The Synthesis of Biaxial Naphthoquinones and an Initial Investigation regarding their Potential as anti-*Mycobacterium tuberculosis* and Apoptotic Agents

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor Philosophiae in the Department of Chemistry, University of the Western Cape.

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work, which has not previously been submitted for a degree at any tertiary educational institution.

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Wendell Peter Swigelaar

2009

ABSTRACT

Tuberculosis and cancer are amongst the most devastating diseases affecting the world today, especially Africa and other less developed countries. Both diseases have the ability to develop resistance against drugs being used as treatment regimes and therefore the search for effective novel drugs remains an ongoing challenge. Diospyrin and its monomer 7-methyljuglone, both naturally occurring quinones, and their synthetic derivatives have been shown to exhibit significant antimycobacterial and anti-tumour activity. The present study focuses on the synthesis of new compounds of similar structures to that of the lead compounds diospyrin and 7-methyljuglone, and their evaluation as potential antimycobacterial and anti-cancer agents via apoptosis.

Three methoxynaphthoquinones were synthesized and coupled to several commercially available boronic acids using the Suzuki methodology. Eight compounds were evaluated against a drug susceptible strain, H37Rv of *Mycobacterium tuberculosis*, using the BACTEC method. Results indicate that the presence of an hydroxyl group *peri* to the quinone carbonyl moiety is crucial for these quinonoidal compounds to be effective as anti-TB agents, since their methyl ether analogues had little effect on the growth rate of the bacteria. Substituents, capable of donating electrons through a resonance electron effect to the carbonyl group, *peri* to the methoxy group, also enhance activity of these molecules.

Several of the synthesized compounds were screened for their ability to induce apoptosis in five human cancer cell lines viz., H157, Hek239T, HeLa, Jurkat, MCF-7 and two non cancerous cell lines viz., CHO (animal) and KMST-6 (human). The methods employed to assess the pro-apoptotic potential of the compounds were APOP*ercentage*™ and DNA fragmentation. The APOP*ercentage*™ assay showed that the activity of the compounds is concentration dependent and also revealed that in addition to the redox ability of the quinone carbonyl groups, the *peri* methoxy or hydroxyl group also plays a role in the apoptotic activity. An independent evaluation, the DNA fragmentation test, confirmed both a degree of selectivity viz., being not too

active against non-cancerous cell lines on the one hand whereas on the other having a high apoptotic potential against all five of the cancer cell lines evaluated.

ABBREVIATIONS

AIDS	Acquired Immuno Deficiency Syndrome
AFB	Acid-fast bacillus
APAF1	Apoptotic protease-activating factor 1
ATP	Adenosiine triphosphate
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase activated recruitment domains
CHO	Chinese Hamster Ovaries
CAN	Cerium (IV) ammonium nitrate
DCM	Dichloromethane
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signalling Complex
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
EAC	Ehrlich Ascites Carcinoma
EBV-EA	Epstein-Barr virus early antigen activation
EMB	Ethambutol
EtOAc	Ethyl acetate
FADD	Fas-activated Death Domain
GI	Growth Index
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HRMS	High Resolution Mass Spectrum
IAP	Inhibitor of Apoptosis Protein

IC_{50}	50% Inhibitory Concentration
INH	Isoniazid
MIC	Minimum Inhibition Concentration
MDR-TB	Multi-drug Resistant TB
MOMP	Mitochondrial Outer Membrane Permeabilization
M. tuberculosis	*Mycobacterium tuberculosis*
NMR	Nuclear Magnetic Resonance
PZA	Pyrazinamide
RGM	Rapidly Growing Mycobacteria
RMP	Rifampicin
SLD	Second-line Drugs
STM	Streptomycin
TB	Tuberculosis
TNF	Tumour Necrosis Factor
TPA	Tumour Promoter 12-*O*-tetradecanoylphorbol-13-acetate
TRADD	Toll Receptor Activated Death Domain
WHO	World Health Organization
XDR-TB	Extensively Drug Resistant TB

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To

Tristan and Teyah

CHAPTER 1

INTRODUCTION

Tuberculosis remains a serious health concern in many parts of the world and has been present in humans since antiquity. It has in all probability co-evolved with humans over many thousands of years, and perhaps since the very beginning of humankind itself. The earliest unambiguous detection of *Mycobacterium tuberculosis* is in the remains of a bison dated 18,000 years ago. [1] Skeletal remains of prehistoric humans (4000 BC) have shown the presence of TB, and tubercular decay has been found in the spines of mummies from 3000–2400 BC. [2] The bacillus causing tuberculosis in humans, was first identified and described by a German doctor, Robert Koch in 1882. He named the organism *Mycobacterium tuberculosis*, which means 'fungus-bacterium' because of the fungus-like membrane that the bacteria produce when cultured *in vitro*.

Fig. 1.1 Scanning electron micrograph of *Mycobacterium tuberculosis*

Fig. 1.2 *Mycobacterium tuberculosis* (stained red in sputum)

Mycobacterium tuberculosis is an aerobic, non-endospore-forming, non-motile, small rod-like bacillus with a cell wall that lacks a phospholipid outer membrane **(Fig. 1.1)**. It is classified as a Gram-positive bacterium since when a Gram stain is performed, the bacteria either stains very weakly Gram-positive or does not retain dye due to the high lipid and mycolic acid content of its cell wall. [3] Once stained however, mycobacteria are not easily decolourised, even with acid-alcohol and are therefore classified as an acid-fast bacillus ("AFB"). The most common staining technique, the Ziehl-Neelsen stain, dyes AFB's a bright red that stand out clearly against a blue background as illustrated above **(Fig. 1.2)**.

There are approximately 70 other species of the genus *Mycobacterium*, many of which are opportunistic pathogens in humans and animals, which include three other TB-causing mycobacteria viz., *M. bovis*, *M. africanum* and *M. microti*. *M. africanum* is not that widespread but in parts of Africa it has proved to be a significant cause of tuberculosis. [4, 5] *Mycobacterium bovis* causes TB in cattle and can also be transmitted by eating contaminated meat and through the consumption of unpasturised milk, whereas *M. microti* is mostly seen in immunodeficient people. Other known pathogenic mycobacteria include *Mycobacterium leprae*, *Mycobacterium avium* and *M. kansasii*. The last two are part of the nontuberculous mycobacteria group. Nontuberculous mycobacteria cause neither TB nor leprosy, but they do cause pulmonary diseases resembling TB. In nature, the bacterium can grow only within the cells of a host organism and have a predilection for lung tissue, due to its rich oxygen supply.

M. tuberculosis is spread through the air by airborne particles and when people suffering from active pulmonary TB cough, sneeze, speak, or spit, they expel these infectious aerosol droplets. Each one of these droplets may transmit the disease, and since the infectious dose concentration of tuberculosis is very low, the inhalation of just a single bacterium can cause a new infection.[6] Infection occurs when a susceptible person inhales droplet nuclei containing *M. tuberculosis* and the organisms reach the pulmonary alveoli of the lungs. Once in the lung, the organisms are taken up by the alveolar macrophages from where they are able to spread through the bloodstream to other tissues and organs causing secondary TB lesions to develop in other parts of the lung, peripheral lymph nodes, kidneys, brain and bone.[7] All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid. [8]

Not everyone infected with TB bacilli will contract the disease. However, infection can occur at any age. The immune system fights off the TB bacilli, which, protected by a thick waxy coat, can lie dormant for many years. About 90% of people infected with *Mycobacterium tuberculosis* develop asymptomatic, latent TB infection if they were to contract the disease. Progression from TB infection to developing the TB disease occurs when the TB bacilli overcome the immune system defenses and begin to multiply. Left untreated, each person with the active form of the TB disease will

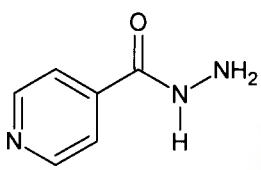
infect on average between 10 and 15 people every year. An individual's risk of infection depends upon exposure to the number of infectious droplets expelled by a carrier, the effectiveness of ventilation, the duration of exposure, the virulence of the *M. tuberculosis* strain, and his or her susceptibility to infection. There are a number of known factors that make people more susceptible to TB infection of which the human immunodeficiency virus (HIV) is the most important. HIV severely weakens the human immune system and makes people more vulnerable to TB infection. TB and HIV infections are both intracellular and known to have a profound influence on the progression of each other. HIV infections bring about a reduction in CD4+ T cells, which play a major role in immunity to TB. Individuals infected with HIV often develop TB before other manifestations of AIDS become apparent.[9] HIV can also facilitate both the progression of latent TB infection to active disease status and relapse of the disease in previously treated patients. Smoking also increases the risk of TB and diabetes mellitus is additionally emerging as an important risk factor in developing countries. [10, 11]

According to the World Health Organization (WHO), TB infection is currently spreading at the rate of one person per second. [12] About one-third of the world's population carry the TB bacteria and it is estimated that 30-60% of adults in developing countries are infected with *Mycobacterium tuberculosis*. Approximately 8-10 million individuals develop clinical TB and 3 million die of the disease each year. [13] In 2004, around 14.6 million people had the active TB disease and 9 million new cases being reported. It has been estimated that from 2003 until the year 2020, nearly one billion people will be newly infected, 200 million will become sick and 70 million will die from TB if it is not controlled. [14] The annual incidence rate varies from 356 cases per 100,000 people in Africa to 41 per 100,000 in the Americas (WHO, 2006). In 2005, Swaziland was the country with the highest estimated incidence of TB with 1262 cases per 100,000 people whereas India had the largest number of infections, with over 1.8 million cases.

In South Africa, TB is the most commonly notified disease and the fifth largest cause of death among the black population. South Africa is currently fourth on the WHO list of 22 high-burden countries responsible for 80% of the world's TB burden, and also has the second-highest prevalence of TB per capita in the world, at 998 cases per 100

000 population. [15] TB control is hampered by the HIV epidemic which is why the term "co-epidemic" or "dual-epidemic" is often used to describe their interrelationship, and is one of the main reasons for the rapid increase in TB in South Africa. This is now compounded by escalating rates of multi-drug resistance (MDR) and the emergence of extensively drug-resistant TB (XDR-TB).

Drug treatment for TB has been available since streptomycin was produced in 1944. This was followed by isoniazid in 1952, and rifampicin in 1966. The most common drugs used to treat TB infections are isoniazid (INH) **1**, rifampicin (RMP) **2**, pyrazinamide (PZA) **3**, ethambutol (EMB) **4**, and streptomycin (STM) **5**.



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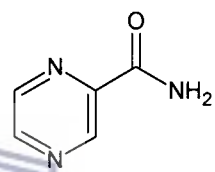

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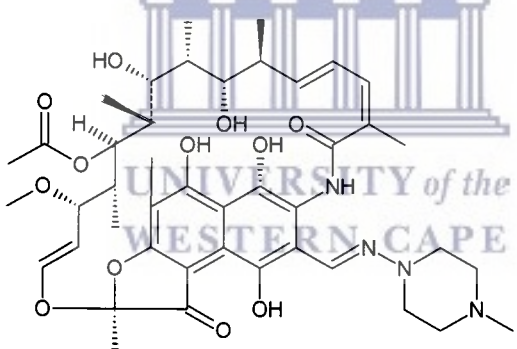

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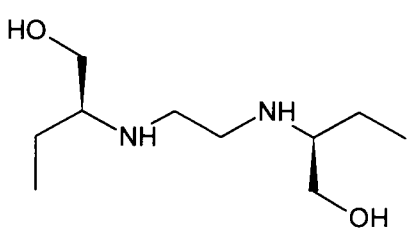

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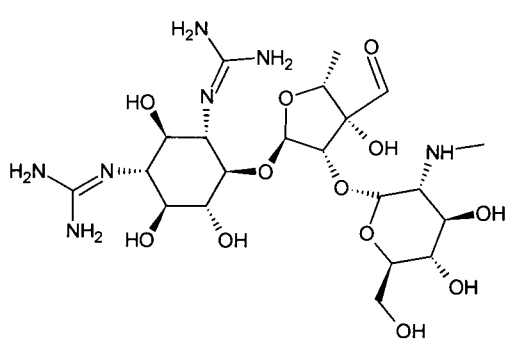

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Tuberculosis has been treated with combination therapy for over fifty years. Drugs are not used singly, except in latent TB, and regimens that use only single drugs, viz., monotherapy, result in the rapid development of resistance and treatment failure. The

frequency of spontaneous mutations that confer resistance to an individual drug are well known: 1 in $1x10^7$ for EMB, 1 in $1x10^8$ for STM and INH, and 1 in $1x10^{10}$ for RMP. [16] The rationale for using multiple drugs to treat TB is based on simple probability. While mutants resistant to a single drug may be fairly easily generated by monotherapy, the probability of developing mutants that are resisitant to multiple drugs decreases exponentially by increasing the number of drugs to which *M. tuberculosis* is simultaneously exposed.[17] Clinical studies provided unambiguous evidence of how the administration of multiple drugs bears a significantly lower chance of both disease reappearance and development of drug resistant strains compared with monotherapy. [17]

There are other theoretical reasons for supporting combination therapy. The different drugs in the regimen have different modes of action. Isoniazid **1** has the strongest early bactericidal action, is active against replicating bacteria, and thus significantly contributes to rapidly making patients non-infectious. Rifampicin **2** has unique antibacterial properties against bacilli that are no longer in the active phase of replication and has a sterilizing effect. Pyrazinamide **3** is only weakly bactericidal, but is very effective against bacteria located in acidic environments, inside macrophages, or in areas of acute inflammation. Ethambutol **4** is bacteriostatic at low doses, but is used in TB treatment at higher bactericidal doses and is the best tolerated of the 1st-line drugs. Streptomycin **5**, the most commonly used aminoglycoside antibiotic, is bactericidal and interferes with bacterial protein synthesis.

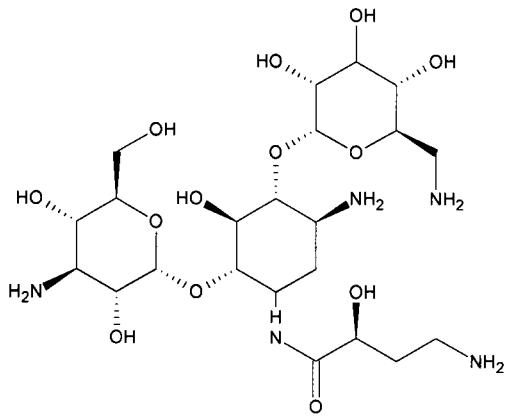
Treatment of TB depends on whether a person has either the active TB or latent TB form. TB can be diagnosed in several different ways, including chest x-rays, analysis of sputum, and skin tests. A person with a positive skin test, a normal chest x-ray, and no symptoms most likely has only a few TB germs in an inactive state and is not contagious (latent TB). The usual prescription recommended in such a case is isoniazid **1** and is taken for six to twelve months to prevent the TB from becoming active in the future. A person with a positive skin test along with an abnormal chest x-ray and sputum evidencing TB bacteria (positive smear test) has active TB and is contagious. Treatment for active TB consists of three or four drugs that are effective against the organism in the initial phase of therapy. The standard therapy for pulmonary TB includes isoniazid **1** and rifampicin **2** for 6 months along with

pyrazinamide **3**, plus a fourth drug, ethambutol or streptomycin, for the first 2 months. In some cases a fourth drug, ethambutol **4** or streptomycin **5** is also used, concurrently, for the first two months.

However, patients often stop taking drugs as soon as the symptoms are alleviated. As a result of this, the bacilli become imminent and resurface as drug-resistant tuberculosis. This suboptimal use of antituberculosis medications creates a selective milieu in the host's tissues where the initially scantly drug-resistant mutants are able to replicate, eventually replacing the original drug-susceptible *M. tuberculosis* population.[16] While a host of genetic factors may contribute, drug resistance primarily results from a) TB patients not adhering to their prescribed drug regimens, b) health professionals prescribing an incorrect treatment regimen, c) an unreliable drug supply interrupting the patient's treatment.

The widely adopted acronym MDR-TB (multi-drug resistant tuberculosis) indicates the presence of *M. tuberculosis*, resistant to both isoniazid **1** and rifampicin **2** with or without resistance to other drugs. [18] Treatment of MDR-TB is complex and requires more and different medications for a longer period of time. Patients with MDR TB must be treated with a combination of "second-line" drugs (SLD), which are not only significantly more expensive (>US$ 250 000 per case), but also much more toxic and less effective than the drugs used in standard therapy. [19] Although these drugs are active against TB, it is primarily used for MDR-TB treatment regimens.

There are six classes of second-line drugs of which the aminoglycosides and fluoroquinolones are the most important. Amikacin **6** and kanamycin **7** are the two most widely used aminoglycosides in the treatment of MDR-TB and may even remain effective if streptomycin resistance has developed. Alternatively, capreomycin, a polypeptide, is used and it is somewhat better tolerated than the aminoglycosides when prolonged administration is required.



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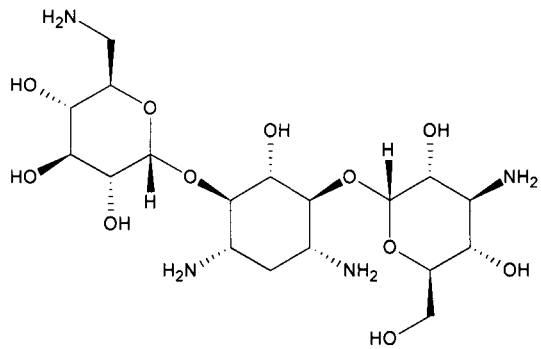

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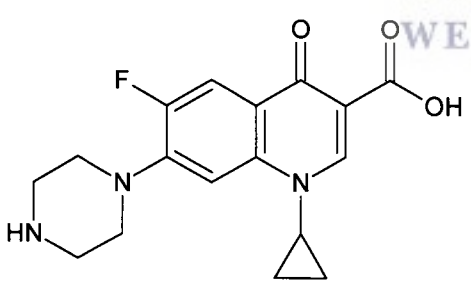
The four fluoroquinolones currently recommended are ciprofloxacin **8**, ofloxacin **9**, levofloxacin **10**, and sparfloxacin **11**. [20] The quinolones are broad-spectrum antibiotics and have few serious adverse effects. Ciprofloxacin **8** and ofloxacin **9** have similar potency, while levofloxacin **10**, the L-isomer of ofloxacin, has approximately twice the potency. Sparfloxacin **11** has even greater potency than levofloxacin however, photosensitivity reactions may occur, and patients must be instructed to avoid the sunlight. [21]



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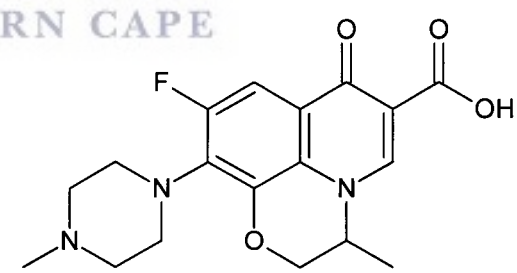

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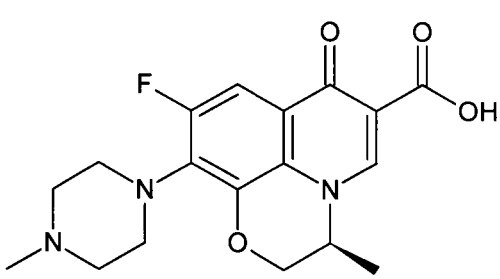

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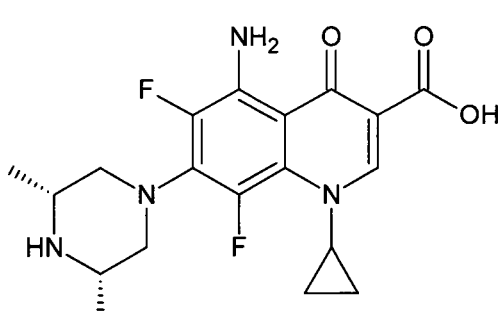

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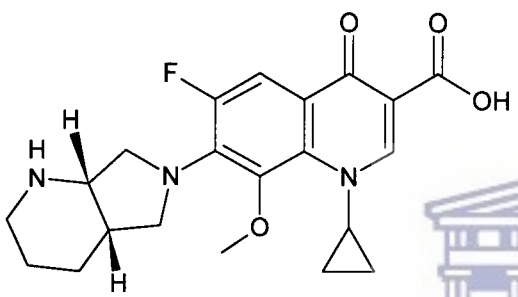
The newer fluoroquinolones, moxifloxacin **12** and gatifloxacin **13**, appear almost as active as INH and RMP, but the long term tolerability and safety of these two compounds have not been established as is known for the other quinolones. [22] Unfortunately, resistance to one fluoroquinolone usually means resistance to the others (cross-resistance). Other 2nd-line drugs include ethionamide, cycloserine, and para-aminosalicylic acid (PAS). These are less effective and more toxic than the 1st-line drugs but useful in treatment of MDR-TB. Patients with MDR strains should receive therapy based on individual drug susceptibility tests (DST), including residual first-line (SM, EMB, PZA) and second-line drugs.



