*]furans / Biocatalysis / Catechols / C–C coupling / Cytostatic effects / Cytotoxic effects / GI_{50} / LC_{50} / Laccase / Michael addition / TGI

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Introduction

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (13% of all deaths) in 2008 (http://www.who.int/mediacentre/factsheets/fs297/en/). Amongst the most deadly cancers are lung, stomach, liver, colon and breast cancer. It is projected that worldwide deaths from cancer will continue to rise to an estimated 13.1 million in 2030 (http://www.who.int/mediacentre/factsheets/fs297/en/).

The primary treatment for many cancers is chemotherapy. The development of multidrug resistance to chemotherapeutic drugs is a main obstacle for the successful treatment of malignant tumours. Overexpression of the ATP-binding cassette (ABC) transporters that actively pump drugs out of tumour cells is one of the best known mechanisms of multidrug resistance [1, 2].

The number of effective drugs available for treating malignant tumours has been reduced by the development of chemoresistance and this has led to a search for therapeutic alternatives through the discovery of new classes of anticancer compounds.

The hydroxylated benzo[*b*]furan moiety has attracted much attention due to its wide range of biological activities [3–7]. This group of compounds act as antifungal agents, antioxidant agents, 5-lipoxygenase inhibitors, cyclooxygenase-2 inhibitors, Na^+ and K^+-ATPase inhibitors and modulators of the estrogen receptor [8–12]. Examples of benzofuran scaffolds that exhibit anticancer activity are shown in Fig. 1.

Usnic acid **1**, a common and abundant lichen metabolite, showed activity against the wild-type p53 breast cancer cell line MCF7, the breast cancer cell line MDA-MB-231 and the

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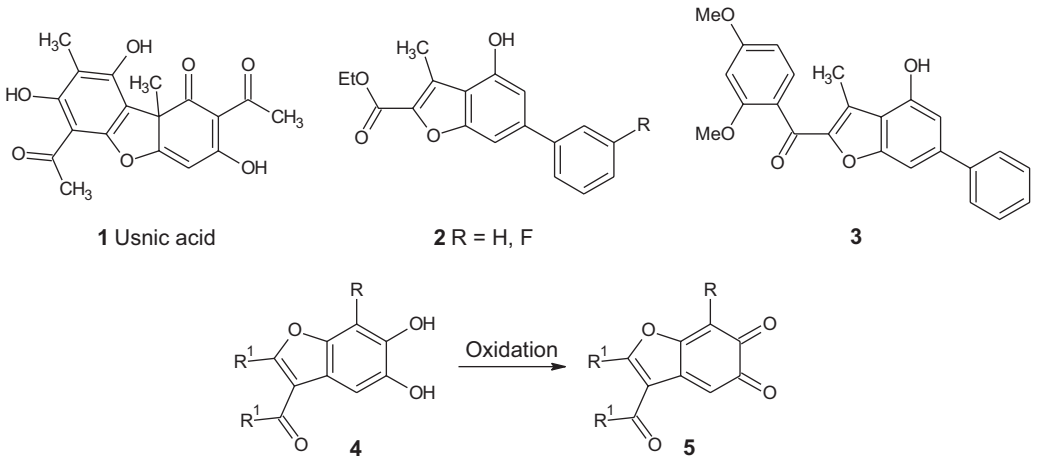


Figure 1. Benzofuran scaffolds.

lung cancer cell line H1299 [13]. Benzofuran derivatives **2** and **3** (Fig. 1) were identified as anticancer agents of which derivatives displayed selective cytotoxicity against a tumourigenic cell line [14]. Laccases (EC 1.10.3.1) are enzymes that are widely distributed in plants and fungi. They are characterised by a multinuclear copper-containing active site and have been classified as oxidoreductases. Laccases catalyse the oxidation of a broad range of substrates such as phenols, *o*- and *p*-diphenols, aminophenols, methoxyphenols, aryl thiols, anilines, polyphenols, polyamines and lignin-derivatives [15–17]. In the monoelectronic oxidation of substrates molecular oxygen is used and water is produced as the only by-product (http://www.who.int/mediacentre/factsheets/fs297/en/) [1, 2, 15–17]. Laccases have been successfully applied in organic synthesis which has culminated in several reports in the field of green chemistry [18].

In our laboratories, we have been interested in the use of enzymes for the development of green methods of synthesis, and for accessing compounds that have pharmaceutical value. We have previously reported on the synthesis of diaminobenzoquinones [19a] and aminonaphthoquinones [19b] via C–N bond formation as well as 1,4-naphthoquinone-2,3-*bis*-sulfides [19c] via C–S bond formation using commercial laccases (Denilite II Base on an inert support, and Novozyme 51003) from Novozymes [19]. In this article, we report on the synthesis of 5,6-dihydroxylated benzo[*b*]furan derivatives using another commercial laccase (Suberase® from Novozymes) and on the anticancer activity of the synthesised compounds. We anticipated that the 5,6-dihydroxylated benzo[*b*]furans **4** possessing *ortho* phenolic substituents could be metabolised to the *o*-quinonoid structure **5** in cancer cells (Fig. 1). Since quinonoid compounds display potent biological properties such as antibacterial, anticancer, antifungal and antimalarial activity [20], we postulated that the benzo[*b*]furan **5** could have similar biological activities. This study is, to the best of our knowledge, the first on the synthesis of a variety of 5,6-dihydroxylated benzo[*b*]furans **4** and catechol derivatives using the commercial laccase, Suberase®, as well as the first report on the anticancer activity of the synthesised compounds.

Results and discussion

Synthesis

The catechols (**6a–c**) and the 1,3-dicarbonyls (**7a–f**) used in this study are depicted in Fig. 2.

The method used for the synthesis entailed reacting one equivalent of the catechol with one equivalent of the 1,3-dicarbonyl at room temperature using Suberase® in a vessel open to air at pH 7.15 (Scheme 1). A pH of 7.15 was chosen because it would make the reaction medium sufficiently basic to deprotonate the alpha-proton from the 1,3-dicarbonyls and thus facilitate the Michael addition reaction with the *in situ*-generated *o*-quinone.

The first approach, Method A, entailed reacting the catechols **6a–c** with the 1,3-dicarbonyls **7a–f** at room temperature (rt) at pH 7.15 for 24 h. The second approach, Method B, entailed conducting the reaction under the same conditions but for a longer time (44 h) to determine whether a longer reaction time would improve the yield of the product. In the third approach, Method C, a co-solvent, DMF, was added to the reaction mixture to improve the solubility of the organic substrates. The number of equivalents of the 1,3-dicarbonyl was also increased so that a ratio of 1,3-dicarbonyl to catechol was 4:1. These reactions were conducted for 42 h. The results of the investigations with Methods A to C are depicted in Table 1.