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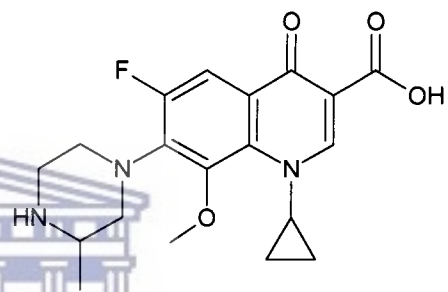

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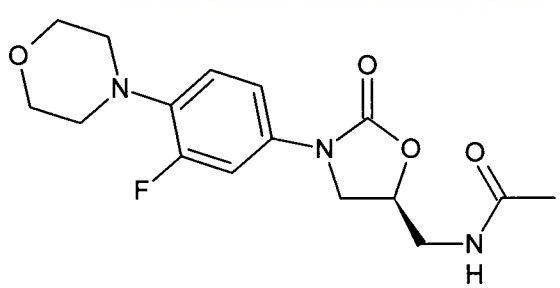
It is estimated that 424,000 cases of MDR-TB occur annually, representing 14% of the global burden of TB. [23]The highest rates were in the former USSR, the Baltic states, China, India, Argentina and the Dominican Republic in the Americas, and Cote d'Ivoire in Africa. According to a recent mathematical model, in South Africa, adults between the ages of 15–49, will number 278,154 TB deaths and 54,765 incident MDR-TB cases between the years of 2008 and 2017, if current control measures are continued. [24]

MDR-TB needs to be treated with SLD and extensively drug-resistant tuberculosis (XDR-TB) can develop when SLDs are either misused or mismanaged, thus rendering them ineffective. XDR-TB is defined as MDR-TB that is resistant to quinolones and also to any one of kanamycin, capreomycin, or amikacin (WHO 2006). In 2008 an epidemic of XDR-TB broke out in South Africa, which was a cause for grave concern. The outbreak was first reported as a cluster of 53 patients in a rural hospital in KwaZulu-Natal (KZN) of whom 52 died and since then, a wide spread of cases have been reported in most provinces in South Africa. [25]

Of particular concern to the medical fraternity in the initial outbreak in KZN, was that the mean survival period from sputum specimen collection to patient death was only 16 days and in addition that the majority of these patients had never previously received treatment for tuberculosis. This implies that they had been newly infected by XDR-TB strains, and that resistance obviously could not develop during treatment. It was concluded that in this particular case the spread of the disease was closely associated with a high prevalence of HIV and poor infection control.[26] However, world wide mismanagement of cases coupled with poor patient compliance with drug treatment cannot be discarded as major contributing factors.

TB strains that fulfil the current definition of XDR-TB have been identified retrospectively, and according to data (WHO, 2008), 49 countries have confirmed cases of XDR-TB, especially those in the former Soviet Union and Asia. It is estimated that there are about 40,000 of such cases per year. Despite early fears that this strain of TB was untreatable, recent studies have shown that XDR-TB can be treated through the use of aggressive regimens. [27] Because XDR-TB is resistant to first- and second-line drugs, treatment options are seriously limited. One of the first priorities is to perform drug susceptibility tests for all the necessary second-line drugs and to rapidly diagnose and correctly identify the presence of XDR-TB.



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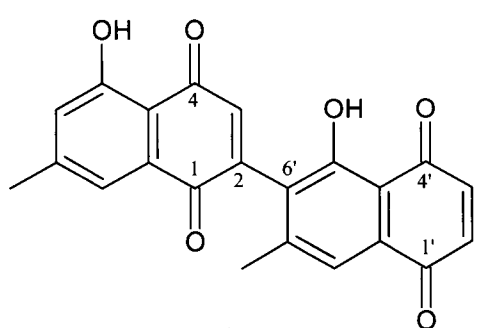
Linezolid **14**, a member of the oxazolidinone class of drugs, has shown good activity against different species of mycobacteria, including resistant strains, both *in vitro* and in animal studies and has been frequently used in the treatment against MDR- and XDR-TB. [28] However, data on the clinical efficacy and the tolerability of linezolid **14** in the treatment against *M. tuberculosis* are lacking, and its long-term use may be compromised by its high cost and serious adverse reactions. [29]

The resurgence of tuberculosis as a major disease in many parts of the world has prompted the search for novel compounds active against the causative organism, *Mycobacterium tuberculosis*. For many years, man has used plant extracts and decoctions to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various illnesses. Over the past decade there has been a proliferation in the literature on the antimycobacterial properties of plant extracts.

Twenty South African medicinal plants used to treat pulmonary diseases were screened for activity against drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis*. [30] A preliminary screening, using the agar plate method, showed that 14 of the 20 acetone extracts exhibited inhibitory activity at a concentration of 0.5 mg/ml against the drug-sensitive strain of *Mycobacterium tuberculosis*, H37Rv. Only six of the water extracts showed activity at concentrations ranging from 0.5-5.0 mg/ml and was the highest in *Euclea natalensis* (0.5 mg/ml).

Evaluation of the 14 active acetone extracts, using the radiometric method, showed that 12 inhibited growth of the H37Rv strain at a concentration of 0.5 mg/ml, while all 14 exhibited inhibitory activity against the drug-resistant strain (CCK028469V) at a concentration of 1.0 mg/ml. The minimum inhibition concentration (MIC) of a few plants, such as *Ekebergia capensis, Euclea natalensis, Nidorella anomala* and *Polygama myrtifolia,* was found to be 0.1 mg/ml for both the drug-sensitive and drug-resistant strains of *M. tuberculosis*. Out of the 20 medicinal plants investigated by Lall *et al.* [31], *Euclea natalensis* exhibited the best activity against drug sensitive and drug-resistant strains of *Mycobacterium tuberculosis*. They isolated and identified the active principle in Euclea *natalensis* to be the binaphthoquinone, known as diospyrin **15**.



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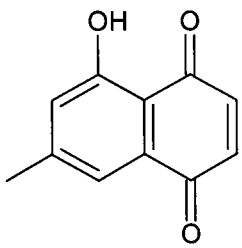
Euclea natalensis A.DC. , a tree of the Ebenaceae family is used extensively in oral health care, for chest complains, bronchitis, pleurisy, chronic asthma, urinary tract infections, veneral diseases etc. by the indigenous people of South Africa. The subtropical genus *Euclea* is also well known as a source of naphthoquinones; monomers such as 7-methyljuglone **16**, complex dimers and trimers, such as diospyrin **15** and galpinone **17** but, until recently, there have been no reports concerning their antimycobacterial activity.



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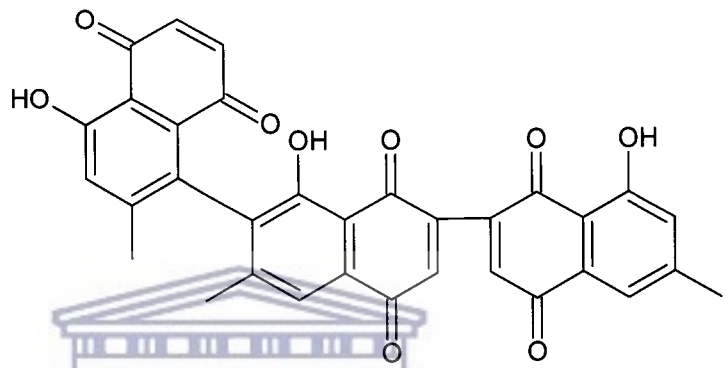
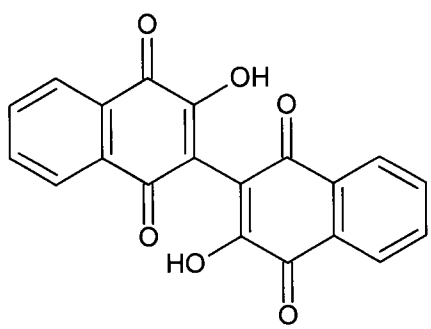


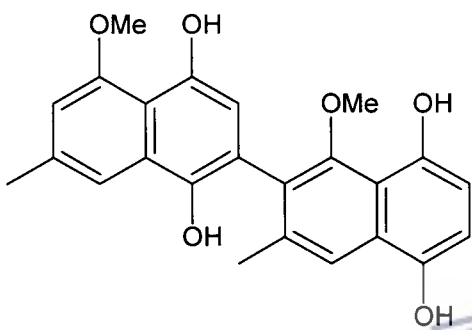
17

In continuation of their work Lall *et al.*[32] synthesized three derivatives of diospyrin to determine whether these structural changes would enhance the antimycobacterial activity of diospyrin **15**. They also evaluated a structural analogue of diospyrin **15**, i.e. the dimer of lawsone **18**, for its potential inhibitory activity against *Mycobacterium tuberculosis*, employing the rapid radiometric method, *in vitro*.

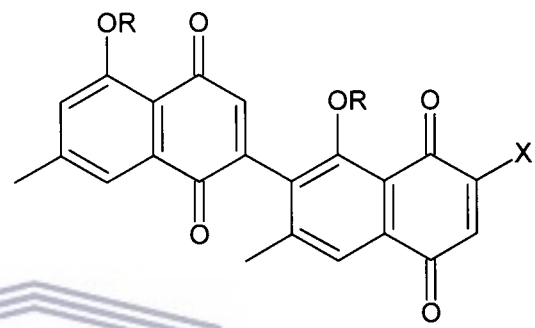


18

The latter and the two derivatives, i.e. diospyrin dimethyl ether hydroquinone **19** and diospyrin diethyl ether **20**, did not show any activity against any strain at the highest concentration tested. The aminoacetate derivative **22**, of diospyrin dimethyl ether **21**, was found to be more active than diospyrin **15** against a drug-susceptible strain, H37RV, of *M. tuberculosis*. The MIC of **22** was between 10-50 mg/L and that of diospyrin **15** 100mg/L. The same aminoacetate derivative **22** also exhibited an MIC of 50 mg/L, for a few multi-drug resistant strains of *M. tuberculosis*.



19

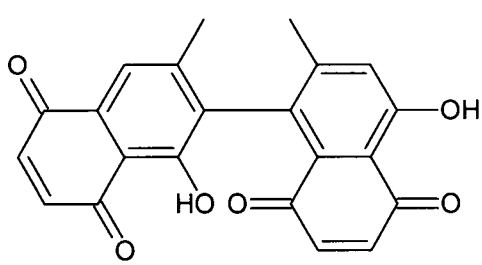


20 : R = C_2H_5 ; X = H

21 : R = CH_3 ; X = H

22 : R = CH_3 ; X = $NHCOCH_3$

In 2006, van der Kooy *et al.*[33] evaluated and reported the activity of six naphthoquinones, isolated from the root extracts of *Euclea natalensis*, against *M. tuberculosis*. The results showed that the MIC values of diospyrin **15** (8.0 µg/ml), isodiospyrin **23** (10.0 µg/ml) and neodiospyrin **24** (10.0 µg/ml) exhibited good activity when compared to the known antimycobacterial drugs, isoniazid (0.062 µg/ml), rifampicin (0.125 µg/ml) and ethambutol (1.25 µg/ml). The activity of 7-methyljuglone **16** (0.5 µg/ml) was found to be comparable to rifampicin and better than ethambutol.



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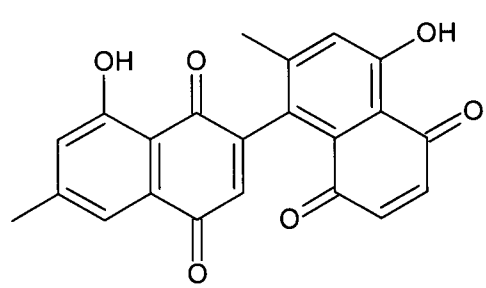
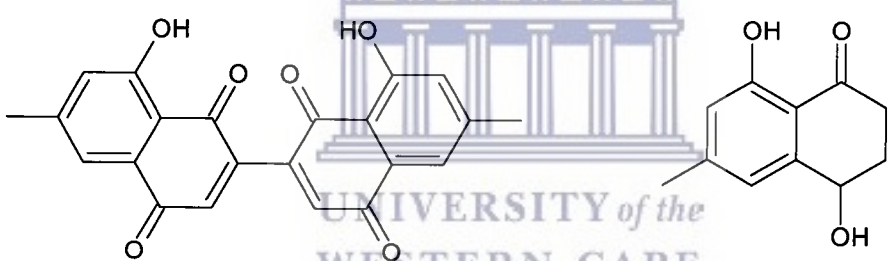


24

Mamegakinone **25** and shinanolone **26** showed the least activity, with both having the same MIC value of 100 μg/ml. [33] The MICs of the naphthoquinones indicate that the ketone groups on C1 and C4 are important for antimycobacterial activity, as can be seen by comparing the activity of 7-methyljuglone **16** with that of shinanolone **26**. The lack of aromaticity between carbons 1 and 4 might also play a role in decreasing the activity.

The reduction of activity for the dimeric compared to the monomeric molecules of 7-methyljuglone might be explained by the larger size of the former molecules and the likelihood that the ketone groups are hindered to fully participate in chemical reactions by the three dimensional shape of the molecule which could inhibit hydrogen-bonding as just one example. Mamegakinone **25** in which the two naphthyl nuclei are almost at right angles is extremely congested in this region, which might be the reason for its low antimycobacterial activity.

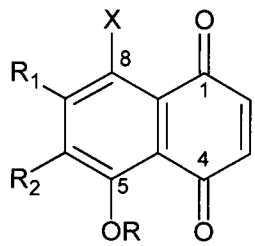


25 **26**

27 **28**

In view of these observations, Mahapatra and co-workers [34] synthesized and evaluated a series of synthetic and plant-derived naphthoquinone derivatives of 7-methyljuglone **16** for antibacterial activity evaluation against *Mycobacterium tuberculosis*. Of these, 7-methyljuglone **16** was the most potent (MIC = 0.5 μg/ml) followed by juglone **27** (MIC = 1.0 μg/ml) and the 5-acetoxy derivative **28** (MIC = 2.5 μg/ml), the last being 5-fold less active. Most of the MICs of the other derivatives varied between 5.0 μg/ml and ≥20 μg/ml.

In the halide series, **29-31**, the activity increased with an increase in halide bulkiness and decrease in halide electronegativity (the MICs being 10.0, 10.0, 5.0 μg/ml, respectively).

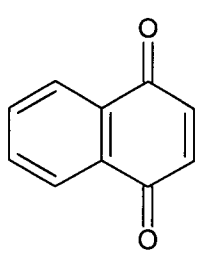


29 : $R = R_2 = H$; $R_1 = CH_3$; $X = F$

30 : $R = R_2 = H$; $R_1 = CH_3$; $X = Cl$

31 : $R = R_2 = H$; $R_1 = CH_3$; $X = Br$

The alkoxy derivatives, R = alkyl, showed a decrease in activity compared to their hydroxy precursors, which again illustrates the importance of having a hydroxyl and carbonyl group in the relative peri positions. This was also found to be the case in the diospyrin analogues study. [32] Previous studies have shown that mono- or dihydroxy substitution, at C_5 or C_5 and C_8, of naphthoquinones results in higher toxicity as compared to the parent 1,4-naphthoquinone due to increased efficiency of redox cycling. [35] The derivatives without the quinone motif exhibited reduced activity and cytotoxicity, implying that the quinone motif contributes both to biological activity and cytotoxicity.



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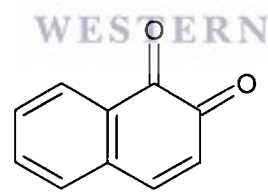

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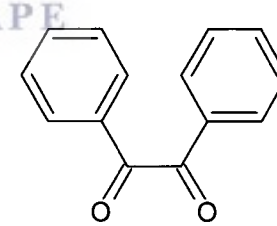
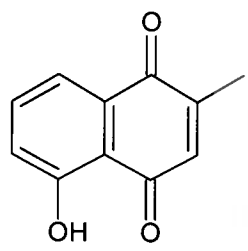


34

Tran *et al.* [36] observed similar results in an earlier study, in which twenty-eight compounds were screened for activity against four species of mycobacteria. The 1,4-diketone motif in the quinones exhibited a strong inhibitory activity, with the 1,2-naphthoquinone **32** being 8-fold less active than 1,4-naphthoquinone **33**. Compounds without this quinone moiety as part of the total structure had much lower activity. When the diketone moiety itself was retained but not part of the aromatic ring, the activity fell, as with benzil **34**, suggesting that the ketone groups themselves are not toxic.

While the addition of a second aromatic ring mildly improved activity (MIC of benzoquinone was 463 μM, while that of **33** was 316 μM against *M. smegmatis*), adding a third aromatic ring strongly decreased activity (anthraquinone has an MIC of 30,700 μM against *M. smegmatis*). Addition of a hydroxyl group to the second ring, peri to the quinone motif, enhanced activity. Plumbagin **35** was reported [36] to be the most active synthesized compound against rapidly growing mycobacteria (RGM) and the *M. avium* complex (MAC), with an MIC of 66 μM, while juglone **27** had an MIC against MAC of 72 μM. For comparison, kanamycin **7** has an MIC against *M. smegmatis* of 4 μM, and against *M. avium* of 26 μM. Naphthazarin **36**, which has two hydroxyl groups on the second ring, was found to be 2-fold more effective than the mono hydroxyl analogue **35**.



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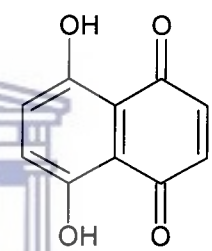

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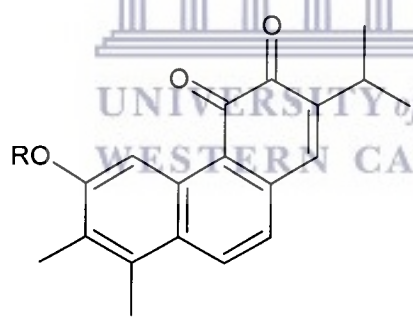


37 : R = CH_3

38 : R = H

Other quinones exhibiting activity against *Mycobacterium tuberculosis* include multiorthoquinone **37** and 12-demethylmultiorthoquinone **38** isolated from *Salvia multicaulis*. [37] The MIC values of a few compounds such as allicin isolated from garlic oil, [38] hypargenin F, from the roots of *Salvia hypargeia*, [39] triterpenes from *barrichia frutesecen* [40] were found to be higher than that of diospyrin, whilst compounds such as ambroxol, a semi-synthetic derivative of vasicine from the Indian shrub *Adhatoda vasica*, [41] and alkaloids isolated from *Galipea afficinalis* [42] showed better activity than diospyrin against *Mycobacterium tuberculosis.*

Diospyrin has also been isolated from other species of *Euclea* viz., *E. pseudebenus, E. crispa, E. divinorum* and *E. schimperi*, and from the *Diospyros* species, *D. mannii, D. montana, D. chamaethamus* and *D. piscatorial.* [31]

The bisnaphthoquinone diospyrin **15** was first isolated in 1961 by Kapil and Dhar as an orange-red constituent of ***Diospyros montana***, of the ***Ebenaceae*** species, a small or medium sized poisonous tree widely found in the tropics. [43] The structure of **15** was first proposed by Ganguly and Govindachari in 1966 as a dimer of 7-methyljuglone **16** linked between C-2′ and C-3, which implied the two naphtoquinone halves are linked through the quinone moieties. [44] However, in subsequent studies by Sidhu and Pardhasaradi the correct structure of diospyrin was established and demonstrated to have a linkage between C-2 and C-6′. [45, 46] Diospyrin **15** is optically inactive, which implies that there is no restricted rotation around the connecting bond between C-2 and C-6′. [47] The arguments for the proposed structure of diospyrin **15** were based on NMR spectra and recently the unambiguous structure has been established crystallographically **(Fig. 1.3)**. [48]

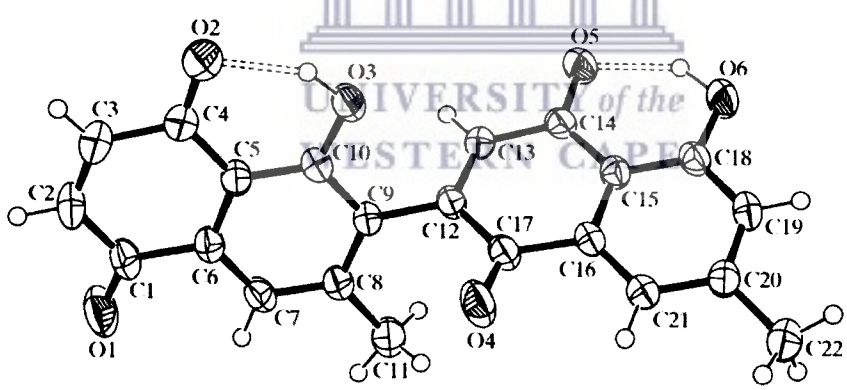


Fig. 1.3: Crystal structure of diospyrin showing intra-molecular H-bonding.

In the crystal, the two ring systems are not coplanar with the angle between their least-squares planes being 59.74°. The length of the bond between the two rings is 1.494 Å, suggesting that is essentially a single bond. A surprising feature is that the bulky C11 methyl group lies close to atom 04 rather than, as might be expected, close to the much smaller H atom attached to atom C13. As a result, atom O4 is significantly displaced from the least-squares plane of its naphthoquinonyl unit

C12-C22/O5-O6. Conversely, atom C11 shows no significant deviation from the C1-C11/O1-O3 least-squares plane.

The two OH groups of diospyrin participate in bifurcated intra- and intermolecular hydrogen bonds to C=O acceptors. The intramolecular O-H...O bonds are much shorter and stronger than the intermolecular links. This difference results in an 'unbalanced' hydrogen-bonding network, in which atoms O2 and O5 accept two hydrogen bonds each, one intramolecular and one intermolecular, and atoms O1 and O4 do not accept any conventional hydrogen bonds **(Fig. 1.4)**. Together the O-H...O bonds generate infinite stacks of molecules that are dissymmetric, but crystal symmetry generates a racemic mixture that is consistent with the lack of optical activity shown by diospyrin **15** in solution. [47] The interconversion of the two enantiomeric forms would be expected to occur readily in solution by analogy with the behaviour of trisubstituted biphenyls, which undergo rapid racemization in solution. [49]

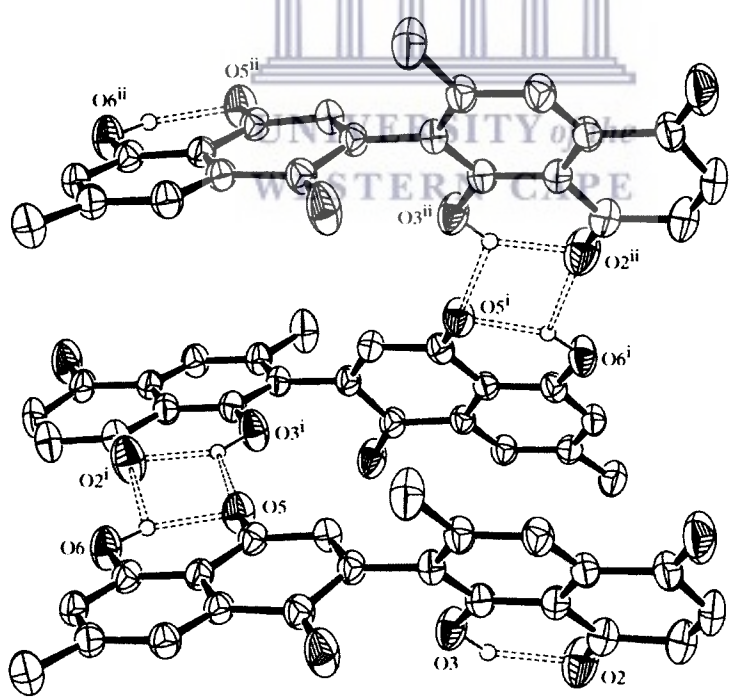


Fig. 1.4: Crystal structure of diospyrin showing intra-molecular and intermolecular H-bonding.

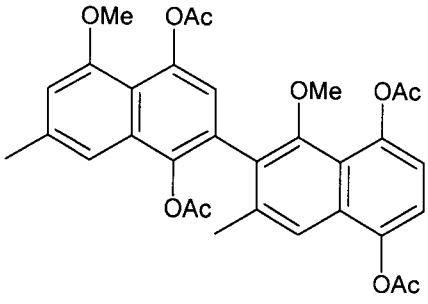
Quinonoid compounds are widely distributed in nature, mainly as secondary metabolites, in all respiring animal and plant cells. Some of these quinones play important roles in the biochemistry of energy production for their natural hosts, whilst many others show pronounced cytotoxic and allergenic actions that might enable the host to defend themselves against invading pathogens. [50] A number of natural quinonoids and their synthetic analogues have been found to possess significant antitumour activity by virtue of their facile redox cycling capacity, and are currently the second largest class of antitumour agents in use. [51]

It has been shown that the crude ethanoloic extract of the stem bark of ***Diospyros montana*** produces significant regression in the growth of ***Ehrlich Ascites Carcinoma*** (EAC) in Swiss Albino mice and the haematological parameters of tumour bearing mice could be significantly restored towards the normal range. [52] Earlier studies identified diospyrin **15** as a biologically active principle present in the bark extract of *Diospyros montana*. [53] The *in vitro* studies indicated marked effects that small doses of diospyrin **15** have on EAC cell surfaces, leading to agglutination and exocytosis. Higher doses caused disruption of intracellular material leading to total lysis. Several experiments in this study demonstrated significant inhibition of *in vivo* growth of EAC and an increase in the life span of the tumour bearing mice, which confirmed the effect of diospyrin **15** against EAC.

Diospyrin **15** and its two derivatives viz. diospyrin dimethyl ether hydroquinone **19** and diospyrin dimethyl ether **21** were tested for their inhibitory activities towards EAC *in vivo*.[54] The growth of EAC was significantly inhibited by all three compounds. However, the derivatives **19** and **21** were far more effective than diospyrin **15**. The tetraacetate derivative **39** and **19** were also evaluated for their activity against EAC and Sarcoma 180 and it was noted that the activity of **39** was lower than that of **19**. [55]

Subsequently, Pal *et al.* [56] synthesized a series of aminonaphthoquinones in fairly good yield, starting with diospyrin **15**, isolated form *Diospyros montana.* The aminoacetate derivative **40** showed the maximum increase in life-span (~93 %) with respect to the untreated control in Swiss A. mice bearing Ehrlich Ascites Carcinoma

(EAC) and exhibited the lowest inhibitory concentrations (IC_{50} for **40** = 0.06 μM, for **22** = 0.07 μM and for **41** = 0.09μM) *in vitro*. [57]



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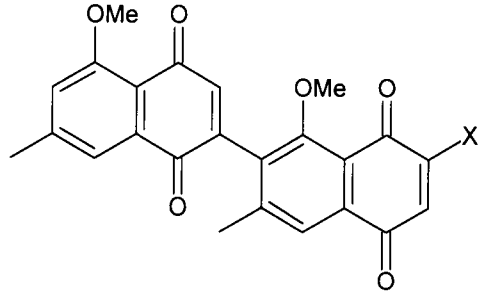
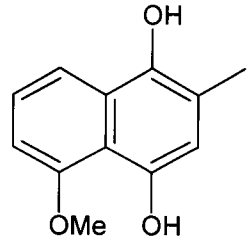


22 : X = $NHCOCH_3$

40 : X = $NHOCC(CH_3)_3$

41 : X = $NHCH_2CO_2Et$

Hazra *et al.* [58] in a similar approach prepared the hydroquinonoid derivative **42** of plumbagin **35** to examine whether the structural changes would improve the effectiveness and perhaps reduce its toxicity. Plumbagin **35**, a regiochemical monomer of diospyrin **15**, was found to possess anticancer activity against chemically induced fibrosarcoma and hepatoma in rats. [59, 60] This 5-hydroxynaphthoquinone, isolated as the major constituent of *Plumbago* species, however causes toxic side effects in mice as manifested by diarrhoea and adverse pathological prognosis thereby limiting its anticancer activity usefulness. [61]

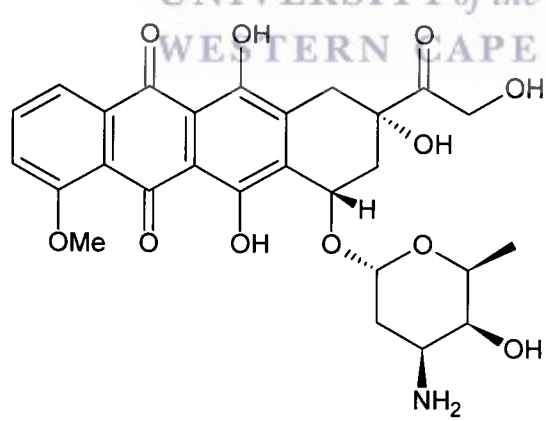


42

Synthetic modification of the plumbagin nucleus **35** did not however produce any substantial enhancement of its antitumour activity against Ehrlich Ascites Carcinoma *in vivo*, the only slight improvement being a slight increase in the life span of the tumour-bearing mice treated with the hydroquinonoid derivative **42**.

In 2002, Chakrabarty *et al.* [62] evaluated diospyrin **15** and three of its synthetic derivatives, i.e. diospyrin dimethyl ether hydroquinone **19**, diospyrin diethyl ether **20**, and diospyrin dimethyl ether **21**, for their activity on four human cancer cell lines viz., acute myeloblastic leukaemia (HL-60), chronic myelogenic leukaemia (K-562), breast adenocarcinomia (MCF-7) and cervical epithelial carcinoma (HeLa). The screening studies showed that diospyrin diethyl ether **20** was more active than the parent diospyrin **15** and its other derivatives.

The IC_{50} (50 % inhibitory concentration) values in HL-60 were > 100 μM for **15**, 64 μM for **21**, 54 μM for **19** and 30 μM for **20**, and the corresponding values in K-562 cells were > 100 μM, >100 μM, 65 μM and 40 μM, respectively, compared to doxorubicin **43**, a quinonoid antitumour drug, whose IC_{50} was found to be 1 μM in both leukemic cell lines. The diethyl ether derivative **20** inhibited growth in a concentration dependent manner and was equally effective in all cell lines. Compound **20** was not cytotoxic toward normal human lymphocytes, suggesting its action is specific for tumour cells. On microscopic examination cells treated with diospyrin diethyl ether **20** exhibited characteristic morphological features of apoptosis, such as cell shrinkage and formation of apoptotic bodies.

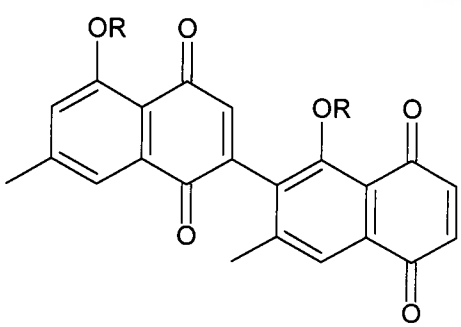


43

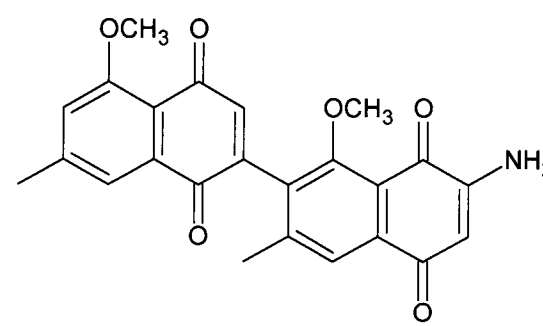
Diospyrin 15 and its derivatives have also been shown to be particularly active against protozoan parasites, e.g. *Leishmania*, *Trypanosoma* and *Plasmodium* species. [63, 64] Leishmaniasis manifests itself as a spectrum of diseases, ranging from benign cutaneous through metastatisizing mucocutaneous forms to the often fatal visceralizing forms. The visceral form of the disease is caused by *Leishmania donovani*, whilst *Leishmania major* causes cutaneous leishmaniasis.

Diospyrin **15** exhibited a significant inhibitory effect on the growth of *Leishmania donovani* promastigotes, by inhibiting the catalytic activity of the DNA topoisomerase I of the parasite. DNA topoisomerases are omnipresent enzymes that control many vital cellular processes by making reversible DNA breaks and have been classified into two types. The type I enzymes make a temporary nick in single stranded DNA, whereas the type II cause double-stranded DNA to undergo fission. These breaks in the strands cause the DNA strands to relax during replication and chromosomal separation. Ray *et al.* [65] has shown that the inhibition by diospyrin **15** is relatively specific as the compound requires 10-fold higher concentrations to inhibit DNA topoisomerase I from calf thymus and it does not inhibit DNA topoisomerase II of *L. donovani* at this concentration.

Diospyrin **15** with its synthesized derivatives, viz. alkyl ethers **20**, **21** and **44**, hydroquinonoid **19**, the amino derivative **45**, and the dimer of lawsone **18** were evaluated for their inhibitory activity against *Leishmania major* promastigotes *in vitro*. [66] The inhibitory effect of all the compounds was found to be concentration dependent, with diospyrin **15** being only slightly active at a concentration of 5 µg/mL and causing only 22% inhibition of the parasite growth, while all of the synthetic derivatives produced more than 98 % inhibition under comparable conditions.



44 : R = $CH(CH_3)_2$



45

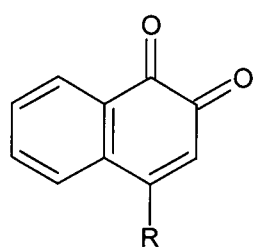
The dimeric naphthoquinonoid analogue **18** however, did not show any inhibitory activity up to a concentration of 5 µg/mL. Amongst the derivatives, the dimethyl ether **21** and its hydroquinonoid analogue **19** were the most active compounds, causing nearly 98% inhibition at a concentration of 2.5 µg/mL. These results corroborate earlier studies where compounds **19** and **21** were also found to exhibit significantly higher inhibition of *L. donovani* compared to that of diospyrin **15** and certain other

derivatives. [63] A nearly 20-fold reduction of activity was observed in the IC_{50} value of diospyrin **15** when it was converted to the hydroquinonoid form **19**, whereas the same chemical transformation carried out on plumbagin **35**, to its hydroquinonoid derivative **42**, resulted in a marginal viz., 1.5-times increase in the IC_{50} value.

While diospyrin **15** specifically inhibits the type I topoisomerase of *L. donovani* in preference to the type II [65], plumbagin **35** was found to induce topoisomerase II-mediated mammalian DNA-cleavage *in vitro*. [67] It is suggested that the difference in activity may be ascribed to the regiospecific positions of the methyl and hydroxy groups in diospyrin **15** compared to plumbagin **35**. [58]

In 1989, Konoshima and co-workers [68] reported the inhibitory effects of over 50 quinones on Epstein-Barr virus early antigen activation produced by the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In continuation of their studies a number of naphthoquinone derivatives occurring in the *Diospyros virginiana* and other selected plant genera, together with several synthetic naphthoquinones, were evaluated for *in vitro* anti-tumour promoting effects on EBV-EA activation. [69]

Amongst the 1,2-naphthoquinones, 4-amino-1,2-naphthoquinone **46**, was the most active showing about 30% and 75% inhibition of antigen activation at 10 and 100 times the TPA concentration respectively. The other two viz., 1,2-naphthoquinone **32** and 1,2-naphthoquinone-4-sulfonic acid **47** were relatively less active but were interestingly non-toxic to the cells.



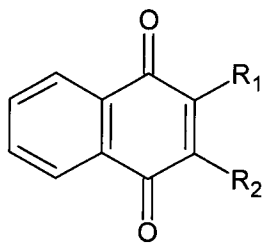
32 : R = H

46 : R = NH_2

47 : R = SO_3K

In the 1,4-naphthoquinone series, the parent 1,4-naphthoquinone **33** was the most active compound showing over 40% inhibition at 10 times the TPA concentration and over 85% at 100 times the TPA concentration, but was unfortunately toxic to cells and cell viability was poor at these concentrations.

The range of 1,4-naphthoquinones that had substituents in the quinone ring, e.g. 2-hydroxy-1,4-naphthoquinone **48** (lawsone) and 2-amino-1,4-naphthoquinone **49** were active showing about 35% inhibition at 100 times the TPA concentration, whereas lapachol **50**, which is the 3-(3'-methyl-2-butenyl) analogue of lawsone and 3-methyl lawsone **51**, were slightly less active than lawsone.



33 : $R_1 = R_2 = H$

48 : $R_1 = OH$; $R_2 = H$

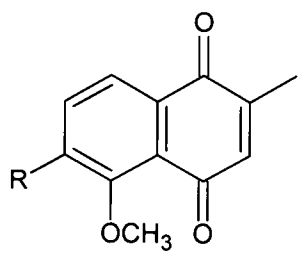
49 : $R_1 = NH_2$; $R_2 = H$

50 : $R_1 = OH$; $R_2 = CH_2CH{=}C(CH_3)_2$

51 : $R_1 = OH$; $R_2 = CH_3$

Amongst the 1,4-naphtoquinones substituted in the aryl ring, a structural isomer of lawsone viz., 5-hydroxy-1,4-naphthoquinone **27** (juglone), was more active than lawsone showing approximately 30% inhibition and 50% inhibition at 10 and 100 times the TPA concentrations, respectively. However, as in the case of the parent 1,4-naphthoquinone **33**, juglone **27** showed concomitant decrease in cell viability with increase in activity. It could thus be concluded that a hydroxyl substituent at position 5 increases the activity compared to the hydroxyl group in position 2 as in lawsone **19**. Interestingly, plumbagin **35**, the 2-methyl analogue of juglone did not show any activity even at 500 times the TPA concentrations.

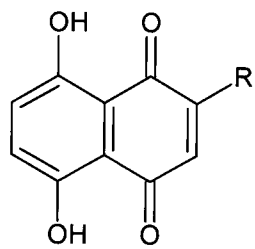
Methylation of the phenolic hydroxy group in plumbagin returned activity with *O*-methylplumbagin **52** exhibiting 80% inhibition at 100 times the TPA concentration. Introduction of a hydroxyl group at position 6 as in 2-methyl-5-methoxy-6-hydroxy-1,4-naphthoquinone **53** resulted in a decrease in activity with concomitant increase in cell viability.



52 : R = H

53 : R = OH

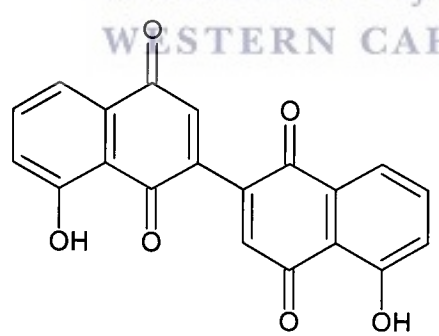
In contrast to plumbagin **35**, 7-methyljuglone **16** was as active as juglone **27** and also showed better cell viability. Amongst all the analogues tested naphthazarin **36**, the 8-hydroxy analogue of juglone, was the most active showing over 55% inhibition of a EBV – EA activation even at 10 times the concentration of TPA. Shikonin **54**, which is an 8-hydroxy juglone analogue with a 1-hydroxy-3-methyl-but-3-ene side chain at position 3, was the most active compound in the entire series with almost 60% inhibition of EBV-EA activation at 10 times the TPA concentration but it also showed a profound cell loss at the above-mentioned concentration.



36 : R = H

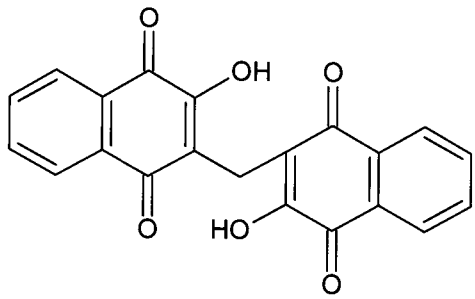
54 : R = $CHOHCH_2CH{=}C(CH_3)_2$

In addition to the monomeric analogues several dimeric napthoquinones were also investigated to evaluate strucure-activity relationship. The oxidative quinone-quinone coupling products of **27**, **48** and **16**, viz. 3,3'-bisjuglone **55**, 3,3'-bislawsone **18** and mamegakinone **25** were respectively less active than their parent monomers.

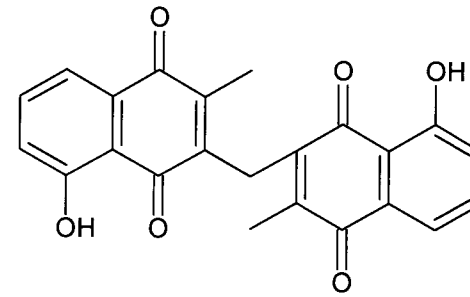


55

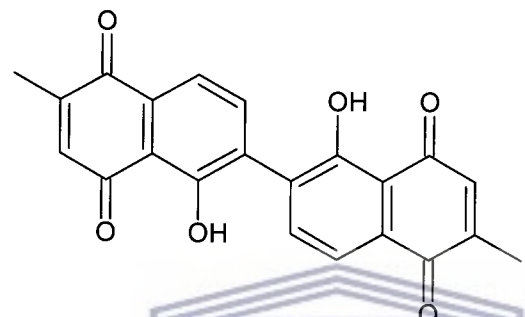
The 3,3'-methylene-bis analogues **56** and **57**, of lawsone and plumbagin showed equipotent activity, but an increased activity compared to their monomeric counterparts. In the case of diospyrin **15**, resulting from quinone-arene oxidative coupling of 7-methyljuglone **16**, the bis-naphthoquinone demonstrated a lower activity than the parent monomer. Both the arene-arene oxidative coupling product of 7-methyljuglone **16**, isodiospyrin **23** and the corresponding coupling product of 2-methyljuglone **58** (elliptinone) exhibited weak activity.



56



57



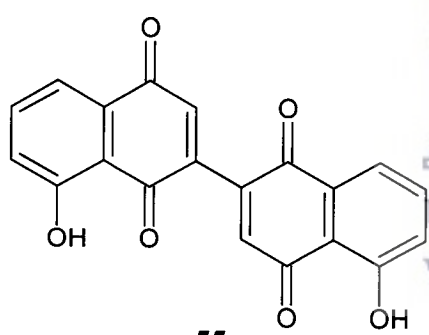
58

In conclusion, diospyrin **15** and its derivatives have been shown to be potent inhibitors of a variety of organisms viz., *Mycobacterium tuberculosis, Leishmania*, *Trypanosoma* and *Plasmodium* species as well as various cancer cell lines. Tuberculosis and cancer are among the most devastating diseases affecting Africa and other less developed countries for which there are few therapeutic options available. Both diseases have the scary ability to develop resistance against the drugs being used as treatment regimes and thus it remains a huge ongoing challenge to purposefully search for new potential drugs to address this scenario.