From these results it can be seen that 5,6-dihydroxylated benzo[*b*]furans can be accessed using all three synthesis methods. For Method A the highest yield that was obtained is 98% for **17** (Entry 15, Table 1) and the lowest is 37% for **15**

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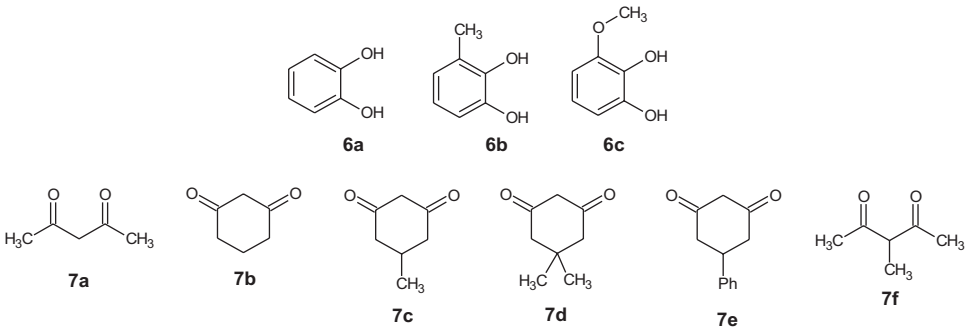


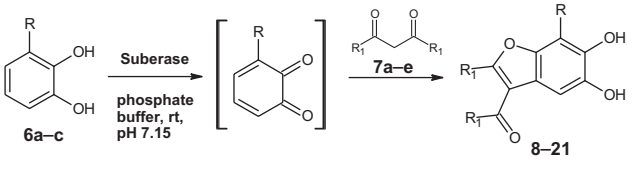
Figure 2. The catechols **6a–c** and 1,3-dicarbonyls **7a–f** used in this study.

(Entry 12, Table 1). For Method B the highest yield was 77% for **18** (Entry 18, Table 1) and the lowest 49% for **8** (Entry 2, Table 1). In the case of Method C, the highest yield was 71% for **14** (Entry 11, Table 1) and the lowest 15% for **21** (Entry 23, Table 1).

When comparing the yield of product using Method A to that obtained using Method B, it can be seen that there is not a significant increase in yield even though the reaction time has almost doubled (Entries 1 and 2, 5 and 6, 17 and 18, Table 1). It is therefore concluded that there is a limit to the quantity of product that can be formed in the reaction. The presence of DMF (Method C) may have deactivated the laccase, Suberase®, resulting in a lower yield of the product. A higher volume of organic solvent increases the solubility of a substrate but significantly decreases the overall reaction rate by deactivating the enzyme [21].

The optimum conditions for synthesising these 5,6-dihydroxylated benzo[*b*]furans **4** using Suberase® is thus that used under Method A.

The enzymatic synthesis of 5,6-dihydroxylated benzo[*b*]furans has been reported previously. The first enzymatic synthesis of compound **9** was reported in 2007 by Witayakran et al. [22] using *Trametes villosa* laccase in phosphate buffer (pH 7.0) with a Lewis acid, scandiumtris(trifluoromethanesulfonate), and sodium lauryl sulfate at 20°C and afforded it in 76% yield. In 2009, Witayakran et al. also reported on a laccase–lipase co-catalytic system for the synthesis of compounds **8** and **9** using phosphate buffer (pH 7.0) with lipase [23]. In this reaction, the Michael addition step was enhanced by lipase addition providing improved yields. Compound **8** was obtained in 60% yield using *Candida rugosa* lipase and laccase from *T. villosa* at 23°C. Compound **9** was obtained in 62% yield using *Candida antarctica* (CALB) lipase and laccase from *T. villosa* at 23°C [23]. We have synthesised compounds **8** and **9** without the use of scandiumtris(trifluoromethanesulfonate) or lipase and achieved a 49% yield of compound **8** (Entry 2, Table 1) and a 50% yield of compound **9** (Entry 3, Table 1).



Scheme 1. Synthesis of the 5,6-dihydroxylated benzo[*b*]furans **8–21**.

Hajdok et al. [24] was the first to report on the synthesis of compounds **10–21** using laccase initiated oxidative domino reactions. One method entailed using a commercial laccase from *Trametes versicolor* in an acetate buffer (pH 4.38) at room temperature while the other method used laccase from *Agaricus bisporus* in phosphate buffer (pH 5.96) also at rt. The latter method was found to be better since it gave the product in higher yield and purity with yields ranging from 71 to 97%. The yields of compounds **10–21** using our methods are 37–98%. The commercial fungal laccase, Suberase® from *Myceliophthora thermophila*, was overall less effective than the laccase from *A. bisporus* which was used by Hajdok et al. [24].

Compounds **13** and **19** have also been synthesised by employing tyrosinase and laccase from *A. bisporus* and were obtained in 39 and 44% yields, respectively [25]. Our method was higher yielding since compound **13** was obtained in 59% yield and compound **19** in 76% yield (Entries 8 and 19, respectively, Table 1; Fig. 3).

There have also been literature reports on the chemical syntheses of 5,6-dihydroxylated benzo[*b*]furans. The first report of compound **8** was a chemical synthesis which afforded **8** in 47% yield using pyridine and sodium metaiodate in ethanol at 20°C [26]. Our methods afforded similar yields for compound **8** (48 and 49%, Entries 1 and 2, respectively, Table 1). Duthaler and Scherrer [27] reported on the chemical synthesis of compound **10** which was obtained in 22% yield using sodium acetate in water. We were able to obtain compound **10** in 65% yield (Entry 4, Table 1).

Electrochemical syntheses have also been reported for the synthesis of 5,6-dihydroxylated benzo[*b*]furans. The first report on the synthesis of **16** was by Grujić et al. [28] in 1976

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Table 1. Synthesised benzo[*b*]furans **8–21** (yield in parentheses) at rt in phosphate buffer at pH 7.15.

Entry	Catechol	Dicarbonyl	Reaction time (h)	Method	Product (%Yield)	Yields of other enzymatic syntheses	Yields of other syntheses
1	**6a**	**7a**	24	A	**8** (48)	60 [23]	47 [26]
2	**6a**	**7a**	44	B	**8** (49)		
3	**6b**	**7a**	24	A	**9** (50)	76 [22], 62 [23]	
4	**6a**	**7b**	24	A	**10** (65)	87 [24]	22 [27]
5	**6b**	**7b**	24	A	**11** (62)	70 [24]	
6	**6b**	**7b**	44	B	**11** (67)		
7	**6c**	**7b**	24	A	**12** (70)	89 [24]	
8	**6a**	**7c**	24	A	**13** (59)	85 [24], 39 [25]	
9	**6a**	**7c**	42	C	**13** (30)		
10	**6b**	**7c**	24	A	**14** (78)	71 [24]	
11	**6b**	**7c**	42	C	**14** (71)		
12	**6c**	**7c**	24	A	**15** (37)	97 [24]	
13	**6a**	**7d**	24	A	**16** (58)	91 [24]	90 [28], 90 [29], 82 [30], 66 [31]
14	**6a**	**7d**	42	C	**16** (40)		
15	**6b**	**7d**	24	A	**17** (98)	92 [24]	87 [30], 81 [31]
16	**6b**	**7d**	42	C	**17** (59)		
17	**6c**	**7d**	24	A	**18** (73)	95 [24]	93 [30], 80 [31]
18	**6c**	**7d**	44	B	**18** (77)		
19	**6a**	**7e**	24	A	**19** (76)	96 [24], 44 [25]	
20	**6a**	**7e**	42	C	**19** (50)		
21	**6b**	**7e**	24	A	**20** (80)	91 [24]	
22	**6c**	**7e**	24	A	**21** (43)	97 [24]	
23	**6c**	**7e**	42	C	**21** (15)		