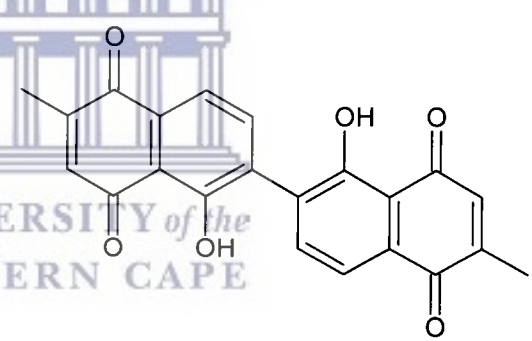
CHAPTER 2

SYNTHESIS OF BINAPHTHYL SYSTEMS

The structures of naturally occurring binaphthoquinones and higher quinones are based on two or more naphthoquinone units being linked together, firstly either between the quinone C=C bond moiety viz., 3,3'-bisjuglone **55**, or secondly between the benzenoid moieties of the molecule, e.g. elliptinone **58**, or thirdly, as in the case of diospyrin **15**, the linkage is between the quinone moiety of the one molecule and the benzenoid moiety of the other molecule. In almost all cases these molecules possess an element of symmetry and it is thus reasonably considered that the biosynthetic pathway to these binaphthoquinones involves oxidative biaryl coupling of a common naphthol precursor intermediate and subsequent oxidation of the resulting binaphthols. [70, 71]



55



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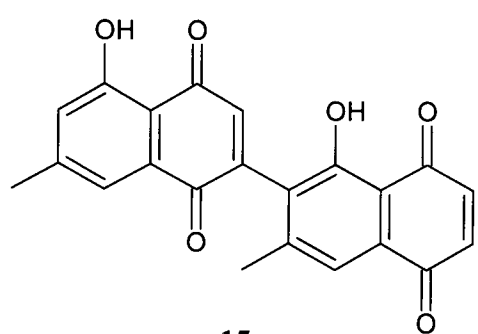
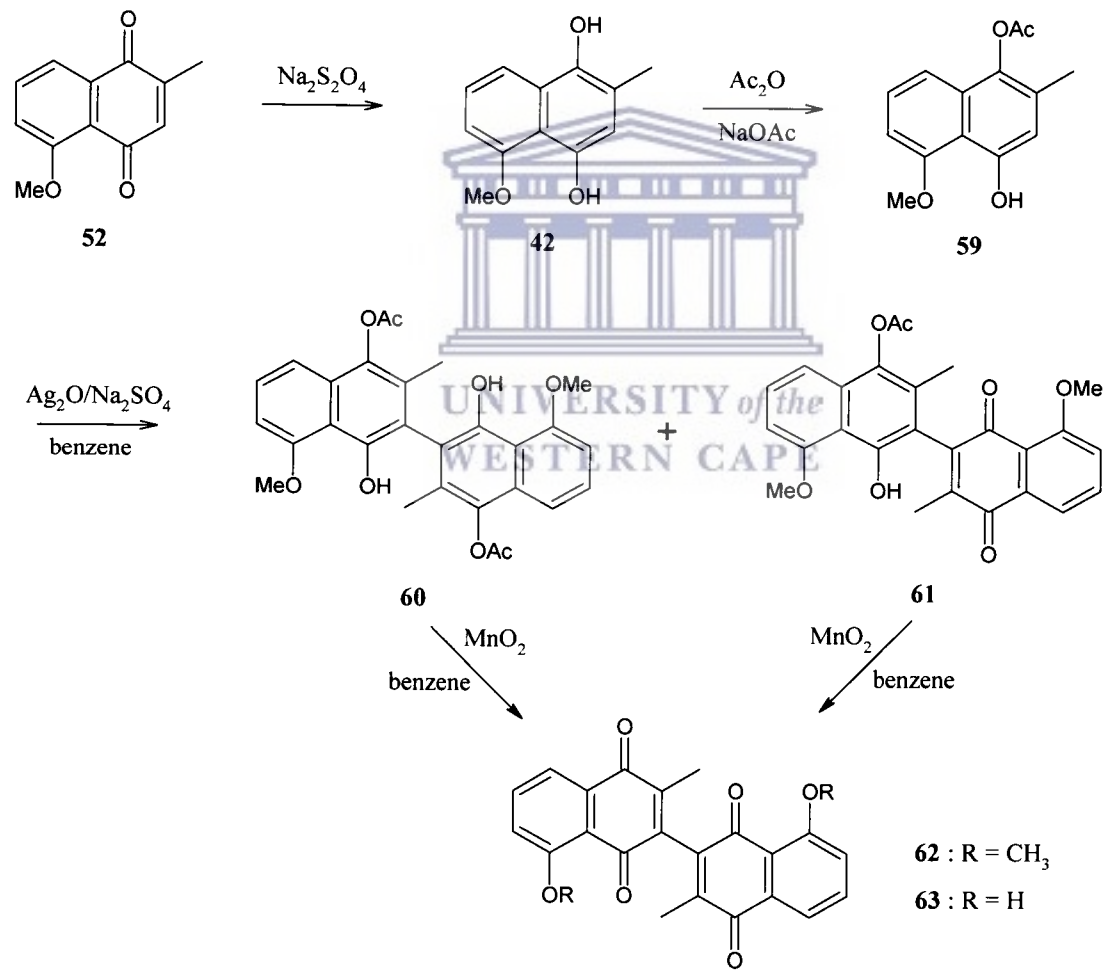


15

Over the years, various research groups have reported on the synthesis of these natural products, due to their diverse array of biological activities. An overview of just some of the synthetic protocols used by these research groups is presented as background material to the project.

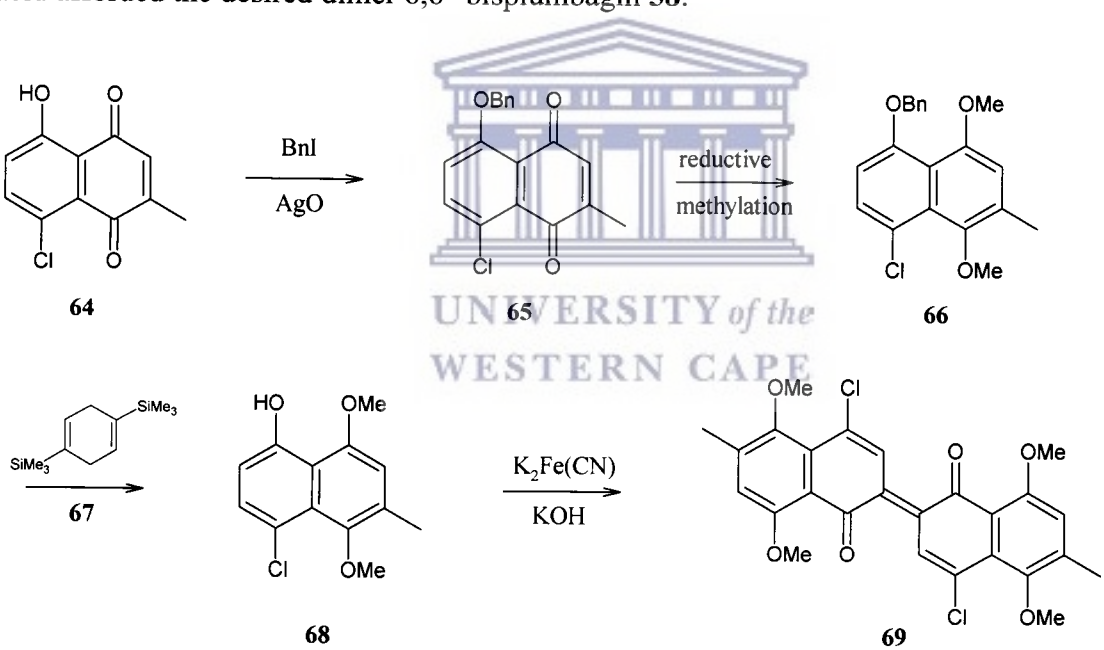
In 1974, Sankaram and Sidhu [72] first reported the synthesis of 3,3'- bisplumbagin **63** as illustrated in **Scheme 2.1**. The first stage of the synthesis involved the reduction of plumbagin methyl ether **52** with aqueous sodium dithionate to the corresponding quinol **42**. Upon acetylation with acetic anhydride and sodium acetate it yielded two products of which the monoacetate **59** was the major product along with the leucodiacetate. Oxidative coupling of monoaceate **59**, using Ag_2O in dry benzene containing anhydrous sodium sulphate, yielded a mixture of binaphthyls **60** and **61** which was converted quantitatively into the binaphthoquinone **62** on treatment with MnO_2 in dry benzene. Demethylation of compound **62** with anhydrous $AlCl_3$ in nitrobenzene yielded 3,3'- bisplumbagin **63**.



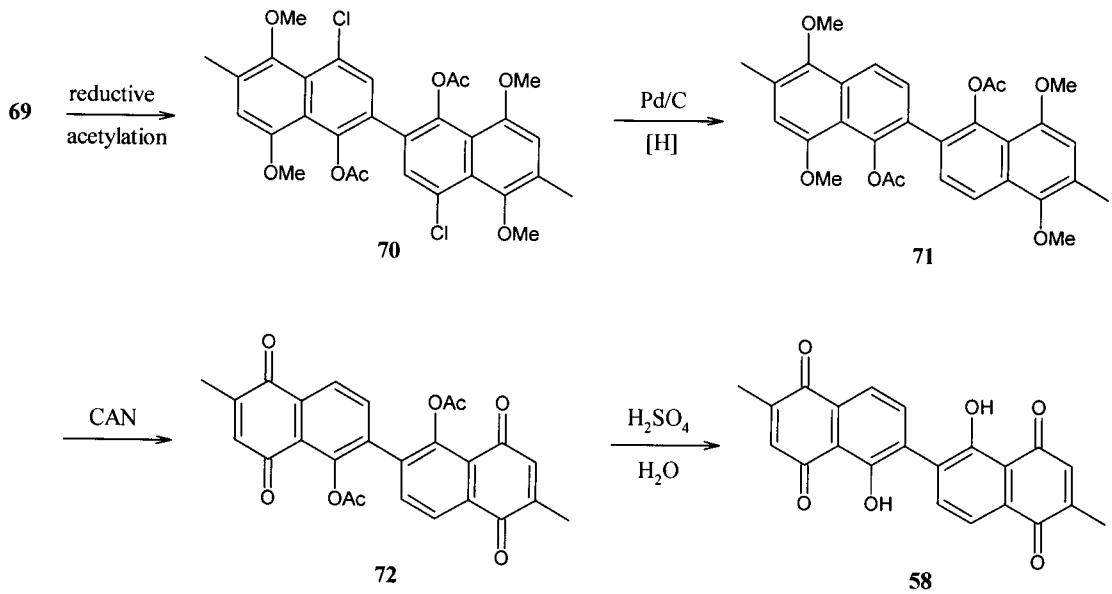
Scheme 2.1

In 1983, Laatsch *et al.* [73] reported the synthesis of 6,6-bisplumbagin, commonly known as elliptinone **58**, according to **Scheme 2.2** shown below.

Treatment of 8-chloroplumbagin **64** with benzyl iodide in the presence of silver oxide yielded quinone **65**, which was subsequently reductively methylated to afford naphthalene **66**. This in turn was chemoselectively debenzylated using diene **67** to afford naphthol **68**. Phenol oxidation of naphthol **68**, using potassium ferricyanide in the presence of potassium hydroxide afforded dimer **69**, which was then reductively acetylated to afford the corresponding dimer **70**. Treatment of this latter dimer with palladium catalyst in the presence of hydrogen gas removed the chlorine atoms to afford the dechlorinated dimer **71**, which was oxidized using cerium ammonium nitrate to yield the corresponding quinone **72**. Finally, deacetylation with sulphuric acid afforded the desired dimer 6,6'-bisplumbagin **58**.

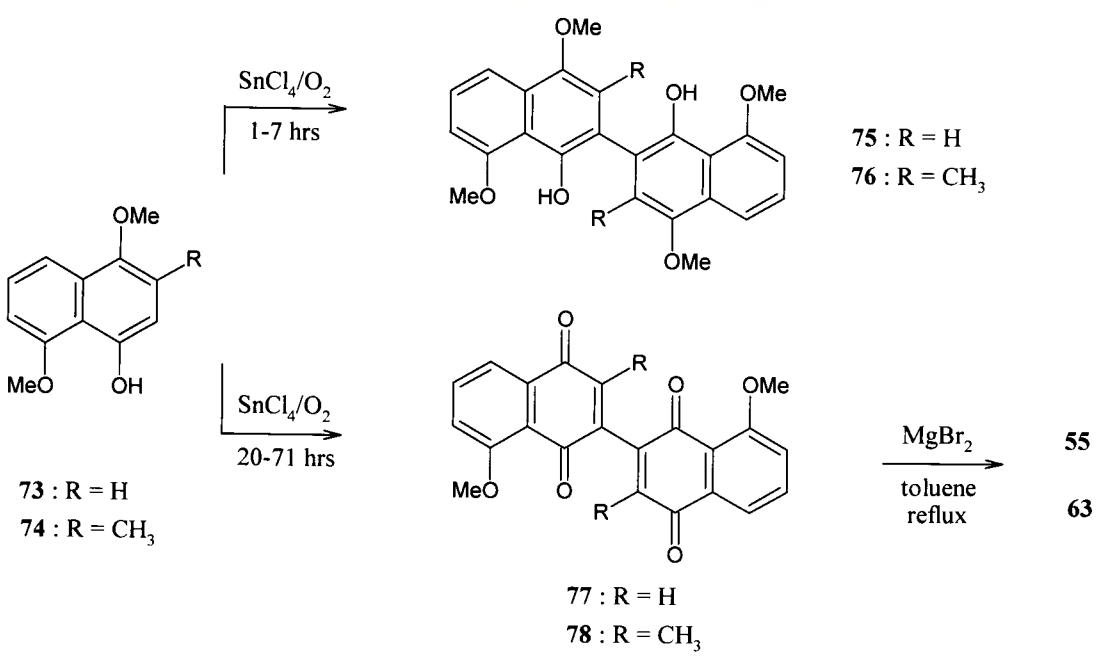


Scheme 2.2 (contd. over)



Scheme 2.2 (contd.)

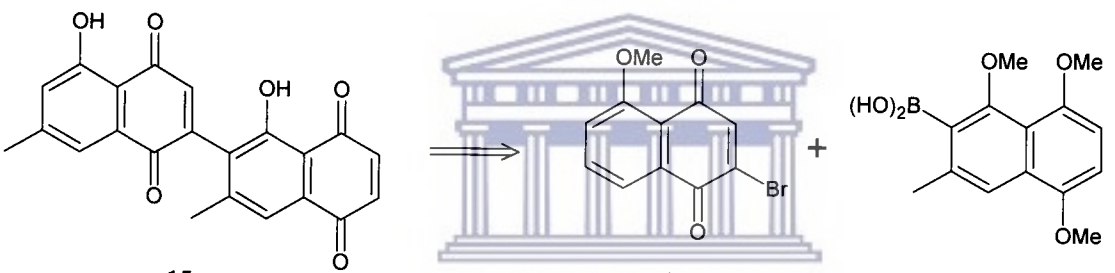
In 2004, Takeya *et al.* [74] reported a simple and more environmentally friendly method for the synthesis of the naturally occurring binaphthoquinones viz., 3,3'-bisjuglone **55** and 3,3'-bisplumbagin **63**, in a single step by means of aerobic oxidative dimerization of 1-naphthols with $SnCl_4$ in the presence of dioxygen. It was found that the nature of the major products changed drastically with the passage of time, as illustrated in **Scheme 2.3** below.



Scheme 2.3

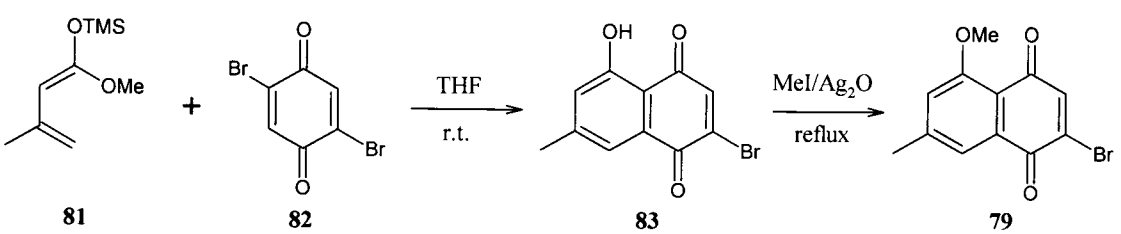
In the cases of naphthols **73** and **74**, binaphthols **75** and **76** were obtained as major products of the reaction for a short duration (1-7 h). However, prolonged reaction (20-71 h) under the same conditions selectively afforded the binaphthoquinones **77** and **78**. Finally, the demethylation of **77** and **78**, with $MgBr_2$ in refluxing toluene, afforded the corresponding natural products viz., 3,3'-bisjuglone **55** and 3,3'-bisplumbagin **63** in 97% and 98% yields, respectively.

In 2000, Yoshida and Mori [75] reported the first total synthesis of diospyrin **15** as illustrated in **Scheme 2.4**, **2.5** and **2.6**. The two naphthoquinone moieties **79** and **80** were synthesized separately, and then coupled by using Suzuki coupling protocols via an organoboronic acid intermediate as shown by the retrosynthetic analysis of diospyrin **15** in **Scheme 2.4**.



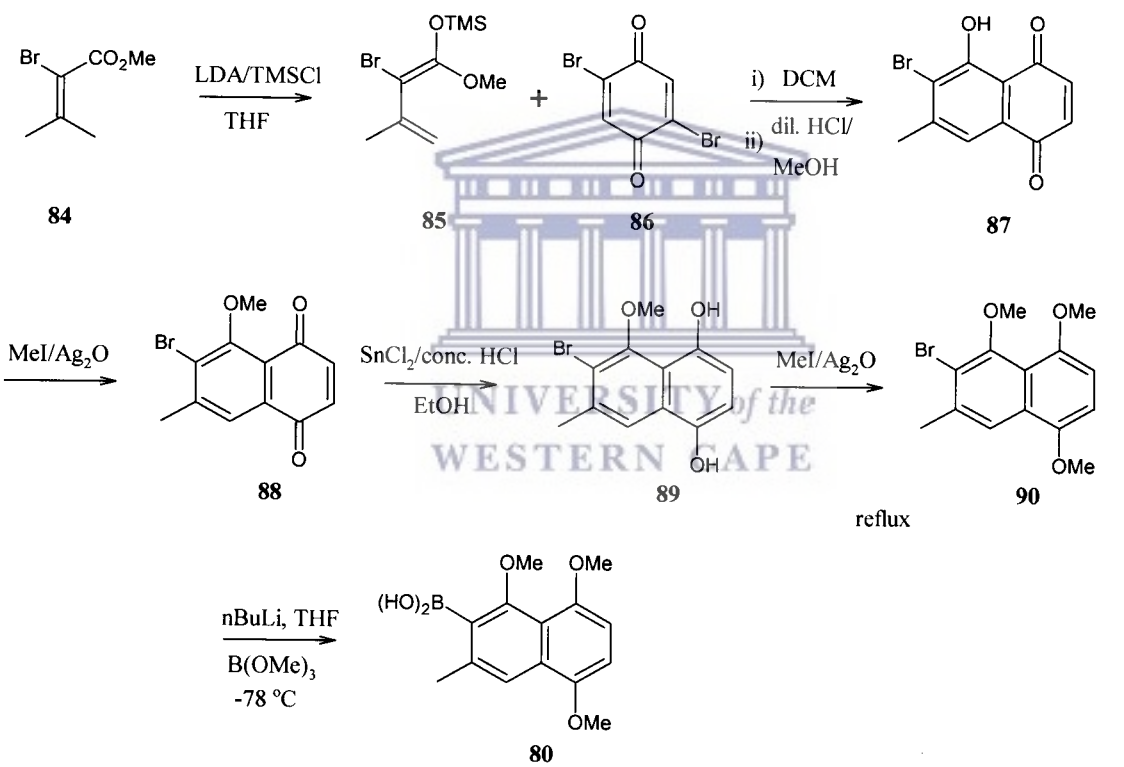
Scheme 2.4

The first stage of the synthesis was to prepare the naphthalene building blocks **79** and **80**. Diels-Alder reaction between 1-methoxy-3-methyl-1-trimethylsilyloxy-1,3-butadiene **81** and 2,5-dibromo-1,4-benzoquinone **82** afforded naphthoquinone **83**, which upon methylation with methyl iodide and silver oxide gave 2-bromo-5-methoxy-7-methyl-1,4-naphthoquinone **80** as depicted in **Scheme 2.5**.



Scheme 2.5

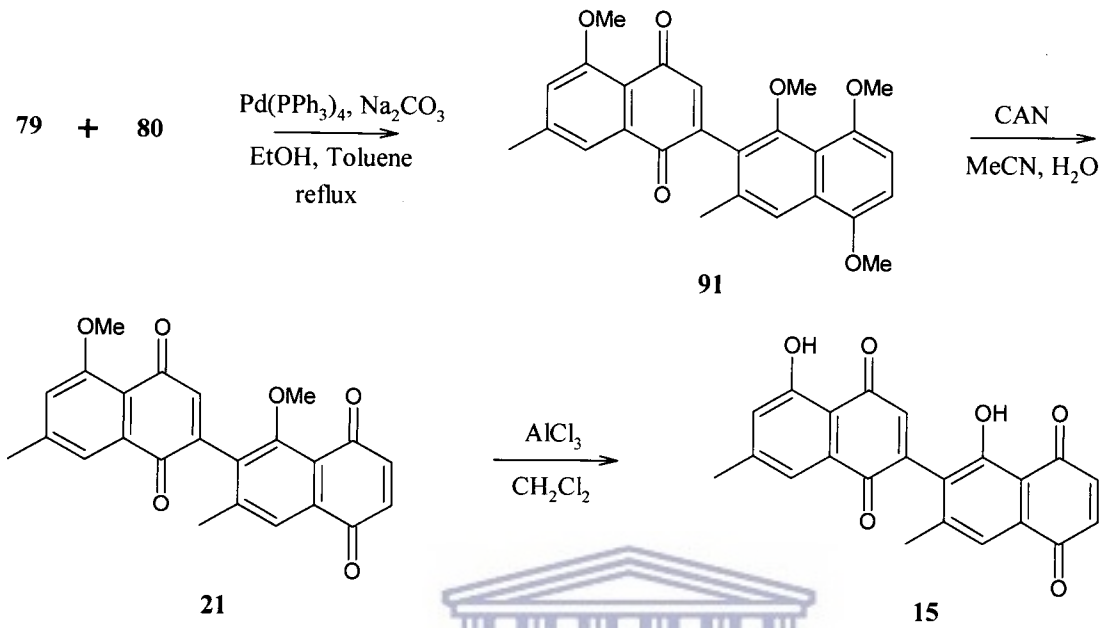
For the synthesis of **80 (Scheme 2.6)**, Diels-Alder methodology was again utilized to good effect and thus, 2-bromo-1-methoxy-3-methyl-1-trimethylsilyloxy-1,3-butadiene **85** was prepared from methyl 2-bromosenecioate **84**. The Diels-Alder reaction between **85** and 1,4-benzoquinone **86** proceeded in dichloromethane, and the initially formed product was then treated with dilute hydrochloric acid to remove the partially remaining trimethylsilyl group, giving a mixture of **87** and **88**. This mixture was fully methylated with methyl iodide and silver oxide to afford pure naphthoquinone **88**. Reduction of **88** with tin(II) chloride furnished diol **89**, which was then methylated to give **90**. The boronic acid **80** was prepared by lithiation of **90** followed by subsequent reaction with trimethyl borate.