Method A – Suberase® (8.0 mL), catechol (2.0 mmol), 1,3-dicarbonyl (2.0 mmol, 1 eq), phosphate buffer (20.0 mL, 0.10 M, pH 7.15), stirring time at rt = 24 h.
Method B – Suberase® (8.0 mL), catechol (2.0 mmol), 1,3-dicarbonyl (2.0 mmol, 1 eq), phosphate buffer (20 mL, 0.10 M, pH 7.15), stirring time at rt = 44 h.
Method C – Suberase® (4.5 mL), catechol (0.60 mmol), 1,3-dicarbonyl (2.4 mmol, 4 eq), phosphate buffer (4.0 mL, 0.10 M, pH 7.15) and DMF (2.0 mL), stirring time at rt = 42 h.

and afforded it in 90% yield. Later Tabaković et al. [29] also reported on the electrochemical synthesis of **16** in 90% yield in water. Another electrochemical synthesis of **16** using sodium acetate in water by Nematollahi et al. [30] afforded it in 82% yield. The electrochemical synthesis of **16** by Davarani et al. [31] only afforded a 66% yield. We could only obtain a 58% yield for **16** (Entry 13, Table 1). Nematollahi et al. [30] also reported on the electrochemical synthesis of **17** and **18** which were obtained in 87 and 93% yields, respectively, also using sodium acetate in water as a reaction medium. The electrochemical synthesis of **17** and **18** in a sodium acetate solution by Davarani et al. [31] afforded these compounds in slightly lower yields, 81 and 80%, respectively. Compound **17** was obtained in 98% yield (Entry 15, Table 1) which is higher than the yields obtained by electrochemical synthesis, while compound **18** was obtained in 77% yield (Entry 18, Table 1) which was lower than that obtained by electrochemical syntheses.

The synthesis of two novel catechol derivatives **22** and **23** was also investigated (Scheme 2).

These reactions were conducted using Method A which was used for the synthesis of the 5,6-dihydroxylated benzo[*b*]-furans **8–21**. In this case, the aim was to achieve only C–C bond formation using the 1,3-dicarbonyl **7f**. The purpose was to determine whether anticancer activity would still be observed without the formation of the furan ring. The results of the investigation are shown in Table 2.

Both reactions proceeded in mediocre yield to afford **22** and **23** (Entries 1 and 2, respectively). A proposed mechanism involving an *o*-quinone intermediate of **22** and **23** is shown in Fig. 4 below.

Anticancer evaluation

Screening was conducted against renal (TK10), melanoma (UACC62), breast (MCF7) and cervical (HeLa) cancer cell lines using the Sulforhodamine B (SRB) assay to determine the growth inhibitory effects of the compounds [32]. These cell lines have been used routinely at the U.S. National Cancer Institute for screening for new anticancer agents and were derived from tumours that have different sensitivities to chemotherapeutic drugs [33]. Etoposide, an anticancer agent, was used as a positive control. It is known to be an inhibitor of topoisomerase, particularly topoisomerase II, and aids in DNA unwinding which causes the DNA strands to break

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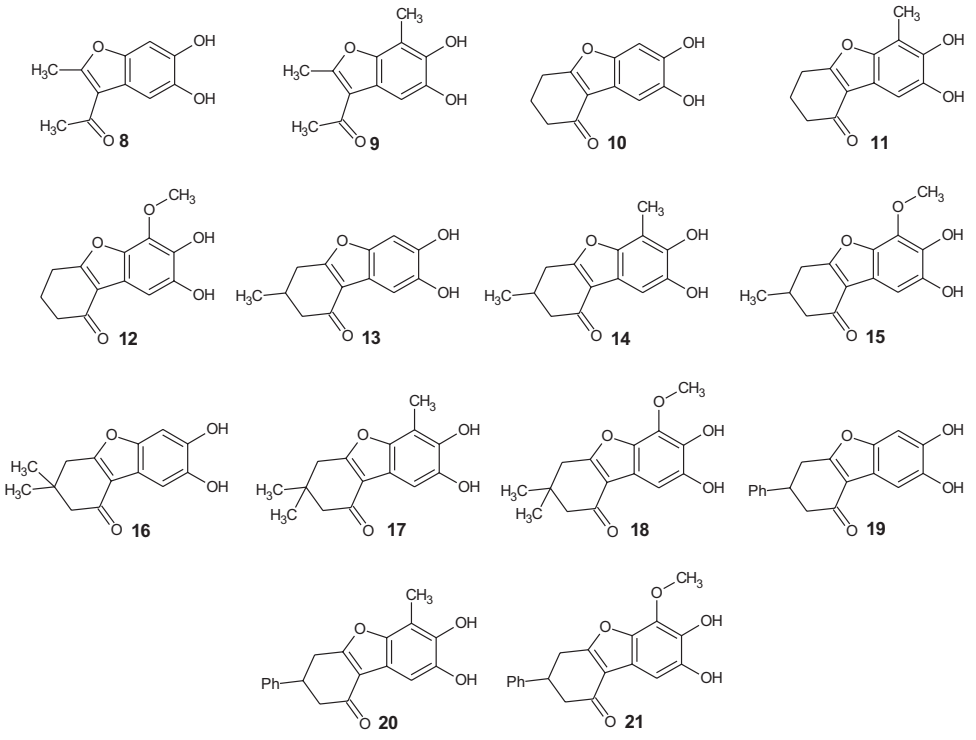
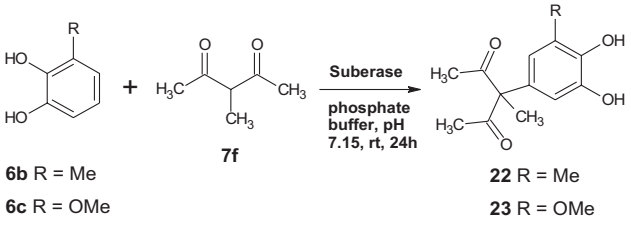


Figure 3. Structures of the 5,6-dihydroxylated benzo[*b*]furans **8**–**21** synthesised at rt.

[34]. Three parameters were determined during the screening process: 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI) and the lethal concentration that kills 50% of cells (LC_{50}). The results of this investigation are shown in Table 3 from which it can be seen that several compounds exhibited potent cytostatic effects.

The GI_{50} values of the catechols **6a–c**, the catechol derivative **22** and selected benzo[*b*]furans were compared to that of etoposide. No selection criteria were used for the compounds that were screened. Screening against the TK10 cell line



Scheme 2. Synthesis of C-C coupled products **22** and **23**.

Table 2. Synthesised catechol derivatives **23** and **24** (yield in parentheses) at rt in phosphate buffer at pH 7.15.

Entry	Catechol	Dicarbonyl	Reaction time (h)	Product (Yield)
1	**6b**	**7f**	24	22 (80)
2	**6c**	**7f**	24	23 (55)

Method A – Suberase® (8.0 mL), catechol (2.0 mmol), 1,3-dicarbonyl (2.0 mmol, 1 eq), phosphate buffer (20.0 mL, 0.10 M, pH 7.15), stirring time at rt = 24 h.

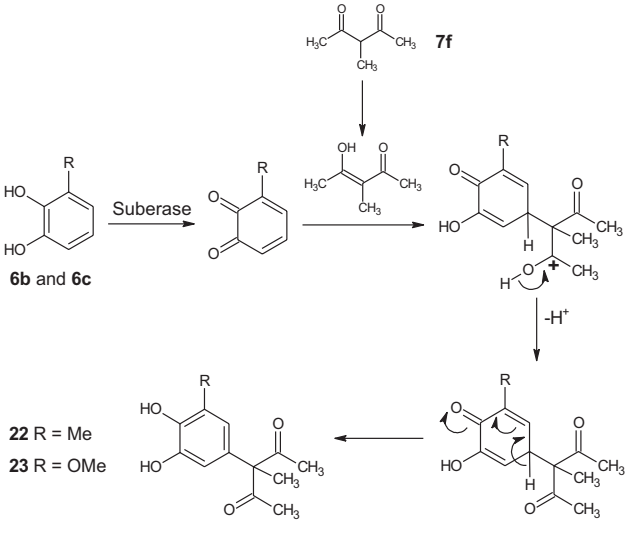


Figure 4. A proposed mechanism for C–C bond formation for the catechol derivatives **22** and **23**.

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showed that only **21** had potent activity (GI_{50} = 9.73 μM, Entry 12) which was not as good as that of etoposide (GI_{50} = 7.19 μM, Entry 14).

Most of the compounds exhibited potent growth inhibitory activity against the UACC62 cell line. The potent activity exhibited by **15** (GI_{50} = 0.78 μM, Entry 9) and **21** (GI_{50} = 0.77 μM, Entry 12) was slightly better activity than that of etoposide (GI_{50} = 0.89 μM, Entry 14).

Moreover, the same two compounds, **15** (GI_{50} = 8.79 μM, Entry 9) and **21** (GI_{50} = 9.30 μM, Entry 12), also exhibited potent growth inhibitory activity against the MCF7 cell line but this was not as good as that of etoposide (GI_{50} = 0.56 μM, Entry 14).

Most compounds exhibited weak TGI activity and three were inactive against the TK10 cell line. The activities of **18**, **20** and **21** (Entries 10–12, respectively) were slightly better (TGI = 46.14–48.25 μM) than that of etoposide (TGI = 49.74 μM, Entry 14).

The compounds also exhibited moderate to weak activity against the UACC62 cell line with most compounds, **11**, **12**, **14**, **15**, **18** and **21**, exhibiting better activity (TGI = 18.32–51.06 μM) than that of etoposide (TGI = 52.71 μM, Entry 14). The best activity was observed for **15** (TGI = 18.32 μM, Entry 9) which was almost threefold better than that of etoposide.

The test compounds exhibited weak activity against the MCF7 cell line, but the cytostatic effects of these compounds were better than that of etoposide which was inactive (TGI > 100 μM, Entry 14).

The LC_{50} values of the compounds were also compared to that of etoposide to get an idea of the cytotoxic effects of these compounds against the different cell lines. Screening against the TK10 cell line showed that most of the compounds were more lethal than etoposide (LC_{50} > 100 μM) with **18** (LC_{50} = 73.59 μM, Entry 10) being the most lethal.

Again, most of the compounds were more lethal than etoposide (LC_{50} > 100 μM) against the UACC62 cell line and **15** (LC_{50} = 60.32 μM, Entry 9) was the most lethal. Apart from **18**, none of the test compounds were lethal for HeLa cells, indicating a degree of selectivity between cell lines.

The compounds also exhibited more lethal cytotoxic effects against the MCF7 cell line than that of etoposide (LC_{50} > 100 μM, Entry 14) and **21** (LC_{50} = 86.93 μM, Entry 12) was the most lethal. Apart from **18**, none of the test compounds were lethal for HeLa cells indicating a degree of selectivity between cell lines.

The catechols **6a–c** and the catechol derivative **22** (Entry 13) did not exhibit any significant anticancer activity. From the results it can be seen that the GI_{50} concentrations of benzo[*b*]-furan **9** (Entry 4) are almost half of those of **22** (Entry 13) against the renal (TK10), melanoma (UACC62) and breast (MCF7) cancer cell lines. It may thus be concluded that the presence of the furan ring enhances anticancer activity. The benzo[*b*]furans were most effective against the melanoma (UACC62) cell line. Only one benzo[*b*]furan, **21**, exhibited growth inhibitory activity against all three cancer cell lines which may be attributed to the presence of the methoxy group on the benzene ring. When the methoxy group was replaced with a methyl group, as in **20**, growth activity against the renal (TK10) and breast (MCF7) cell lines was diminished. The benzo[*b*]furan **15** exhibited activity against melanoma (UACC62) and breast (MCF7) cell lines. The replace-

Table 3. *In vitro* anticancer screening of the compounds against renal (TK10), melanoma (UACC62), breast (MCF7) and HeLa cancer cells expressed as GI_{50}, TGI and LC_{50} values (μM).