Scheme 2.6

The Suzuki coupling between **79** and **80** was successfully achieved under the standard conditions employing tetrakis(triphenylphosphane)palladium(0) as the catalyst in the presence of aqueous sodium carbonate to give the coupling product **91** as orange red needles in 53% yield **(Scheme 2.7)**. Oxidative demethylation of **91** with cerium ammonium nitrate afforded diospyrin dimethyl ether **21** as yellow needles. Further

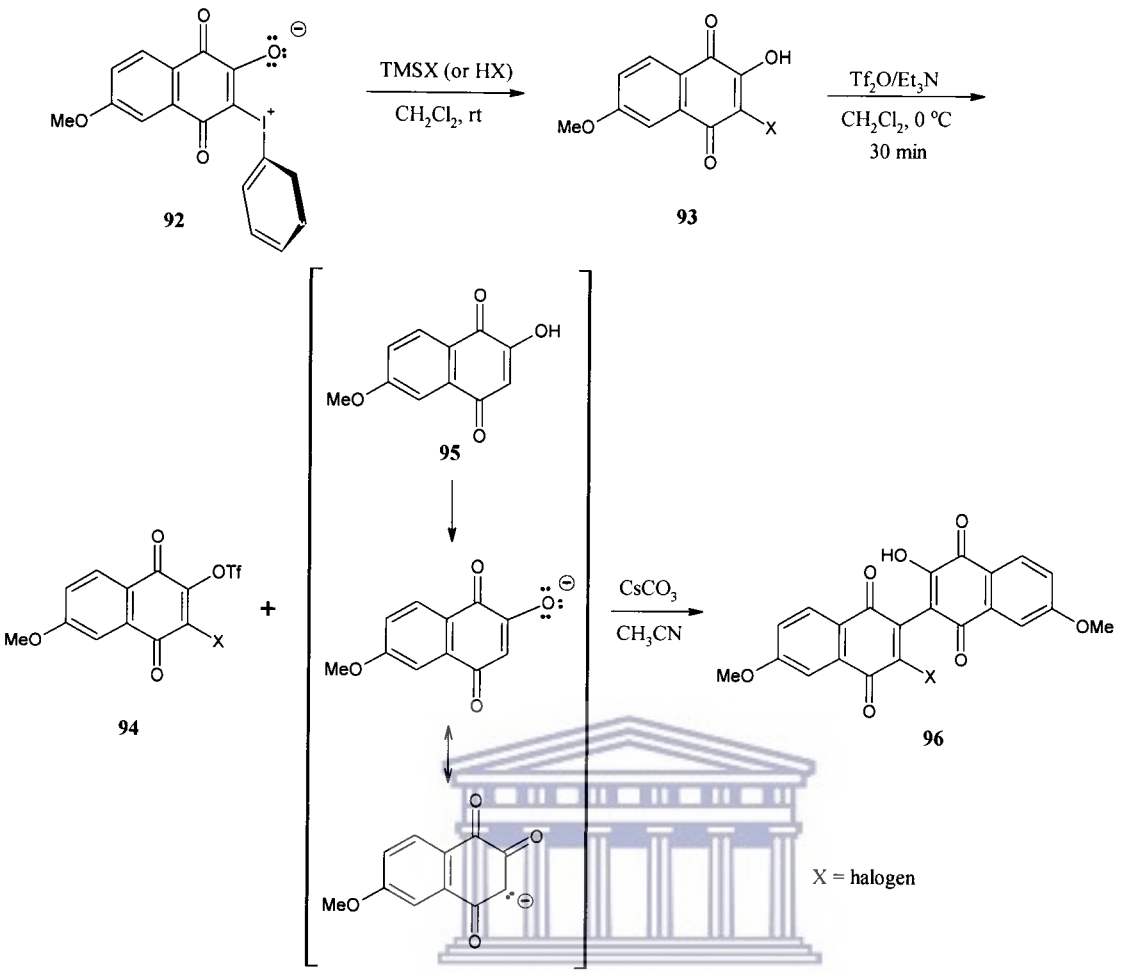
demethylation of **21** with aluminium trichloride in dichloromethane at room temperature yielded diospyrin **15**.



Scheme 2.7

In 2006, Stagliano *et al.* [76] reported the synthesis of unsymmetrical binaphthoquinones using halotriflates and hydroxynaphthoquinones as depicted in **Scheme 2.8** below.

The reaction proceeds via Michael addition of the carbanion to the triflated carbon followed by the elimination of the triflate group. The halonaphthoquinone triflates **94** were prepared by reacting the readily available quinone 1,4-dipoles **92** [77] with either trimethyl silyl halides or the corresponding hydrohalic acids to afford the hydronaphthoquinones **93**, which upon treatment with triflic anhydride provided the desired quinone triflates **94**. A mixture of **94**, hydroxynaphthoquinone **95**, and $CsCO_3$ was then stirred in acetonitrile for 4-6 days to afford the biquinone **96** in good yield. Further substitution of the halogen by hydroxyquinone anions afforded trimeric quinones. [78]

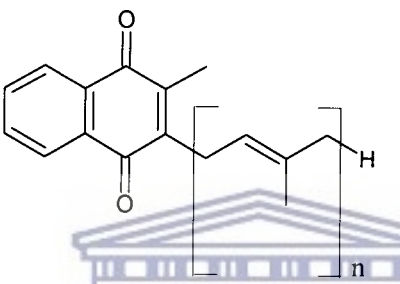


Scheme 2.8

CHAPTER 3

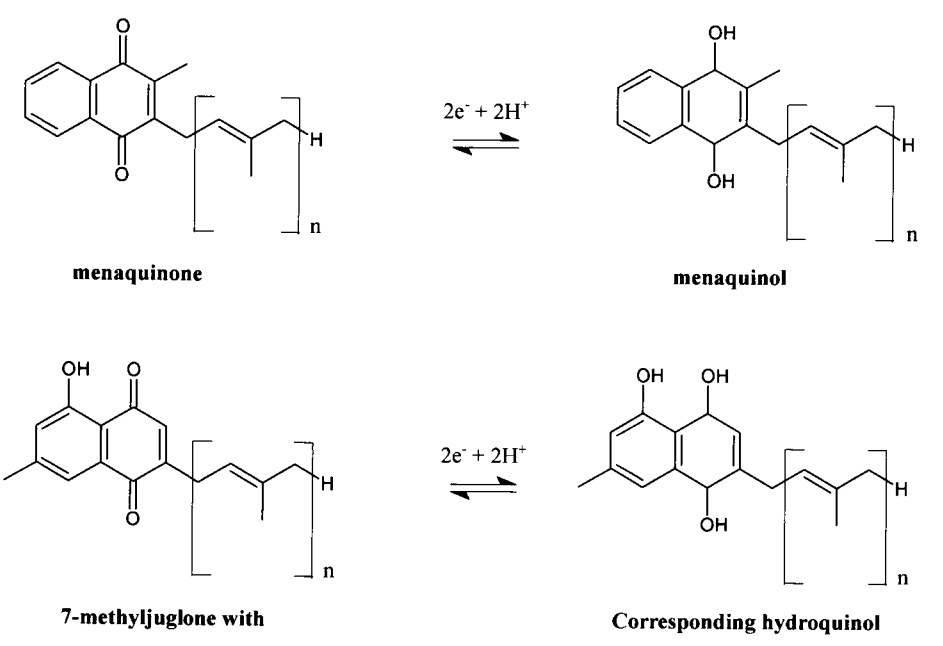
AIM

Diospyrin **15** and its monomer 7-methyljuglone **16**, both naturally occurring naphthoquinone compounds, have been shown to exhibit significant antimycobacterial and anti-tumour activity. It has been suggested that the activity of 7-methyljuglone **16** against *M. tuberculosis* could be attributed to the structural similarities between 7-methyljuglone **16** and menaquinone **97**. [33]



97

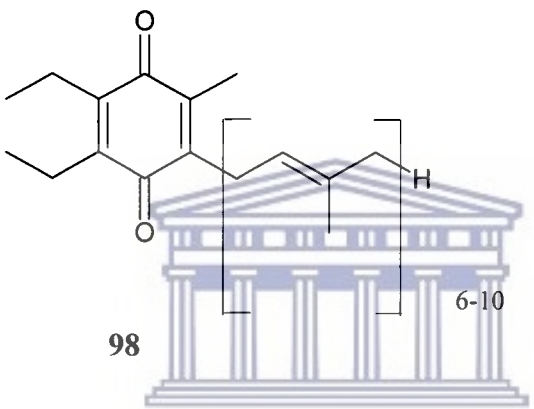
Mycobacterium contains the natural redox cycler menaquinone **97**, which mediates electron transfer between different membrane-bound enzymes of the respiratory chain. [79] Hence, it is feasible to postulate that 7-methyljuglone **16** can interact with enzymes in the mycobacterial electron transport chain **(Scheme 3.1)**.



Scheme 3.1

Due to the difference in the redox potential of the incorporated 7-methyljuglone **16**, the electron flow might be slowed or stopped. It is also possible that 7-methyljuglone **16** binds to the *Men* enzymes, responsible for the formation of menaquinone, and inhibits the addition of the hydrophobic isoprene side chain to carbon 2. This will influence ATP production and result in a detrimental effect on the organism.

Most bacteria and mammals make use of ubiquinone **98** to fulfill this function. *M. tuberculosis* lacks ubiquinone **98** and makes use of only menaquinone **97** in the electron transport chain. [80] It is therefore an attractive drug target because it lacks a human homologue.



98

The main focus of this project was the development of a general synthetic protocol for the synthesis of precursors for a cross-coupling route to biaryl molecules that can be further manipulated chemically into compounds related to the active molecules isolated from plants. The aim was then to synthesize biaryl systems, having a similarity to that of the lead compound diospyrin **15**, using the framework of juglone **27** as our monomer building block and the evaluation of their biological activity against different strains of *Mycobacterium tuberculosis* as well as cancer cell lines that might be accessed. The reason for this was that juglone **27** represents the nor-methyl analogue of 7-methyljuglone **16** and its regioisomer plumbagin **35**, both of which have been shown to possess antibacterial and anti-tumour properties.

By synthesizing compounds related in some aspects to the lead molecules found in nature and evaluating them on a comparative basis to the lead molecules a structure versus activity profile would become available which when studied will guide a more focused approach in deciding on specific targets for synthetic protocol development.

In addition to examining what effect the different structural changes of the naphthyl moieties might have on the biological activity of the binaphthoquinone systems, it was also a means to investigate what effect the linkage between the two naphthyl moieties has on the their activity. Thus, our aim was to synthesize binaphtho-systems with one moiety having a naphthoquinone structure linked, to a second aryl system through a C-C bond at the C=C moiety of the naphthoquinone. The envisaged route towards the synthesis of these biaryl compounds would essentially involve Suzuki coupling reactions.

It was proposed that the evaluation of the different analogues against the drug-susceptible clinical strain, HR37v of *M. tuberculosis*, using the BACTEC method, would be done in conjunction with senior and responsible colleagues using the facility available at the Stellenbosch Medical School near the UWC. Further investigation of the potential of these compounds to induce apoptosis in six cancer cell lines viz., H157, Hek239T, HeLa, HT29, Jurkat, MCF7 and two normal cell lines viz., CHO and KMST-6 was also proposed. These evaluations would be conducted, by the author, at the Biotechnology department at the University of the Western Cape.

CHAPTER 4

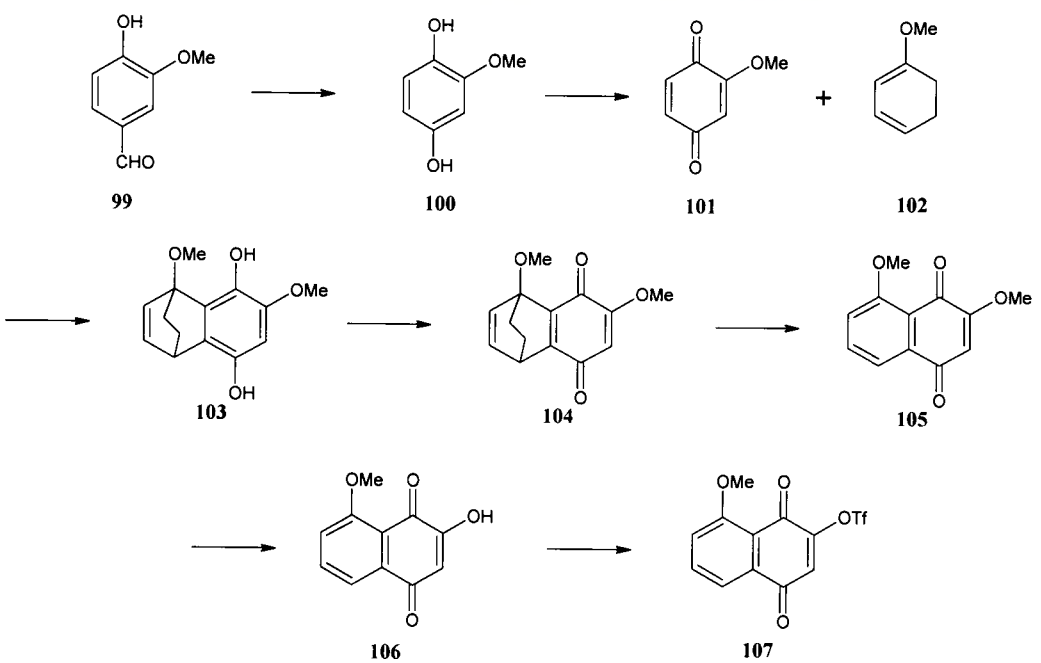
RESULTS AND DISCUSSION

4.1 Synthesis of naphthalene precursors

The first objective was to synthesize several naphthyl precursors, based on the framework of the naturally occurring naphthoquinone, juglone **27**, to be employed as building blocks for the targeted binaphthyl systems utilizing either Suzuki or Stille cross coupling reactions or a Michael addition reaction.

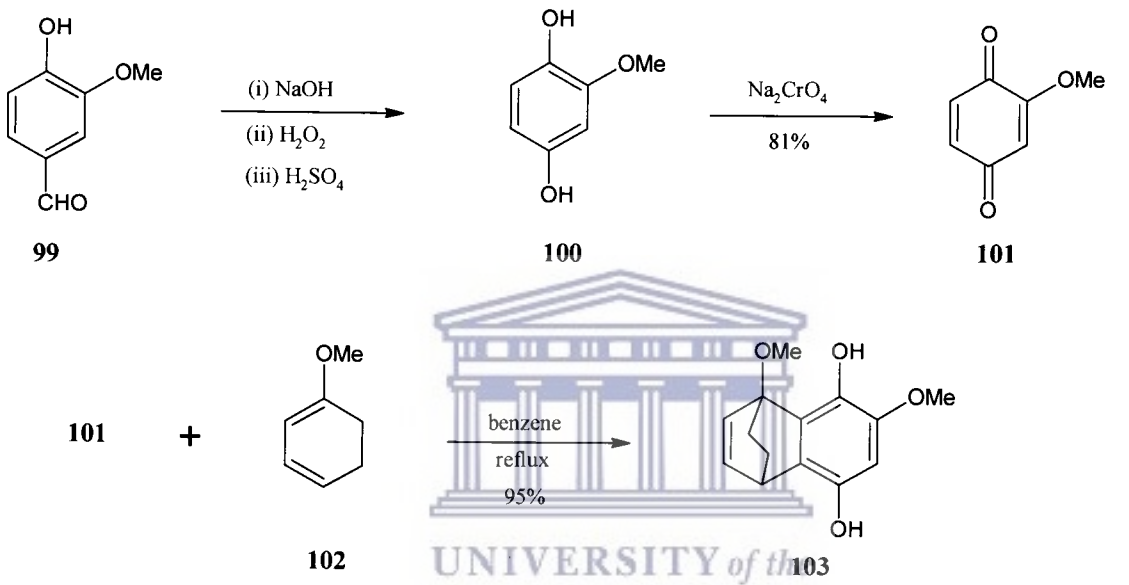
4.1.1 Synthesis of 8-Methoxy-2-trifluoromethanesulphonyloxy-1,4-naphtho-quinone

With the coupling procedure, used by Stagliaono *et al.*, [76] in mind, we set out to synthesize naphthoquinone triflate **107** and couple this to its naphthol precursor **106** which had previously been synthesized by Giles and Roos [81] in 1976. Much later in 2002, Ameer and co-workers [82] synthesized the same naphthol **106** using a similar method to that of Giles and Roos but in an improved yield. Thus, utilizing Ameer's method, we proceeded with the preparation of naphthol **106**. **(Scheme 4.1)**



Scheme 4.1

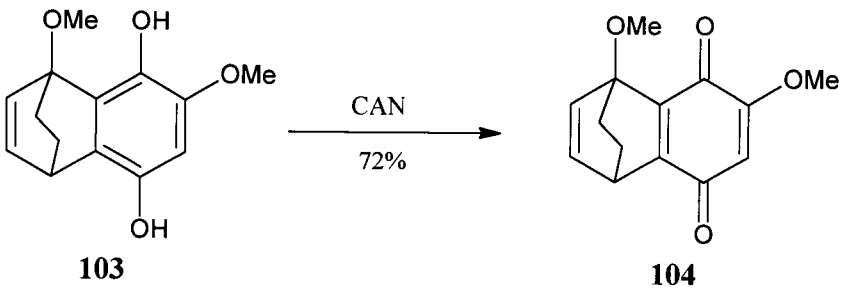
Baeyer-Villiger [83] oxidation and subsequent acid hydrolysis of the intermediate formylester derived from vanillin **99** afforded phenol **100** which was readily oxidized to 2-methoxybenzoquinone **101** in 81% yield **(Scheme 4.2)**. Diels-Alder condensation between quinone **101** and the commercially available diene **102** was effected in boiling benzene to afford a crude product which was simply passed through a column containing silica gel and afforded the enolized adduct **103** in 95% yield as shown in **Scheme 4.2** below.



Scheme 4.2

Evidence for the product of enolization **103** was clear from the IR spectrum in which displayed a broad absorption at 3400-2500 cm^{-1} for the hydroxyl groups. The ^1H NMR spectrum (d_6-acetone) supported the structure through two D_2O exchangeable hydrogens at δ 2.82 and δ 8.29 and the absence of the C=O carbon peaks in the ^{13}C NMR spectrum. A HRMS ($M^+ + 1$) of 249.1133 supported the molecular formula $C_{14}H_{17}O_4$ (required 249.1127).

Oxidation of phenol **103** with aqueous cerium(IV) ammonium nitrate produced the bridged quinone **104**, depicted in **Scheme 4.3** below. The presence of the two C=O carbon peaks at δ 177.4 and δ 183.4 in the ^{13}C NMR spectrum and the absence of any absorption around 3500-2500 cm^{-1} in the IR spectrum confirmed the structure of quinone **104**.



103 **104**

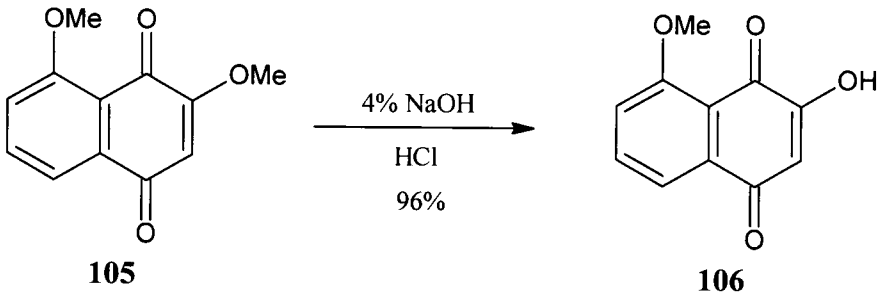
Scheme 4.3

Pyrolysis of naphthoquinone **104**, as reported by Ameer *et al.*, [82] afforded quinone **105 (Scheme 4.4)**, in which the disappearance of the 4-proton multiplet in the ^{1}H NMR spectrum supported the assigned structure of quinone **105**. The two methoxy groups at positions C-2 and C-8 resonated at δ 3.88 and δ 4.01 respectively, whilst the singlet at δ 6.10 was assigned to H-3. The HRMS (M^+ + 1) of 219.0654 confirmed a molecular formula of $C_{12}H_{11}O_4$ (required 219.0657).

104 **105**

Scheme 4.4

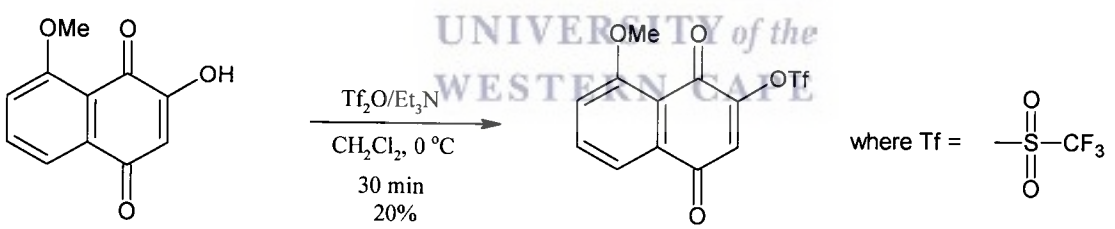
The naphthoquinone **105** was subsequently chemoselectively demethylated at C-2, under basic conditions, [82] to produce hydroquinone **106 (Scheme 4.5)** in an overall yield of 91% for the last two steps. The disappearance of the 3-proton singlet at δ 3.88, which was assigned to the C-2 methoxy group, and the addition of a 1-proton signal in the aromatic region in the ^{1}H NMR spectrum supported the assigned structure to quinone **106**. The downfield shift of the H-3 proton, from δ 6.10 for quinone **105** to δ 6.29 for quinone **106** in the ^{1}H NMR spectrum, substantiated the transformation of the C-2 methoxy group to the hydroxyl group. The HRMS (M^+ + 1) of 205.0504 corresponds to a molecular formula of $C_{11}H_9O_4$ (required 205.0501).



105 **106**

Scheme 4.5

The desired naphthoquinone triflate **92** was obtained by treating quinone **106** with triflic anhydride in the presence of triethyl amine at 0 °C in dichloromethane according to the method of Stagliano *et al.*, [76] **(Scheme 4.6)**. A significant downfield shift of H-3, from δ 6.29 to δ 6.84 in the respective ^{1}H NMR spectra of compounds **106** and **107**, suggested the substitution of the –OH group by the triflate substituent, which as expected, would result in the deshielding of H-3. A quartet at δ 117.8 with *J* 318.5, in the ^{13}C NMR spectrum, due to the coupling of the carbon of the triflate group containing the magnetic nuclei of the fluorine atoms, also supports the structure assigned to quinone **107**.