Entry	Compound	Renal (TK10)			Melanoma (UACC62)			Breast (MCF7)			Cervical (HeLa)		
		GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
1	**6a**	68.02	>100	>100	45.00	>100	>100	52.41	>100	>100	–[a]	–[a]	–[a]
2	**6b**	38.14	75.72	>100	15.18	46.22	77.26	27.34	64.31	>100	–[a]	–[a]	–[a]
3	**6c**	51.63	96.98	>100	23.64	50.26	76.87	27.55	60.68	93.81	–[a]	–[a]	–[a]
4	**9**	41.05	69.94	98.82	32.74	60.84	88.94	35.10	66.12	97.14	60.70	>100	>100
5	**10**	65.00	>100	>100	37.73	68.65	99.57	35.53	64.19	92.85	49.09	>100	>100
6	**11**	24.25	54.74	85.23	23.63	51.06	78.49	25.97	61.87	97.78	44.55	>100	>100
7	**12**	52.47	>100	>100	6.04	33.15	>100	15.85	54.74	93.63	56.46	>100	>100
8	**14**	16.56	55.32	94.09	6.50	33.85	74.35	25.55	68.82	>100	25.93	>100	>100
9	**15**	26.54	51.65	76.75	0.78	18.32	60.32	8.79	48.79	95.11	26.68	69.67	>100
10	**18**	18.69	46.14	73.59	6.79	28.93	66.27	28.44	60.90	93.35	34.37	62.15	89.93
11	**20**	18.60	48.25	77.90	9.76	>100	>100	17.96	60.79	>100	47.28	>100	>100
12	**21**	9.73	47.15	85.35	0.77	23.60	64.58	9.30	46.93	86.93	22.32	73.19	>100
13	**22**	86.99	>100	>100	51.29	>100	>100	68.03	>100	>100	–[a]	–[a]	–[a]
14	Etoposide	7.19	49.74	>100	0.89	52.71	>100	0.56	>100	>100	3.56	40.18	87.54

Inactive, i: GI_{50} or TGI > 100 μM; weak activity, w: > 30 μM GI_{50} or TGI < 100 μM; moderate activity, m: < 30 μM GI_{50} or TGI > 10 μM; potent activity, p: GI_{50} or TGI < 10 μM.
[a] Not screened.

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ment of the methoxy group with that of a methyl, as in **14**, also resulted in diminished activity and in this case against the breast (MCF7) cancer cell line. When comparing the structure of **21** to that of **15** it appears that the replacement of the methyl group with that of a phenyl specifically affords growth inhibitory activity against the renal (TK10) cancer cell line. It was concluded that the phenyl and methoxy groups are essential for activity against all three cancer cell lines.

Conclusions

The fungal laccase from *M. thermophila*, commercially available as an inexpensive preparation known as Suberase®, can be used in the catalytic synthesis of 5,6-dihydroxylated benzo[*b*]furan and catechol derivatives under mild and environmentally friendly reaction conditions. The yields are, in some cases, similar to or better than that obtained by other enzymatic, chemical, or electrochemical synthesis. This method has eliminated the use of the Lewis acid, scandiumtris(trifluoromethanesulfonate), and lipase which was used in previous laccase methods.

The 5,6-dihydroxylated benzo[*b*]furans exhibit potent cytostatic effects against the three cancer cell lines but are most effective against UACC62 (melanoma) with two compounds exhibiting better activity than etoposide based on the GI_{50} concentrations. The 5,6-dihydroxylated benzo[*b*]furans generally have better TGI activity than that of etoposide.

These results warrant further studies of the 5,6-dihydroxylated benzo[*b*]furans for their application in anticancer therapy. These studies will entail further synthesis of 5,6-dihydroxylated benzo[*b*]furans and evaluation of their anticancer activity in addition to structural modification of hits to determine whether the anticancer activity can be optimised.

Experimental

General

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Mercury 400 MHz spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instruments at 100 MHz. Chemical shifts are reported in parts per million (ppm) relative to the solvent peaks and coupling constants are given in Hertz (Hz). A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. The PDA detector was used for all purity determinations (Maxplot 200–500 nm). All chemicals for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) while ultra-pure solvents were purchased from Honeywell (Burdick & Jackson, Muskegon, USA). Ultra-pure water was generated using a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France). Reactions were monitored by thin layer chromatography (TLC) on aluminium-backed Merck silica gel 60 F254 plates. Gravity column chromatography was performed using Merck Silica Gel 60 (70–230 mesh). Melting points were determined using a Glassco melting point apparatus and are uncorrected.

All chemicals were reagent grade materials. The 1,3-dicarbonyls were purchased from Sigma–Aldrich, South Africa. Suberase® (10757.8 PCU/mL) is a fungal laccase from *M. thermophila* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain. The enzymatic preparation is supplied as a brown liquid which is completely miscible with water. Suberase® was obtained from Novozymes in South Africa.

General methods for the synthesis of the benzo[*b*]furan derivatives

Method A

The laccase (Suberase®, 2.0 mL) was added to a mixture of the catechol (2.0 mmol), 1,3-dicarbonyl (2.0 mmol) and phosphate buffer (20.0 mL, 0.10 M, pH 7.15) in a 250-mL round-bottom flask stirred under air at rt. More laccase (2.0 mL) was added after 2, 18 and 20 h. The mixture was vigorously stirred under air until the substrates were consumed as judged by TLC. After stirring the reaction mixture was acidified with 32% HCl to pH 4.0. The mixture was extracted with EtOAc and washed with water (20.0 mL). The organic phases were then combined and the solvent evaporated. The residue, a powder, was purified by washing with EtOAc or recrystallising from a combination of MeOH and EtOAc.

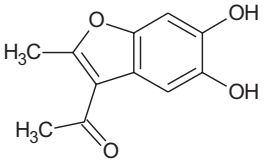
Method B

Same as Method B except that more laccase (2.0 mL) was added after 4, 24 and 28 h.

Method C

The laccase (Suberase®, 1.5 mL) was added to a mixture of the catechol (0.60 mmol), 1,3-dicarbonyl (2.40 mmol), phosphate buffer (4.0 mL, 0.10 M, pH 7.15) and DMF (2.0 mL) in a test tube stirred under air at rt. More laccase (1.5 mL) was added after 2 h and then again after 4 h. The mixture was vigorously stirred under air until the substrates had been consumed as judged by TLC. After stirring the reaction mixture was transferred to a separating funnel and the mixture extracted with EtOAc and washed with water (20.0 mL). The organic phases were combined, the solvent evaporated and the residue (a powder) purified by flash chromatography.

1-(5,6-Dihydroxy-2-methyl-1-benzofuran-3-yl)ethanone ***8***



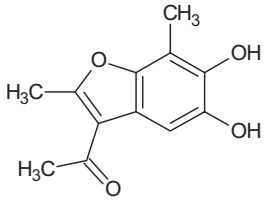
Method A

Stirring time 24 h. The product was recrystallised from 5% MeOH in EtOAc solution at 35°C to afford a dark-brown powder (198 mg, 48%) (Found: M–H$^+$, 205.0518. $C_{11}H_9O_4$ requires M–H, 205.0501). UPLC 95.5%. R_f 0.40 (EtOAc/hexane, 1:1). mp 200–203°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.51 (3H, s, CH_3), 2.68 (3H, s, CH_3), 6.91 (1H, s, ArH), 7.33 (1H, s, ArH), 8.94 (1H, br s, OH), 9.06 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.3, 30.8, 97.8, 106.4, 117.2, 117.2, 143.6, 144.2, 147.0, 160.7, 193.9.

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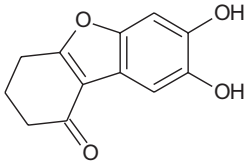
Method B
Stirring time 44 h. The product was washed with EtOAc to afford a black powder (202.0 mg, 49%).

1-(5,6-Dihydroxy-2,7-dimethyl-1-benzofuran-3-yl)-ethanone ***9***



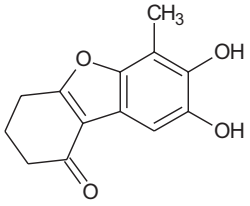
Method A
Stirring time 24 h. The product was washed with EtOAc to afford a red-brown powder (220 mg, 50%) (Found: $M-H^+$, 219.0615. $C_{12}H_{11}O_4$ requires M–H, 219.0657). UPLC 97.3%. R_f 0.46 (EtOAc/hexane, 1:1). mp 234°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.19 (3H, s, CH_3), 2.45 (3H, s, CH_3), 2.64 (3H, s, CH_3), 7.17 (1H, s, ArH), 8.38 (1H, br s, OH), 9.25 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 8.9, 15.4, 30.7, 103.2, 107.1, 116.2, 117.4, 141.8, 143.1, 146.5, 160.5, 193.9.

7,8-Dihydroxy-3,4-dihydrodibenzo[b,d]furan-1(2H)-one ***10***



Method A
Stirring time 24 h. The product was recrystallised from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (284 mg, 65%) (Found: $M-H^+$, 217.0458. $C_{12}H_9O_4$ requires M–H, 217.0501). UPLC 96.2%. R_f 0.48 (EtOAc/hexane, 1:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.21 (2H, m, CH_2), 2.47 (2H, t J 6.4 Hz, CH_2) 2.96 (2H, t J 6.4 Hz, CH_2), 7.00 (1H, s, ArH), 7.22 (1H, s, ArH), 9.18 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.2, 23.2, 37.3, 98.4, 105.4, 114.4, 115.6, 143.7, 144.4, 147.9, 169.7, 194.4.

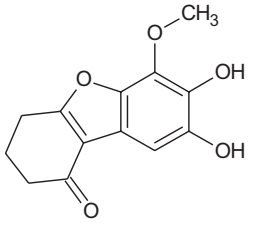
7,8-Dihydroxy-6-methyl-3,4-dihydrodibenzo[b,d]furan-1(2H)-one ***11***



Method A
Stirring time 24 h. The product was recrystallised from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (288 mg, 62%) (Found: $M-H^+$, 231.0683. $C_{13}H_{11}O_4$ requires M–H, 231.0657). UPLC 99.7%. R_f 0.48 (EtOAc/hexane, 1:1). mp 250°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.14 (2H, m, CH_2), 2.26 (3H, s, CH_3), 2.45 (2H, t J 6.1 Hz, CH_2), 2.98 (2H, t J 6.1 Hz, CH_2), 7.11 (s, 1H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0, 22.2, 23.3, 37.9, 102.4, 107.9, 113.5, 115.9, 142.2, 143.4, 147.6, 169.6, 194.6.

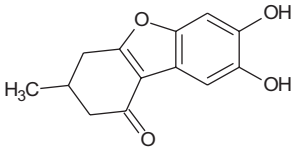
Method B
Stirring time 44 h. The product was washed with EtOAc to afford a brown powder (311.0 mg, 67%).

7,8-Dihydroxy-6-methoxy-3,4-dihydrodibenzo[b,d]furan-1(2H)-one ***12***



Method A
Stirring time 24 h. The product was recrystallised from 5% MeoH in EtOAc solution at 35°C to afford a brown powder (348 mg, 70%) (Found: $M-H^+$, 247.0557. $C_{13}H_{11}O_5$ requires M–H, 247.0606). UPLC 97.0%. R_f 0.38 (EtOAc/hexane, 2:1). mp 232°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.14 (2H, m, CH_2), 2.46 (2H, t J 6.4 Hz, CH_2), 3.00 (2H, t J 6.4 Hz, CH_2), 3.94 (3H, s, CH_3), 7.00 (1H, s, ArH), 8.71 (1H, br s, OH), 9.24 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.1, 23.2, 37.4, 60.4, 99.9, 114.8, 115.6, 133.3, 136.2, 140.3, 145.0, 169.9, 194.5.

7,8-Dihydroxy-3-methyl-3,4-dihydrodibenzo[b,d]furan-1(2H)-one ***13***

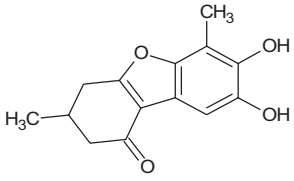


Method A
Stirring time 24 h. The product was washed with EtOAc to afford a brown powder (274 mg, 59%) (Found: $M-H^+$, 231.0610. $C_{13}H_{11}O_4$ requires M–H, 231.0657). UPLC 95.3%. R_f 0.38 (EtOAc/hexane, 1:1). mp 263°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.11 (3H, d, J 6.0 Hz, CH_3), 2.30 (1H, dd, J 4.6, 12.8 Hz, CH), 2.43 (2H, dd J 3.6, 12.8 Hz, CH_2), 2.68 (1H, m, CH), 3.01 (1H, dd J 4.6, 12.8 Hz, CH), 6.98 (1H, s, ArH), 7.21 (1H, s, ArH) and 9.15 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.7, 30.3, 40.0, 45.6, 98.5, 105.3, 114.3, 115.3, 143.7, 144.4, 148.2, 169.4 and 194.0.

Method C
Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 0.5:9.5, 1:9, 2.5:7.5, 1:1) to afford a brown powder (84 mg, 30%).

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7,8-Dihydroxy-3,6-dimethyl-3,4-dihydrodibenzo[b,d]furan-1(2H)-one ***14***



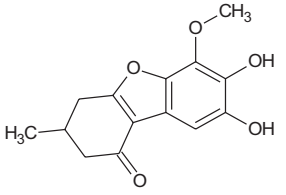
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a light-brown powder (384 mg, 78%) (Found: M–H^+, 245.0817. $C_{14}H_{13}O_4$ requires M–H, 245.0814). UPLC 96.0%. R_f 0.74 (EtOAc/hexane, 1:1). mp 165–168°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.11 (3H, d J 6.0 Hz, CH_3), 2.25 (3H, s, CH_3), 2.29 (1H, dd J 4.7, 12.4 Hz, CH_2), 2.43 (2H, dd J 3.4, 12.8 Hz, CH_2), 2.68 (1H, m, CH), 3.03 (1H, dd J 4.7, 12.4 Hz, CH), 7.11 (1H, s, ArH), 8.47 (1H, br s, OH), 9.38 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0, 20.7, 30.3, 31.0, 45.6, 102.3, 107.9, 113.4, 115.5, 142.1, 143.3, 147.8, 169.2, 194.1.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 0.5:9.5, 1:9, 1:1; EtOAc) to afford a light-brown powder (209.0 mg, 71%).