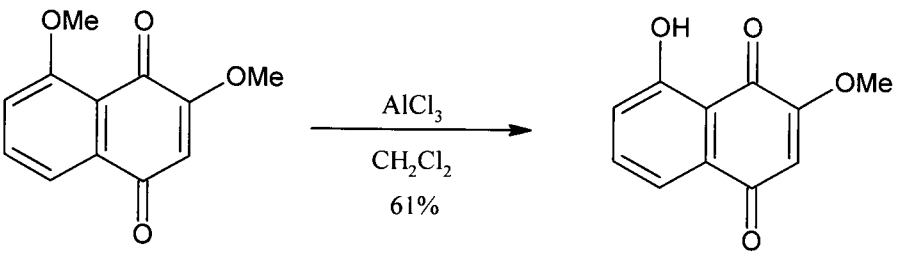


106 **107**

Scheme 4.6

We also prepared the regioisomeric 2-methoxyjuglone **108** by chemoselectively demethylating quinone **105** at position C-8, adopting the method used by Syper *et al.* [84] Thus, a solution of methoxyquinone **105** in dry CH_2Cl_2 at 25 °C was treated with aluminium trichloride **(Scheme 4.7)**. The mixture was stirred at room temperature for 24h, poured into water, and then acidified with dilute HCl to afford the hydroxyquinone **108** in 61% yield.

The structure assigned to naphthol **108** was based on the following spectral evidence: replacement of the 3-proton singlet, present at δ 4.01 in the 1H-nmr spectrum of quinone **105**, with a 1-proton singlet at δ 11.75 in the 1H-nmr spectrum of quinol **108** supported the fact that the C-5 methoxy group had been transformed into an hydroxyl group. The HRMS of 204.0418 corresponds to a molecular formula of $C_{11}H_8O_4$ (required 204.0422).

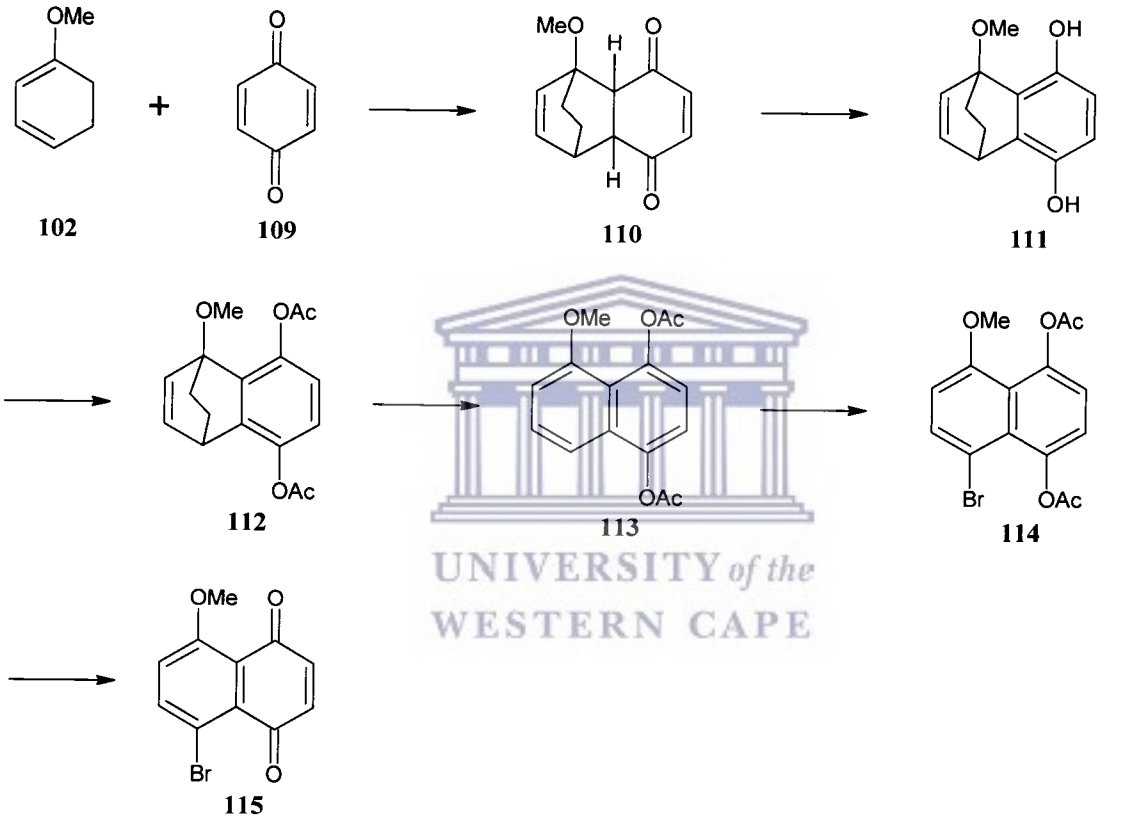


105 **108**

Scheme 4.7

4.1.2. Synthesis of 8-Bromo-5-methoxy-1,4-naphthoquinone

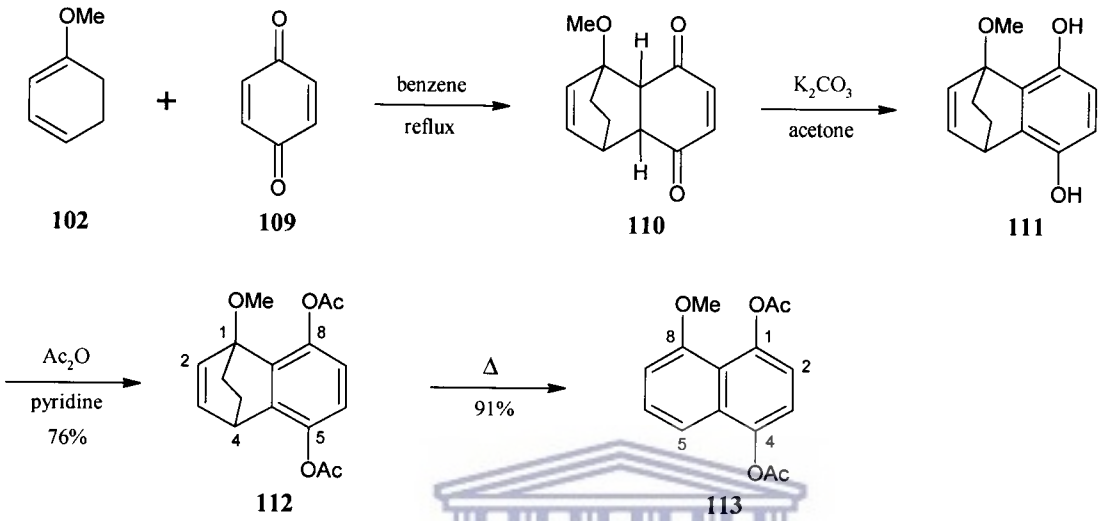
The next compound that we decided to synthesize was the bromonaphthoquinone **115**, in which the bromine atom is attached to the benzenoid half of the molecule. This would eventually enable us to synthesize binaphthyl systems with the linkage being either benzenoid-quinonoid or benzenoid-benzenoid. The proposed synthetic protocol of the target naphthoquinone **115** is illustrated in **Scheme 4.8**.



Scheme 4.8

Utilizing the methodology of Hugo *et al.* [85], naphthalene **113** was prepared in an overall yield of 69% **(Scheme 4.9)**. A mixture of 1-methoxy-1,3-diene **102** and benzoquinone **109** was heated under reflux in benzene under nitrogen to afford compound **110**. The immediate aromatisation using potassium carbonate in acetone, under reflux, and the subsequent acetylation of naphthol **111** produced the diacetoxynaphthalene **112** in 76% yield. In the ^{1}H NMR spectrum, the two 3-proton singlets at δ 2.30 and δ 2.35 are ascribed to the two acetoxy groups, and the 4-proton multiplet at δ 1.54-1.73 is ascribed to the methylene bridge, which confirmed the

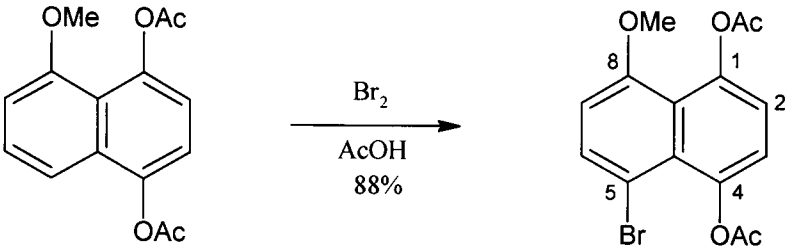
structure of compound **112**. The 3-proton singlet at δ 3.60 was assigned to the methoxy group whilst the 1-proton multiplet at δ 3.90 was designated to H-4. The HRMS (M^+ + 1) of 303.1220 corresponds to a molecular formula of $C_{11}H_9O_4$ (required 303.1232).



Scheme 4.9

Pyrolysis of compound **112**, in an inert atmosphere at 210 °C for 40 min, afforded naphthalene **113** **(Scheme 4.9)** in 91% yield. The disappearance of the 4-proton multiplet, present in the ^1H NMR spectrum of compound **112**, and the downfield shift of H-5 from δ 3.90 to the aromatic region at δ 7.45 supported the structure assigned to naphthalene **113**. The HRMS (M^+ + 1) of 275.0914 confirmed a molecular formula of $C_{15}H_{15}O_5$ (required 275.0919).

The reason for conversion of **110** into specifically the diacetate **113** was to promote bromination at C-5 in the more nucleophilic of the two aryl rings since the acetate groups would be expected to deactivate the ring of attachment to favour bromination at the α-position in the alternative ring. To this end bromination of compound **113** produced the desired bromonaphthalene **114** in acceptable yield, and was effected by treating a solution of **113** in acetic acid with 1 molar equivalent of Br_2 at room temperature **(Scheme 4.10)**.

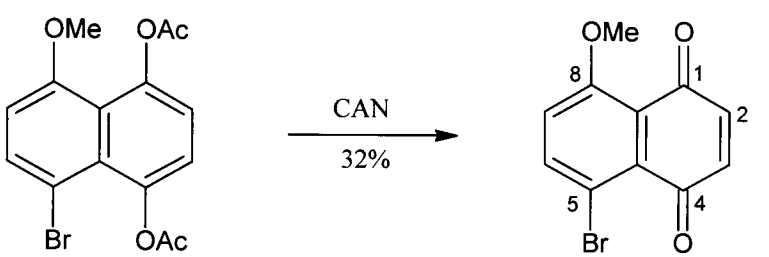


113 **114**

Scheme 4.10

Assignment of structure **114** to the product of bromination is based on the following spectral evidence. In the ^{1}H NMR spectrum the two 1-proton doublets, both with *J* 8.8, at δ 6.69 and δ 7.72 were assigned to H-7 and H-6, respectively. Due to the electron-withdrawing ability of bromine, H-6 is more deshielded than H-7 causing it to resonate downfield relative to H-7, which additionally is adjacent to an electron-donating methoxy group. H-2 and H-3 appeared as two 1-proton doublets, with *J* 8.0 at δ 7.09 and δ 7.19, respectively and the 3-proton singlet at δ 3.90 was assigned to the methoxy group. The 6 protons of the two acetoxy groups resonated at δ 2.36 and δ 2.43. The HRMS (M^+ + 1) of 353.0040 corresponds to a molecular formula of $C_{15}H_{14}O_5Br$ (required 353.0025).

The next step involved the oxidative deacetylation of naphthalene **114** depicted in **Scheme 4.11**. Thus, a suspension of diacetoxynaphthalene **114** in a mixture of acetonitrile and water at 0 °C was treated with cerium(IV) ammonium nitrate in water and stirred for 30 min at 24 °C to afford the naphthoquinone **115**.



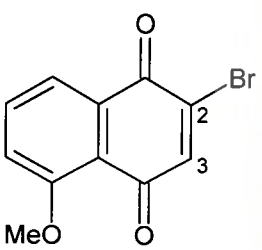
114 **115**

Scheme 4.11

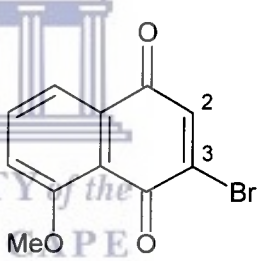
In the ^{1}H NMR spectrum, disappearance of the two upfield 3-proton singlets due to the acetoxy groups, supported the structure assigned to quinone **115**. The upfield shift

of H-3 and H-2 from δ 7.09 and δ 7.19 to δ 6.89 and δ 6.82, respectively in the ^1H nmr spectrum of naphthoquinone **115** also confirmed the absence of the two electron-withdrawing acetoxy groups. The HRMS (M^+ - 1) of 265.9576 corresponds to the formula of $C_{11}H_7BrO_3$ (requires: 265.9578).

Our next objective was to synthesize the two isomeric bromonaphthoquinones **120** [86, 87] and **125** [88]. The rationale behind this thinking was to have molecules with bromine on the quinonoidal half, which would then enable the synthesis of biaryl systems in which the linkage could be either quinonoid-quinonoid or quinonoid-benzenoid. These molecules would also provide an indication as to what effect, all be it steric or electronic, the second naphthalene ring has on the biological activity of the biaryl system when it is attached to C-2, the side of the molecule opposite to the methoxy substituent, and vice versa, i.e. when it is attached to C-3, the same side of the molecule as the methoxy substituent.



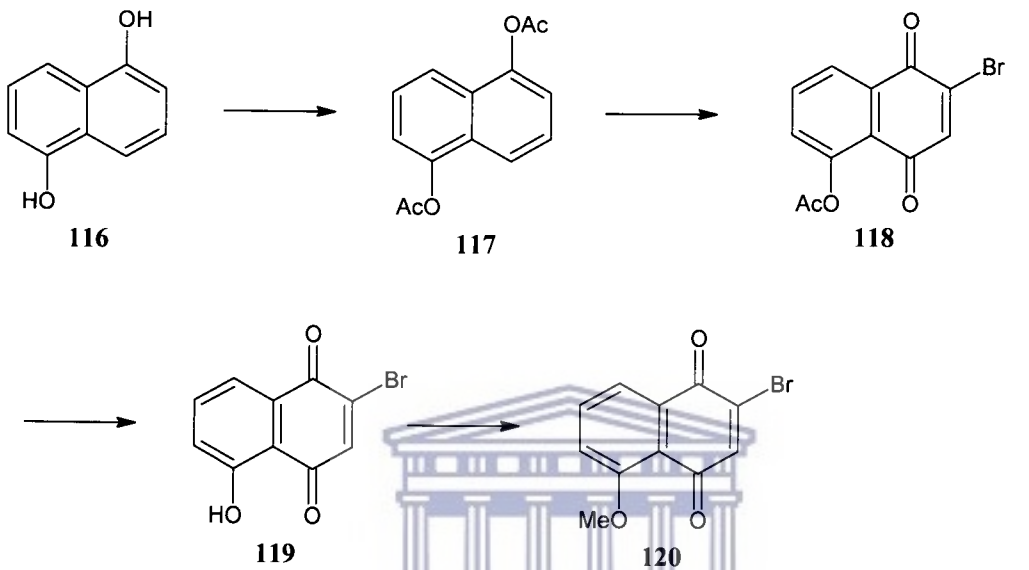
120



125

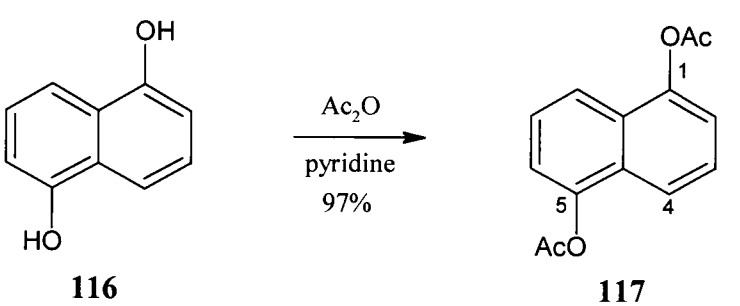
4.1.3. Synthesis of 2-Bromo-5-methoxy-1,4-naphthoquinone

Using a combination of the methodologies of Jung *et al.*, [86] and Nguyen Van *et al.*, [87] the naphthoquinone **120** was synthesized in an overall yield of 78% as outlined in **Scheme 4.12** below.



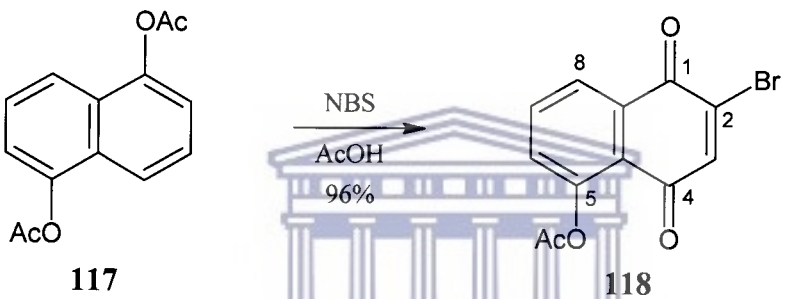
Scheme 4.12

The first step in the synthetic protocol was the acetylation of the commercially available naphthalenediol **116** (**Scheme 4.13**), using acetic anhydride in pyridine to produce the diacetoxynaphthalene **117** with m.p. 158-161 °C (Lit. m.p. 161 °C). [86] In the ^{1}H NMR spectrum the hydrogens on the acetyl groups gave rise to a 6-proton singlet at δ 2.47, due to the symmetry of the molecule.



Scheme 4.13

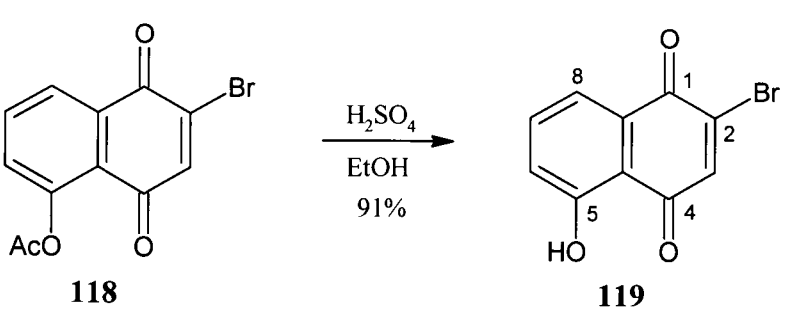
The 1,5-diacetoxynaphthalene **117** was then oxidatively brominated with 4 molar equivalents *N*-bromosuccinimide in aqueous acetic acid to produce 5-acetoxy-2-bromo-1,4-naphthoquinone **118** in 96% yield **(Scheme 4.14)**. [86] The assigned structure for quinone **118** was based on the following spectral evidence. In the ^{1}H NMR spectrum, a 3-proton singlet at δ 2.44 was assigned to the hydrogens of the acetoxy group, and the 1-proton singlet at δ 7.39 to H-3. The doublet of doublets, at δ 7.42 with *J* 8.2 and 1.2 were assigned to H-6 and the other dd at δ 8.14, with *J* 7.8 and 1.2, to H-8. The triplet at δ 7.77 with *J* 8.2 was assigned to H-7. In the ^{13}C NMR the signals at δ 169.2, δ 177.5, and δ 180.9 were assigned to [C=O (ester)], C-1 and C-4.



117 **118**

Scheme 4.14

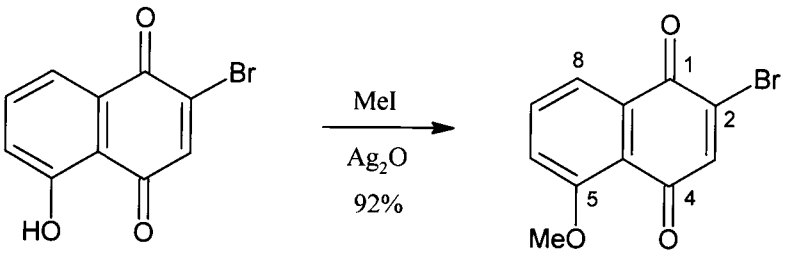
The deacetylation of quinone **118 (Scheme 4.15)** then proceeded by refluxing it in an acidic ethanol solution to afford 2-bromojuglone **119** with m.p. 133-135 °C (Lit. m.p. 135-136 °C). [86] This was confirmed by the replacement of the 3-proton peak at δ 2.44 with a 1-proton singlet at δ 11.78 and the broad absorption peak at 3400-2700 cm^{-1} in the IR spectrum confirmed the presence of an OH group. The HRMS (M^{+} - 1) of 250.9335 corresponds to a molecular formula of $C_{10}H_4O_3Br$ (required 250.9344).



118 **119**

Scheme 4.15

This bromoquinone **119** was then methylated by treating it with methyl iodide in the presence of silver(I) oxide to afford the target methyl ether **120** in 92% yield **(Scheme 4.16)**. [87] The appearance of a 3-proton singlet, due to the methoxy group, at δ 4.02 supported the structure assigned to quinone **120**. The HRMS (M^+ - 1) of 265.9451 confirmed a molecular formula of $C_{11}H_6O_3Br$ (required 265.9578).