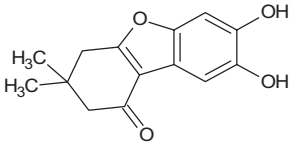
7,8-Dihydroxy-6-methoxy-3-methyl-3,4-dihydrodibenzo-[b,d]furan-1(2H)-one ***15***



Method A

Stirring time 24 h. The product was washed with EtOAc to afford a light-brown powder (194 mg, 37%) (Found: M–H^+, 261.0843. $C_{14}H_{13}O_4$ requires M–H, 261.0763). UPLC 99.6%. R_f 0.50 (EtOAc/hexane, 1:1). mp 179–182°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.12 (3H, d J 6.4 Hz, CH_3), 2.32 (1H, dd J 4.0, 12.4 Hz, CH) 2.44 (2H, dd J 3.4, 13.2 Hz, CH_2), 2.72 (1H, m, CH), 3.06 (1H, dd J 5.0, 12.4 Hz, CH), 3.94 (3H, s, CH_3), 6.97 (1H, s, ArH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.7, 30.3, 31.0, 45.7, 60.4, 99.8, 114.8, 115.3, 133.4, 136.3, 140.6, 145.1, 169.6, 194.1.

7,8-Dihydroxy-3,3-dimethyl-3,4-dihydrodibenzo[b,d]furan-1(2H)-one ***16***



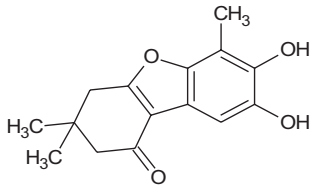
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a yellow powder (286 mg, 58%). (Found: M–H^+, 245.0867. $C_{14}H_{14}O_4$ requires M–H, 245.0814). UPLC 99.8%. R_f 0.64 (EtOAc/hexane, 1:1). mp 278–280°C [lit. [28] 280°C]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.09 (6H, s, 2× CH_3), 2.38 (2H, s, CH_2), 2.88 (2H, s, CH_2), 6.98 (1H, s, ArH), 7.20 (1H, s, ArH), 9.15 (2H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.0 (2× CH_3), 34.9, 36.7, 51.5, 98.5, 105.3, 114.2, 114.4, 143.7, 144.3, 148.3, 168.6, 193.7.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 0.5:9.5, 1:9, 2.5:7.5, 1:1) to afford a yellow powder (118 mg, 40%).

7,8-Dihydroxy-3,3,6-trimethyl-3,4-dihydrodibenzo-[b,d]furan-1(2H)-one ***17***



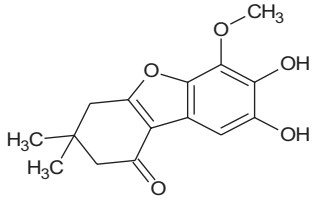
Method A

Stirring time 24 h. The product was recrystallised from a 5% MeOH in EtOAc solution at 35°C to afford a light-brown powder (510 mg, 98%). (Found: M–H^+ 259.0931. $C_{15}H_{15}O_4$ requires M–H, 259.0970). UPLC 96.6%. R_f 0.45 (EtOAc/hexane, 1:2). mp 255°C [lit. [33] 260–262°C]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.09 (6H, s, 2× CH_3), 2.26 (3H, s, CH_3) 2.37 (2H, s, CH_2), 2.90 (2H, s, CH_2), 7.10 (1H, s, ArH), 8.41 (1H, br s, OH), 9.32 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.0, 28.0, 34.9, 36.8, 51.5, 102.4, 107.9, 113.3, 114.7, 142.0, 143.3, 168.5, 193.8.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 5:95, 1:9, 1:1) to afford a light-brown powder (184 mg, 59%).

7,8-Dihydroxy-3,3,6-trimethyl-3,4-dihydrodibenzo-[b,d]furan-1(2H)-one ***18***



Method A

Stirring time 24 h. The product was recrystallised from 5% MeOH in EtOAc solution at 35°C to afford a brown powder (403 mg, 73%). (Found: M–H^+, 275.0922. $C_{15}H_{15}O_5$ requires M–H, 275.0919). UPLC 94.5%. R_f 0.44 (EtOAc/hexane, 1:1). mp 288–

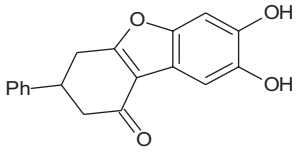
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291°C [lit. [31] 289–291°C]. ^{1}H NMR (400 MHz, MeOH-d_4): δ 1.16 (6H, s, 2× CH_3), 2.43 (2H, s, CH_2) 2.90 (2H, s, CH_2), 4.06 (3H, s, CH_3), 7.01 (1H, s, ArH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.1, 35.0, 36.8, 51.2, 60.4, 99.8, 114.5, 114.7, 113.4, 136.2, 140.8, 145.1, 168.9, 193.9.

Method B

Stirring time 44 h. The product was washed with EtOAc to afford a brown powder (425 mg, 77%).

7,8-Dihydroxy-3-phenyl-3,4-dihydrodibenzo[b,d]furan-1(2H)-one ***19***



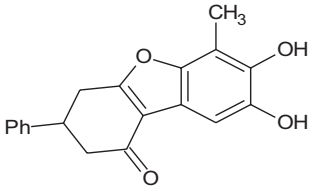
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a white solid (449 mg, 76%). (Found: M–H$^+$, 293.0821. $C_{18}H_{13}O_4$ requires M–H, 293.0814). UPLC 99.8%. R_f 0.50 (EtOAc/hexane, 1:1). mp 240–243°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.58 (1H, dd *J* 3.2, 16.0 Hz, CH), 2.93 (1H, dd *J* 12.4, 16.0 Hz, CH), 3.24 (2H, m, H-11), 3.66 (1H, m, CH), 7.01 (1H, s, ArH), 7.26 (1H, s, ArH), 7.35 (2H, t *J* 7.2, 7.6 Hz, ArH), 7.42 (2H, d *J* 7.6 Hz, ArH), 9.09 (1H, s, CH), 9.13 (1H, s, CH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.7, 44.6, 62.5, 63.4, 68.8, 71.5, 72.3, 73.8, 98.5, 105.4, 114.2, 115.5, 126.9, 128.6, 143.1, 143.9, 144.5, 148.3, 169.0, 193.1.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 5:95, 1:9, 1:1; EtOAc) to afford a white solid (132 mg, 50%).