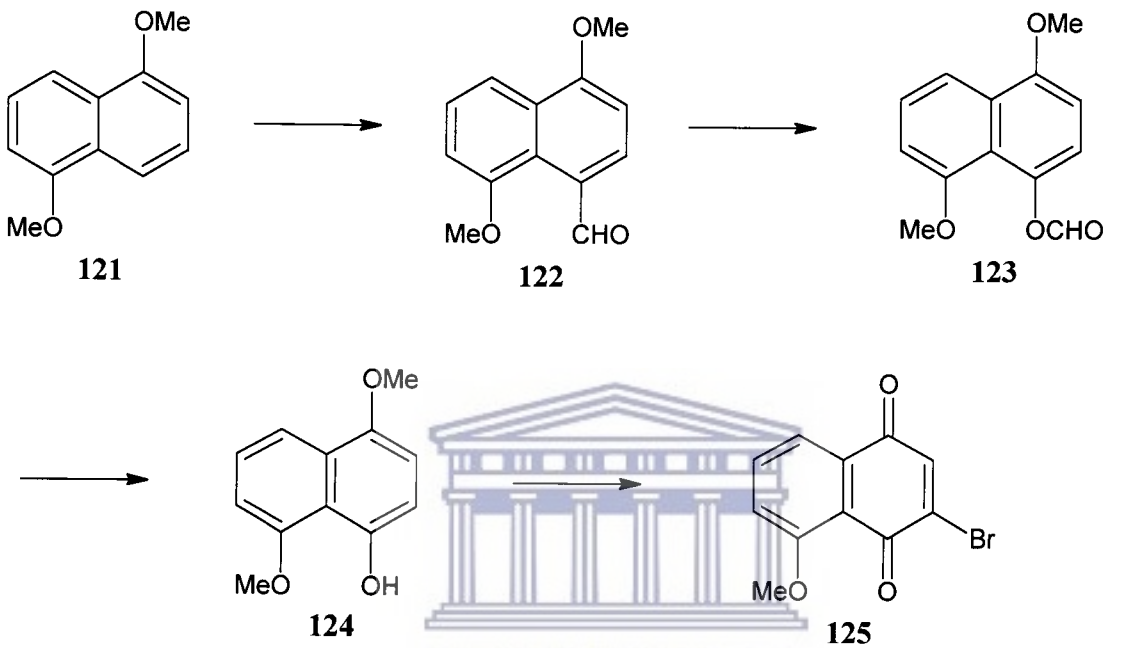


119 **120**

Scheme 4.16

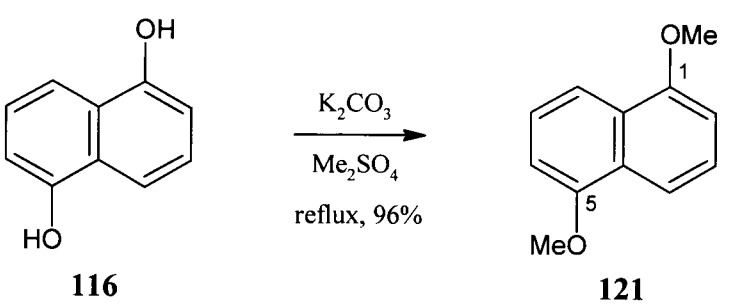
4.1.4 Synthesis of 3-Bromo-5-methoxy-1,4-naphthoquinone

Next we synthesized the isomeric 3-bromonaphthoquinone **125**, with the bromine substituent in a more "peri" position to the methoxy group, according to the method reported by Hannan *et al.*, [88] as shown in **Scheme 4.17**.



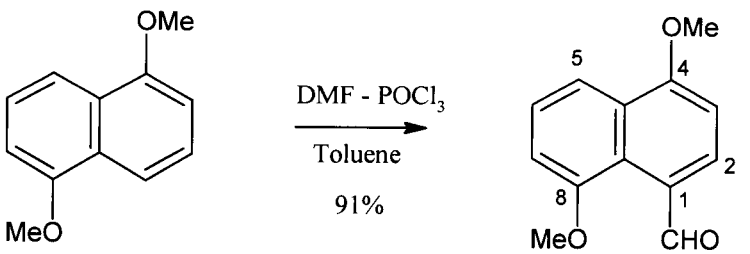
Scheme 4.17

The first step however, was to synthesize 1,5-dimethoxynaphthalene **121** from naphthalenediol **116**. Thus, treatment of a refluxing mixture of **116** and K_2CO_3 in acetone, with dimethylsulphate afforded the dimethoxynaphthalene **121** in 96% yield **(Scheme 4.18)**. The singlet at δ 4.00, integrating for 6 protons, in the ^1H NMR spectrum confirmed the methylation of the diol **116**.



Scheme 4.18

The synthesis of naphthaldehyde **122**, using DMF and $POCl_3$ in toluene, as reported by Hannan and co-workers, [88] afforded **122** in 91% yield **(Scheme 4.19)**. The structure of compound **122** was based on the following spectral evidence. In the ^1H NMR spectrum the two methoxy groups appeared as two singlets at δ 4.01 and δ 4.05, whilst the two doublets, with *J* 7.8 at δ 7.04 and δ 7.96 were assigned to H-3 and H-2, respectively.

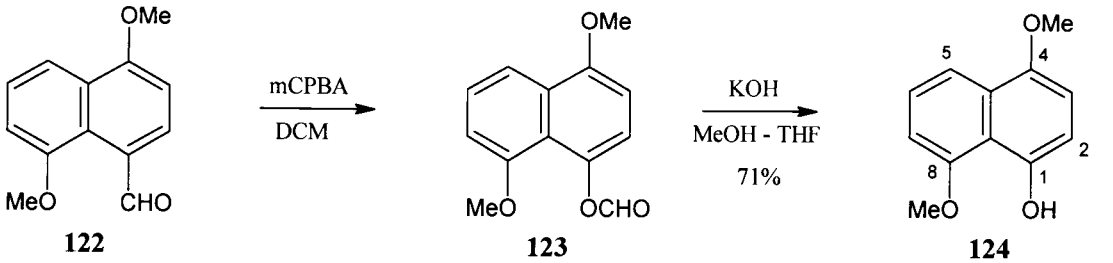


121 **122**

Scheme 4.19

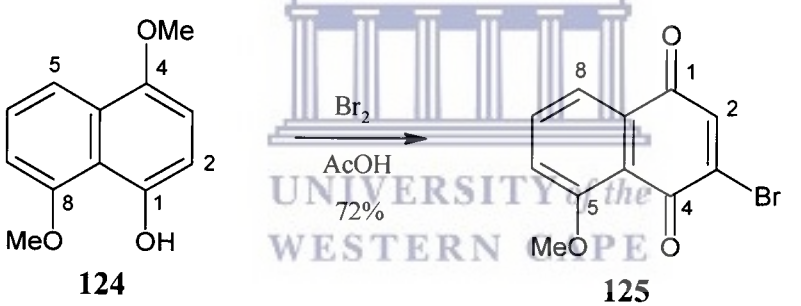
This is ascribed to the difference in their respective neighbouring groups' ability to donate and withdraw electrons. With the –OMe substituent being an electron-donating group and the –CHO substituent an electron-withdrawing group, H-3 resonates upfield from H-2, because it is being more shielded. The other three aromatic protons, i.e. H-5, H-6, and H-7 appeared as a doublet, a triplet, and a doublet, with a coupling constant of *J* 8.4, at δ 8.07, δ 7.45, and δ 6.90, respectively. The 1-proton singlet at δ 11.05 was due to the hydrogen of the aldehyde group. In the ^{13}C NMR spectrum, the peak at δ 194.6 that is typical of a C=O group supported the presence of the aldehyde group. The HRMS (M^+ + 1) of 217.0854 corresponds to a molecular formula of $C_{13}H_{13}O_3$ (required 217.0865).

The Baeyer-Villager oxidation of naphthaldehyde **122** and the subsequent hydrolysis, of the intermediate formyl ester **123** produced naphthol **124** in moderate yield as depicted in **Scheme 4.20**. [88] The 2-proton singlet at δ 6.78, in the ^1H NMR spectrum was assigned to H-2 and H-3, whilst the proton of the hydroxyl group resonated at δ 8.95. The absence of the C=O peak in the ^{13}C NMR spectrum and the presence of a broad absorption peak at 3400-2800 cm^{-1}, due to the O-H stretching frequency, in the IR spectrum confirmed the structure assigned to naphthol **124**. The HRMS (M^+) of 204.0785 confirmed a molecular formula of $C_{13}H_{13}O_3$ (required 204.0786).



Scheme 4.20

The next step in the Hannan [88] reaction sequence involved bromination of naphthol **24** at position 2, using Br_2 in CCl_4 and the subsequent oxidation of the bromonaphthol with CAN to give the naphthoquinone **125**. However, in our hands we found that treating naphthol **124** with bromine in acetic acid afforded the desired quinone **125** with m.p. 154-156 °C (Lit. m.p. 154-155 °C) [88] in a single step and in good yield **(Scheme 4.21)**.

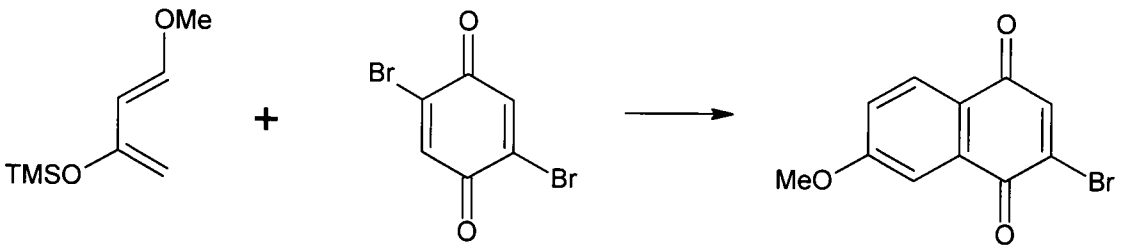


Scheme 4.21

The structure assigned to naphthoquinone **125** was based on the following spectral evidence. In the ^1H NMR and IR spectrum there was no sign of the hydroxyl group and both the ^1H and ^{13}C NMR spectrum showed the presence of only one methoxy group at δ 4.02 and δ 56.5, respectively. The two downfield signals, in the ^{13}C NMR spectrum, at δ 176.1 and δ 182.5 were assigned to the two carbonyl moieties present in the molecule. The HRMS (M^+ - 1) of 265.9575 confirmed a molecular formula of $C_{11}H_6O_3Br$ (required 265.9578).

4.1.5 Synthesis of 2-bromo-7-methoxy-1,4-naphthoquinone

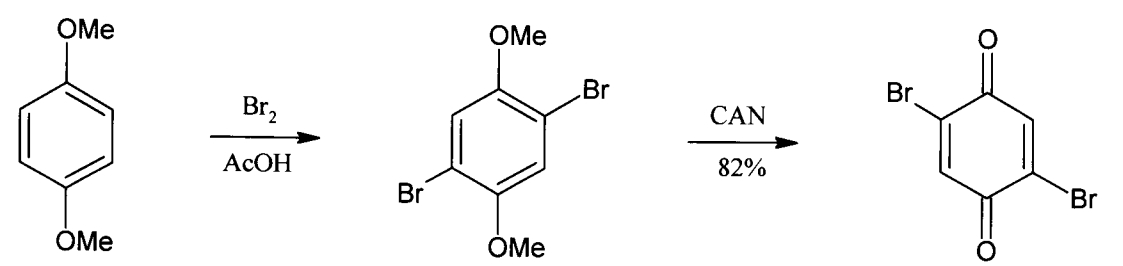
The proposed synthetic protocol of this compound involves Diels-Alder addition of the commercially available 1-methoxy-3-silyloxybutadiene **126** to benzoquinone **127** as illustrated in **Scheme 4.22** below.



126 **127** **128**

Scheme 4.22

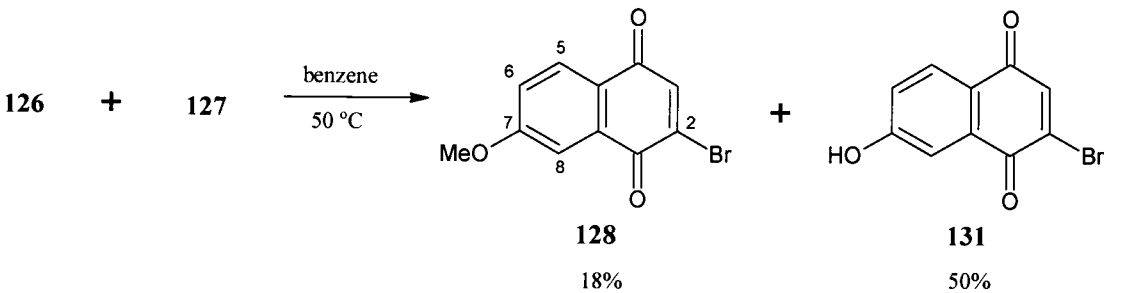
The first step was to synthesize benzoquinone **127** by using the method reported by Alvarado *et al.*, [89] as depicted in **Scheme 4.23** below. A solution of 1,4-dimethoxy-benzene **129** in acetic acid was treated with 2 mole equivalents of bromine in acetic acid to afford the corresponding 2,5-dibromo-1,4-dimethozybenzene **130**. Oxidation of compound **130** with cerium ammonium nitrate afforded the corresponding dibromobenzoquinone **127** in 82% yield. The material was assigned the structure **127** based on a singlet at δ 7.48, which is due to the two symmetrical hydrogens in the ^{1}H NMR spectrum and a m.p. of 159-160 °C (Lit. [89] m.p. 160-161 °C).



129 **130** **127**

Scheme 4.23

Passage of the crude Diels-Alder product through a silica gel column afforded two products, the first of which was the desired trans methylated product **128** in 18% yield **(Scheme 4.24)**.



128 18%

131 50%

Scheme 4.24

A 3-proton signal at δ 3.97 in the ^{1}H NMR spectrum together with the expected methoxy carbon at δ 56.2 in the ^{13}C spectrum was significant supporting evidence. The doublet of a doublet at δ 7.23, with *J* 8.6 and 2.6, was assigned to H-7, whilst the two doublets at δ 7.60 (*J* 2.6) and δ 8.12 (*J* 8.6) were assigned to H-5 and H-8, respectively. The HRMS (M^{+}) of 265.9571 corresponds to a molecular formula of $C_{11}H_7O_3Br$ (required 265.9579). The second product to elute was the corresponding 2-bromo-7-hydroxynaphthalene **131** in 50% yield and this in turn was converted in quantitative yield into the desired methoxy analogue by treatment with methyl iodide and Ag(II) oxide in benzene. In subsequent syntheses the initial crude product mixture was subjected to methylation as described above to afford the naphthoquinone **128** in 80% isolated yield.

4.2 Conclusion

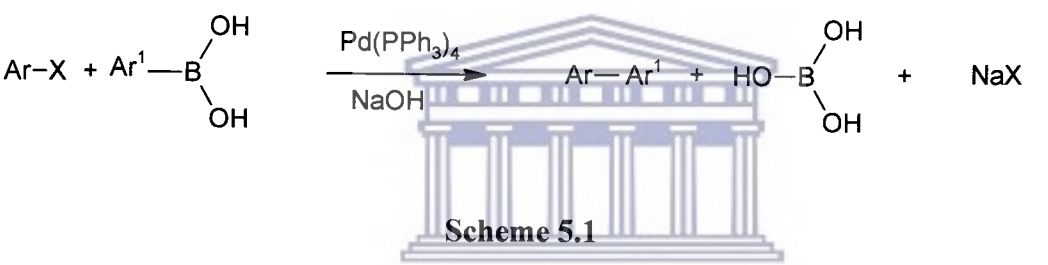
The synthesis of five methoxyquinones was successfully achieved. However, 8-bromo-5-methoxy-1,4-naphthoquinone **115** was afforded in poor yield and the coupling to boronic acids was not further pursued. Hence, only the three remaining bromonaphthoquinones were subsequently used in the coupling reactions. Dimethoxynaphthoquinone **105** was also demethylated at position C-8 to determine whether this would have any influences on its biological activity.

CHAPTER 5

COUPLING REACTIONS

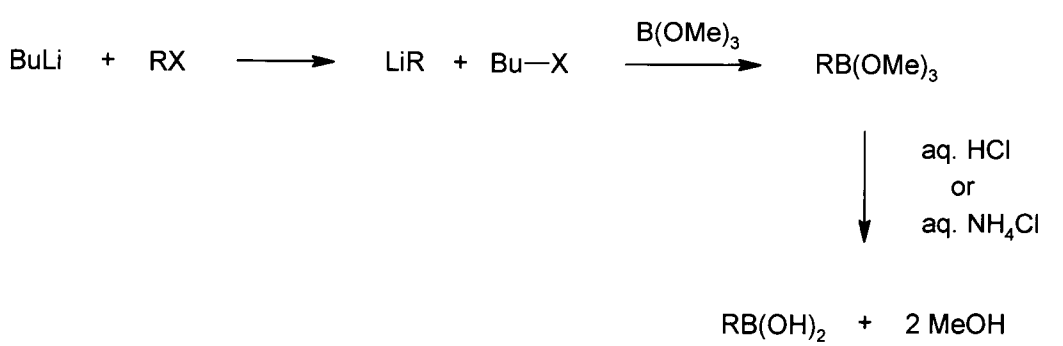
5.1 Suzuki Coupling Mechanism

The Suzuki coupling protocol, which was discovered by Akira Suzuki [90] in 1979, has established itself as one of the most powerful methods for the formation of C-C bonds, especially those involving sp^2-hybridized centres. Consequently, it has been widely applied in the formation of biaryl compounds where it usually involves the Pd(0)-mediated linking of an aryl halide with that of an arylboronic acid as depicted in **Scheme 5.1**..



Scheme 5.1

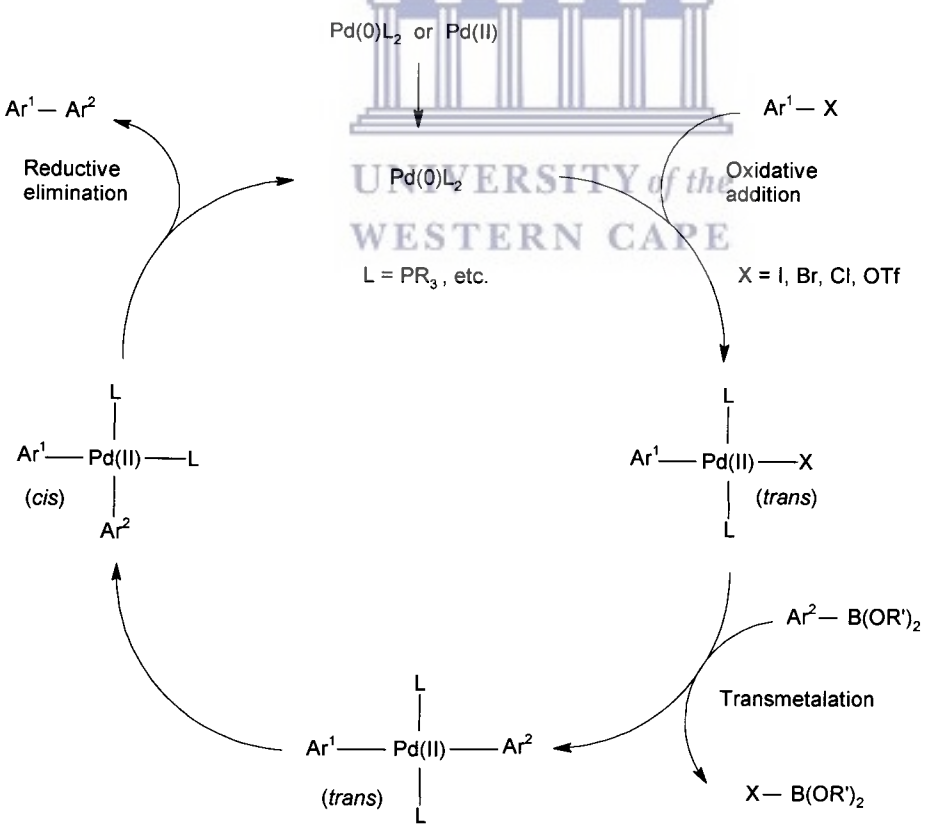
The organohalide is normally either an aryl or alkenyl, whereas the organoborane can be anything, as long as it is relatively stable. Organoboranes are prepared by treating organohalides with butyl lithium to form the corresponding lithiated adducts, which are then reacted with trimethylborate $B(OMe)_3$ to form the boronic acid esters. These are then hydrolysed with either aqueous HCl or aqueous NH_4Cl to form the corresponding boronic acids as shown in **Scheme 5.2** below.



Scheme 5.2

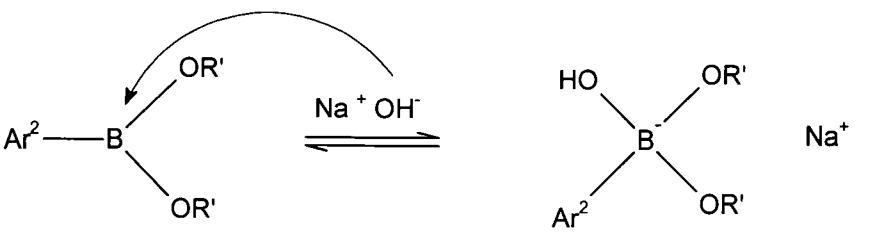
Arylboronic acids are usually quite stable and easy to handle. They are normally stable in air and to a limited amount of heat, and some can even be recrystallised from water and alcohol. Various arylboronic acids can be prepared relatively easily by functionalization reactions of the parent aryl boronic acid since the boronic acid group is inert to many chemical reactions.

The mechanism by which the Suzuki coupling protocol proceeds is known to be complex in its details, but essentially involves the three steps shown in **Scheme 5.3**, viz., oxidative addition, transmetalation and reductive elimination. [91] The first step involves oxidative addition of the electrophilic alkyl or aryl halide to the palladium(0) atom of a two-coordinate complex and is widely assumed to be the turnover-limiting step. [92] The resulting complex then undergoes a nucleophilic substitution reaction in which the organic group attached to boron is transferred to the Pd(II) ion, replacing the halide ion.



Scheme 5.3

This step proceeds only in the presence of bases, and is a requirement of the Suzuki cross-coupling reaction. [90, 93] This is ascribed to the fact that the organic group on boron is not nucleophilic enough for the transfer from boron to the palladium in the transmetalation step because of the strong covalent character of the B-C bond in boron compounds. Therefore, it is necessary to increase the carbanion character of organic groups by the formation of an organoborate having a tetravalent boron atom upon treatment with base **(Scheme 5.4)**.



Scheme 5.4

The diaryl palladium(II) complex then immediately undergoes reductive elimination, yielding the coupled organic product and regenerating the two-coordinated palladium(0) complex as illustrated in **Scheme 5.3** above.

Although the catalyst most often used in the Suzuki cross-coupling reaction is $Pd(PPh_3)_4$, various other palladium catalysts have been utilized, viz., $Pd(dppb)Cl_2$, $Pd(PPh_3)_2Cl_2$, $Pd(OAc)_2$ and $PdCl_2$. Bases are always required and the best results are achieved by use of the relatively weak bases of which Na_2CO_3 is the most frequent. However, other bases, such as Et_3N, $NaHCO_3$ and K_3PO_4, have also successfully been employed. Weak bases are more favourable in the case of sterically unhindered boronic acids since hydrolytic deboronation is suppressed.