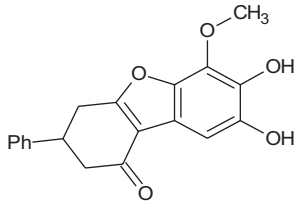
7,8-Dihydroxy-6-methyl-3-phenyl-3,4-dihydrodibenzo-[b,d]furan-1(2H)-one ***20***



Method A

Stirring time 24 h. The product was recrystallised from 5% MeOH in EtOAc solution at 35°C to afford a light-brown powder (279 mg, 80%) (Found: M–H$^+$, 307.0977.1120. $C_{19}H_{15}O_4$ requires M–H$^+$, 307.0970). UPLC 99.0%. R_f 0.45 (EtOAc/hexane, 1:1). mp 250–253°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.28 (3H, s, CH_3), 2.59 (1H, dd *J* 4.0, 16.4 Hz, CH), 2.91 (1H, dd *J* 12.2, 16.2 Hz, CH), 3.28 (1H, m, CH_2), 3.66 (1H, m, H-11 CH), 7.16 (1H, s, ArH), 7.26 (1H, t *J* 7.0 Hz, ArH), 7.35 (2H, t *J* 7.6 Hz, ArH), 7.42 (2H, d *J* 8.0 Hz, ArH), 8.45 (1H, br s, OH), 9.36 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 9.1, 30.7, 44.7, 102.4, 108.0, 113.4, 115.8, 126.9, 127.0, 127.1, 128.6, 142.3, 143.2, 143.4, 148.0, 168.8, 193.2.

7,8-Dihydroxy-6-methoxy-3-phenyl-3,4-dihydrodibenzo-[b,d]furan-1(2H)-one ***21***



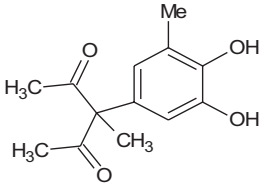
Method A

Stirring time 24 h. The product was washed with EtOAc to afford a dull grey powder (279 mg, 43%). (Found: M–H$^+$, 323.0922. $C_{19}H_{15}O_5$ requires M–H, 323.0919). UPLC 96.8%. R_f 0.38 (EtOAc/hexane, 1:2). mp 165–168°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.57 (1H, dd *J* 4.0, 16.0 Hz, CH), 2.93 (1H, dd *J* 12.0 Hz, 12.4 Hz, CH), 3.26 (2H, m, CH_2), 3.67 (1H, m, CH), 3.95 (3H, s, CH_3), 7.03 (1H, s, ArH), 7.26 (1H, t *J* 7.2 Hz, ArH), 7.35 (2H, t *J* 7.2, 8.0 Hz, ArH), 7.42 (2H, d *J* 8.0 Hz, ArH), 8.76 (1H, br s, OH) and 9.33 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.6, 44.6, 60.4, 99.8, 114.6, 115.5, 126.8, 127.0, 128.5, 133.4, 136.2, 140.6, 143.0, 145.0, 169.0, 193.0.

Method C

Stirring time 42 h. The product was purified by flash chromatography (silica: EtOAc/hexane, 5:95, 1:9, 1:1; EtOAc) to afford a black powder (58.0 mg, 15%).

3-(3,4-Dihydroxy-5-methylphenyl)-3-methylpentane-2,4-dione ***22***

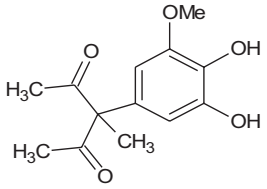


Method A

Stirring time 24 h. The product was washed with EtOAc to afford a dark-brown powder (1.60 g, 80%) (Found: M–H$^+$, 235.0926. $C_{13}H_{14}O_4$ requires M–H, 235.0970. UPLC 98.0%. R_f 0.48 (EtOAc/hexane, 1:1). mp 82–85°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.60 (3H, s, CH_3), 2.03 (6H, s, CH_3), 2.08 (3H, s, CH_3), 6.43 (1H, d *J* 2.0 Hz, ArH), 6.46 (1H, d *J* 2.4 Hz, ArH), 8.36 (1H, br s, OH), 9.29 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.2, 19.0, 27.2, 30.7, 68.8, 112.4, 120.1, 124.7, 127.7, 143.0, 144.8, 207.8.

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3-(3,4-Dihydroxy-5-methoxyphenyl)-3-methylpentane-2,4-dione ***23***



Method A

Stirring time 24 h. The product was washed with EtOAc to afford a dark-brown powder (1.10 g, 55%) (Found: M–H^+, 251.0933. $C_{13}H_{14}O_5$ requires M–H, 251.0919. R_f 0.48 (EtOAc/hexane, 1:1). mp 108–110°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.62 (3H, s, CH_3), 2.05 (6H, s, CH_3), 3.74 (3H, s, OMe), 6.27 (1H, s, ArH), 6.28 (1H, s, ArH), 8.44 (1H, br s, OH), 9.03 (1H, br s, OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.1, 27.3, 56.0, 69.0, 103.1, 108.7, 127.7, 133.9, 145.8, 148.5, 207.7.

In vitro anticancer activity evaluation

Assay background

The growth inhibitory effects of the compounds were tested in a 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using the Sulforhodamine B (SRB) assay [32]. The SRB assay was developed by Skehan and colleagues to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye, sulforhodamine B (Acid Red 52), to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilised for measurement. The SRB assay is performed at CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for this screen.

Materials and method

The human cell lines TK10, UACC62 and MCF7 were obtained from the NCI in a collaborative research program between the CSIR and the NCI. cell lines were routinely maintained as a monolayer cell culture at 37°C, 5% CO_2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50 μg/mL gentamicin.

For the screening experiment the cells (3–19 passages) were inoculated in a 96-well microtitre plate at plating densities of 7–10 000 cells/well and were incubated for 24 h. After 24 h one plate was fixed with TCA to represent a measurement of the cell population for each cell line at the time of drug addition (T0). The other plates with cells were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce five concentrations (0.01–100 μM). Cells without drug addition served as control. The blank contains complete medium without cells. Etoposide was used as a reference standard.

The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at a wavelength of 540 nm using a multiwell spectrophotometer. Optical density measurements were used to calculate the net percentage cell growth.

The optical density of the test well after a 48 h period of exposure to test drug is T_i, the optical density at time zero is T_0, and the control optical density is C. Percentage cell growth is calculated as:

$$\left[\frac{(T_i - T_0)}{(C - T_0)}\right] \times 100 \text{ for concentrations at which } T_i \geq T_0$$

$$\left[\frac{(T_i - T_0)}{T_0}\right] \times 100 \text{ for concentrations at which } T_i < T_0.$$

The results of a five dose screening were reported as TGI (total growth inhibition). The TGI is the concentration of test drug where $100 \times (T - T_0)/(C - T_0) = 0$. The TGI signifies a cytostatic effect.

The biological activities were separated into four categories: inactive (GI_{50} or TGI > 100 μM), weak activity (30 μM < GI_{50} or TGI < 100 μM), moderate activity (10 μM < GI_{50} or TGI < 30 μM) and potent activity (GI_{50} or TGI < 10 μM).

For each tested compound, three response parameters, GI_{50} (50% growth inhibition and signifies the growth inhibitory power of the test agent), TGI (which is the drug concentration resulting in total growth inhibition and signifies the cytostatic effect of the test agent) and LC_{50} (50% lethal concentration and signifies the cytotoxic effect of the test agent), were calculated for each cell line.

We thank Dr Paul Steenkamp for HRMS analysis, the CSIR (Thematic A grant) for financial support and Novozymes SA (Pty) Ltd for a kind donation of the enzyme.

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