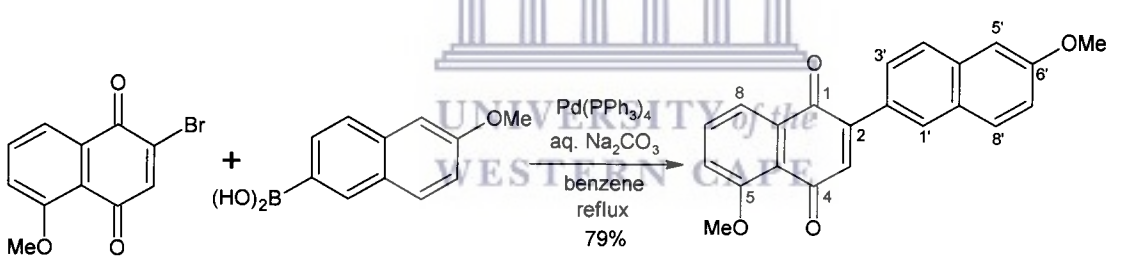
Arylboronic acids that are sterically crowded generally give low yields and hydrolytic deboronation of the C-B bond predominates while, in some cases, it has been reported that both electron-withdrawing and electron-attracting groups accelerate the protonolysis. [94] One of the alternatives is to replace the boronic acids by the corresponding esters, which allows the use of anhydrous conditions in which no protonolysis occurs. [95] Another option is to use strong bases, such as aqueous $Ba(OH)_2$ or NaOH that accelerate the coupling reaction.

5.2 Synthesis of naphthoquinone-naphthalene biaryl systems

With the bromonaphthoquinone precursors in hand, our next objective was to couple them to a variety of commercially available boronic acids using the Suzuki coupling [95] methodology. The subsequent biological evaluations against *Mycobacterium tuberculosis* and various cancer cell lines would then enable us to deduce a structure vs. activity relationship from the results obtained.

5.2.1 Coupling to 6-methoxynaphthalene-2-boronic acid

The first binaphthyl system synthesized was the coupled product between naphthoquinone **120** and 6-methoxynaphthalene-2-boronic acid **132** using the Suzuki coupling [95] methodology **(Scheme 5.5)**. Thus, a mixture of naphthoquinone **120** and $Pd(PPh_3)_4$ in benzene was treated with an aqueous solution of sodium carbonate and boronic acid **132** in benzene at 24 °C, and then heated under reflux for 16 h to afford quinone **133** in 79% isolated yield.

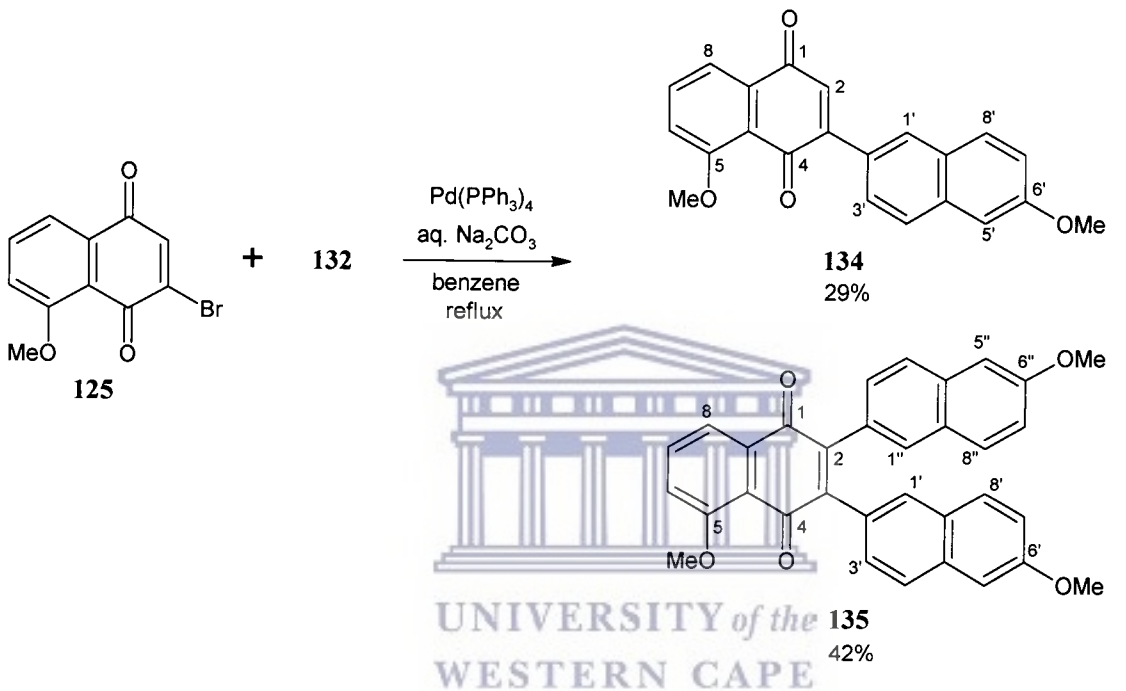


120 **132** **133**

Scheme 5.5

The resulting material was assigned the structure **133** based on the following spectral evidence: the two 3-proton singlets at δ 3.94 and δ 4.04, in the ^{1}H NMR spectrum, were assigned to the methoxy groups at C-6' and C-5 respectively. The upfield shift of H-3 from δ 7.41, in the ^{1}H NMR spectrum of bromoquinone **120**, to δ 7.10 for binaphthylquinone **133** also confirms the replacement of the electron-withdrawing bromine substituent with an electron-donating group, such as the methoxynaphthalene moiety. In the ^{13}C NMR spectrum, the signals at δ 158.8, 159.3, 184.6, and 184.9 were assigned to C-6', C-5, C-1 and C-4. The HRMS (M^+ +1) of 345.1125 supported the molecular formula of $C_{22}H_{17}O_4$ (required 345.1127).

We then proceeded with the Suzuki cross-coupling [95] of the isomeric quinone **125** to boronic acid **132**, which resulted in the formation of two products in this case. The product mixture was separated by column chromatography and the first fraction isolated from the column was the expected product isomeric binaphthylquinone **134**, but in a low yield of 29%. The second band to elute was identified as the trinaphthylquinone system **135** isolated in 42% yield **(Scheme 5.6)**.

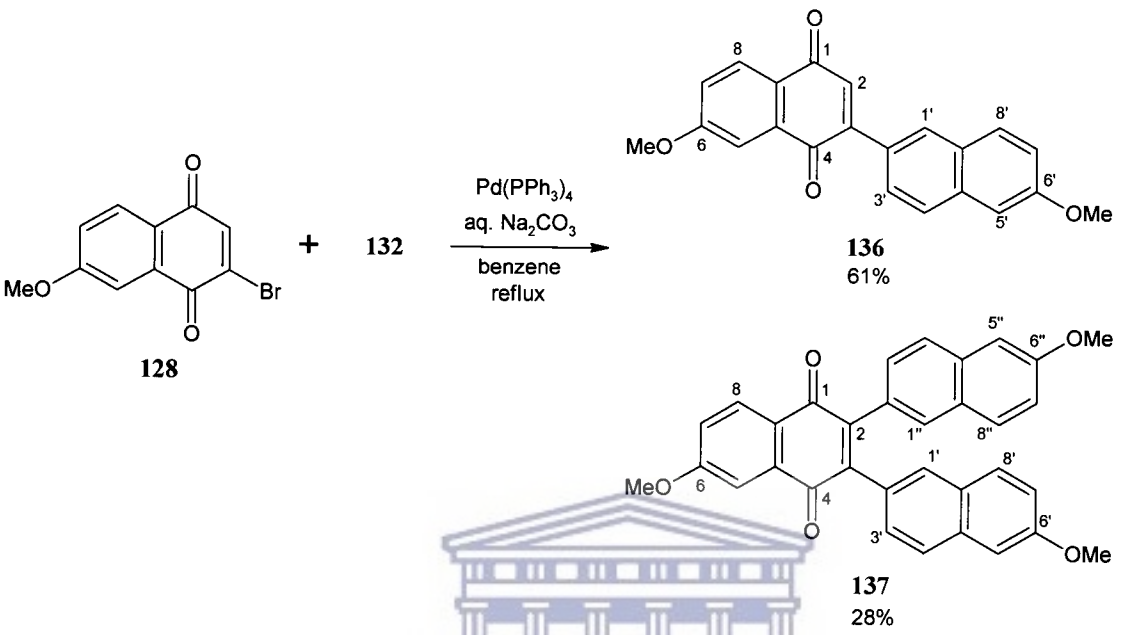


Scheme 5.6

In the ^{1}H NMR spectrum of compound **134** the two 3-proton singlets at δ 3.95 and 4.05 were assigned to 6'-OCH_3 and 5-OCH_3, respectively whilst the 1-proton singlet at δ 7.11 was assigned to H-2. The HRMS (M^+ +1) of 345.1122 supported the molecular formula of $C_{22}H_{17}O_4$ (required 345.1127).

Assignment of structure **135** to the second fraction is based on the following ^{1}H NMR evidence. The 1-proton singlet due to H-2, present in the spectrum of binaphthyl **134**, was absent and a 6-proton singlet, assigned to the two equivalent methoxy groups of C-6' and C-6'', resonated at δ 3.86 in addition to the 3-proton singlet of 6-OCH_3 at δ 4.03. The HRMS (M^+ + 1) of 501.1681 supported a molecular formula of $C_{33}H_{25}O_5$ (requires: 501.1702).

The coupling of the third isomeric naphthoquinone **128** to boronic acid **132**, using the Suzuki [95] methodology also gave rise to two products which were similarly separated by column chromatography and identified as the binaphthylquinone **136** (61%) and trinaphthylquinone **137** (28%) **(Scheme 5.7)**.



Scheme 5.7

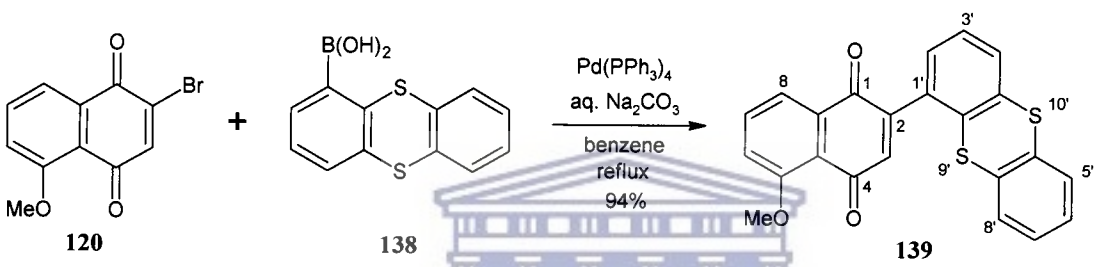
In the ^{1}H NMR spectrum of binaphthylquinone **136** the 1-proton singlet at δ 7.12 was assigned to H-2, whilst the methoxy groups at C-6' and C-6 resonated as 3-proton singlets at δ 3.95 and δ 3.98, respectively. The molecular formula of $C_{22}H_{17}O_4$ for binaphthylquinone **136** was supported by its high resolution mass spectrum, which had a (M^+ +1) peak at *m/z* 345.1121 (required 345.1127).

As in the case of trinaphthyl **135**, the absence of the 1-proton singlet in the ^{1}H NMR spectrum of tri-naphthyl **137**, due to H-2, and the presence of a 6-proton singlet at δ 3.87, assigned to the two equivalent methoxy groups of C-6' and C-6'', in addition to the 3-proton singlet of 6-OCH_3 at δ 3.97 were significant supporting evidence. In the ^{13}C NMR spectrum the signals at δ 158.4, 164.3, 184.2 and 185.2 were assigned to C-6' and C-6'', C-6, C-1 and C-4, whilst all the other equivalent carbons of the two naphthalene moieties appeared as single peaks with double the intensity. The HRMS (M^+ + 1) of 501.1685 confirmed a molecular formula of $C_{33}H_{25}O_5$ (required 501.1702).

5.2.2 Coupling to thianthren-1-boronic acid

Our next aim was to couple the three regioisomeric quinones **120**, **125** and **128** to the commercially available thianthren-1-boronic acid **138**, using the Suzuki coupling [95] methodology.

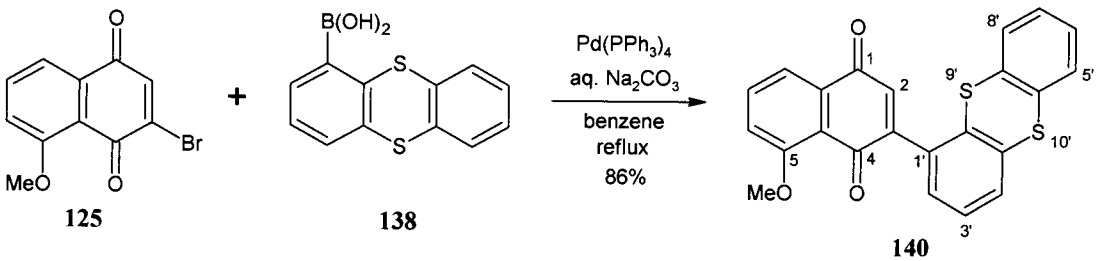
Thus, a mixture of naphthoquinone **120** and $Pd(PPh_3)_4$ in benzene was treated with an aqueous solution of sodium carbonate and thianthren-1-boronic acid **138** in benzene at 24 °C, and then heated under reflux for 16 h to afford quinone **139** in an excellent 94% yield **(Scheme 5.8)**.



Scheme 5.8

The resulting material was assigned the structure **139** based on the following spectral evidence: the methoxy substituent at C-5 appeared as a 3-proton singlet at δ 4.07, whilst the 1-proton singlet resonating at δ 6.87 was assigned to H-3. This significant upfield chemical shift of H-3 from δ 7.41, in the ^{1}H NMR of bromoquinone **120**, to δ 6.87 is ascribed to the anisotropic shielding effect of the adjacent thianthrene moiety. A 6-proton multiplet between δ 7.19-7.41 was assigned to H-6, H-2', H-3', H-4', H-6' and H-7'. The two 1-proton doublet of doublets at δ 7.51 and δ 7.60, with *J* 7.6 and 1.8 Hz, were assigned to H-5' and H-8' respectively. A 1-proton triplet at δ 7.75 with *J* 8.4 Hz was assigned to H-7 and a 1-proton double of a doublet at δ 7.87 with *J* 8.4 and 1.8 Hz to H-8. The HRMS ($M^+ + 1$) of 403.0446 supported a molecular formula of $C_{23}H_{15}O_3S_2$ (requires: 403.0463).

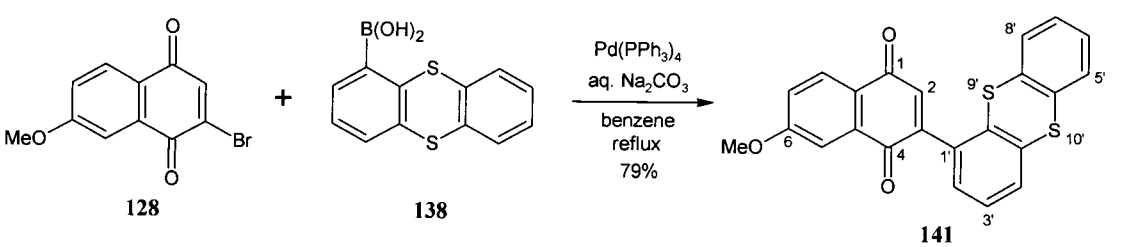
Cross-coupling of quinone **125** to thianthren-1-boronic acid **138** afforded quinone **140** in 86% yield **(Scheme 5.9)**.



Scheme 5.9

The resultant coupled product **140** was again characterized by the upfield chemical shift of the H-2 1-proton singlet from δ 7.45 in the ^{1}H NMR spectrum of bromoquinone **125** to δ 6.88 in the ^{1}H NMR spectrum of binaphthyl **140**. The ^{1}H NMR spectra of compounds **139** and **140** appeared quite similar as was anticipated, but a marked difference between them was observed in the respective melting points of the regioisomers viz., **139** (m.p. 214-217 °C) and **140** (m.p. 195-198 °C). In the ^{13}C NMR spectrum the signals at δ 160.1, 182.5, and 185.1 were assigned to C-5, C-4 and C-1. The HRMS (M^+ + 1) of 403.0470 corresponds to a molecular formula of $C_{23}H_{15}O_3S_2$ (requires: 403.0463).

Suzuki coupling [95] of naphthoquinone **128** to thianthren-1-boronic acid **138** gave the expected quinone **141** in 79% yield **(Scheme 5.10)**.



Scheme 5.10

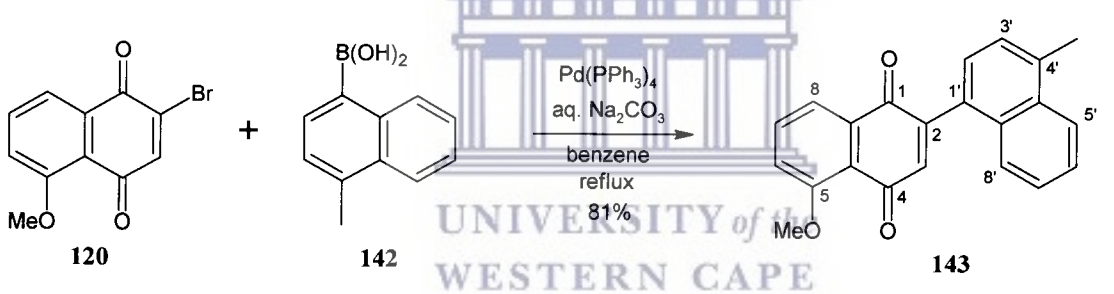
In the ^{1}H NMR spectrum of binaphthyl **141** the 1-proton singlet at δ 6.90 was assigned to H-2, whilst the methoxy group at C-6 resonated as a 3-proton singlet at δ 3.99. The two 1-proton doublet of doublets at δ 7.50 and 7.61 were assigned to H-5' and H-8' respectively whilst H-8 appeared as a 1-proton doublet at δ 8.12 with *J* 8.4 Hz. The molecular formula of $C_{23}H_{15}O_3S_2$ for binaphthylquinone **141** was supported

by its high resolution mass spectrum, which had a (M^+ +1) peak at *m/z* 403.0489 (required 403.0463).

5.2.3 Coupling to 4-methylnaphthalene-1-boronic acid

The two regioisomers **120** and **125** were coupled to the commercially available 4-methylnaphthalene-1-boronic acid **142** utilizing the Suzuki coupling [95] protocol. This was done to evaluate the influence, if any, of the Me group on the activity of the binaphthylquinone products.

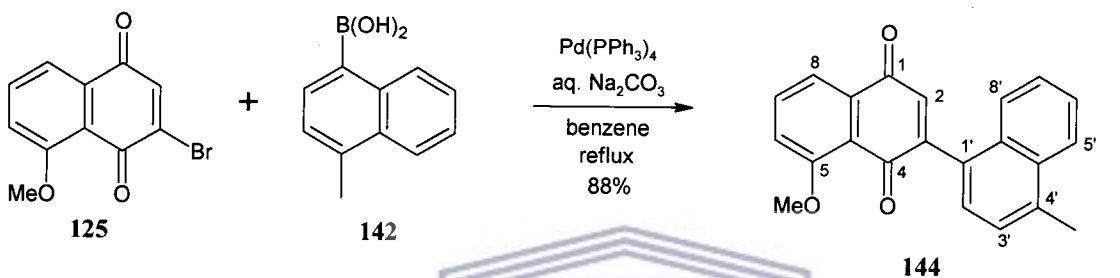
Thus, a mixture of naphthoquinone **120** and $Pd(PPh_3)_4$ in benzene was treated with an aqueous solution of sodium carbonate and 4-methylnaphthalene-1-boronic acid **142** in benzene at 24 °C, and then heating under reflux for 16 h to afford binaphthylquinone **143** in 81% yield **(Scheme 5.11)**.



Scheme 5.11

In the ^1H NMR spectrum of compound **143**, the two 3-proton singlets at δ 2.72 and δ 4.04 were easily assigned to the 4'-CH_3 and 5-OCH_3, whilst the 1-proton singlet resonating at δ 6.99 was assigned to H-3. The 5-proton multiplet at δ 7.30-7.58 was assigned to H-6, H-2', H-3', H-6', and H-7' and the two doublet of doublets at δ 7.65 and δ 8.06, with *J* 7.8 and 1.4 Hz, were assigned to H-5' and H-8', respectively. A 1-proton triplet at δ 7.70 with *J* 8.0 Hz was assigned to H-7 while a doublet of a doublet at δ 7.81 with *J* 8.0 and 1.6 Hz was assigned to H-8. In the ^{13}C NMR spectrum the signals at δ 19.7 and δ 56.6 were assigned to 4'-CH_3 and 5-OCH_3 whilst the two carbonyl carbons, C-1 and C-4, resonated at δ 184.5 and 184.6. The (M^+ + 1) peak at *m/z* 329.1180 in the HRMS supported a molecular formula of $C_{22}H_{17}O_3$ (requires: 329.1178).

The cross-coupling of quinone **125** to 4-methylnaphthalene-1-boronic acid **142** afforded the isomeric binaphthylquinone **144** in 88% yield **(Scheme 5.12)**. Assignment of structure **144** to the coupled product is based on the following spectral evidence: 4'-CH_3 and 5-OCH_3 appeared as 3-proton singlets at δ 2.75 and 3.97 in the ^1H NMR spectrum, whilst the 1-proton singlet at δ 7.02 was assigned to H-2. The doublet of doublets at δ 7.68 and δ 8.05 with *J* 7.8 and 1.4 Hz were assigned to H-5' and H-8'. The HRMS (M^+ + 1) of 329.1187 confirmed a molecular formula of $C_{22}H_{17}O_3$ (required: 329.1178).

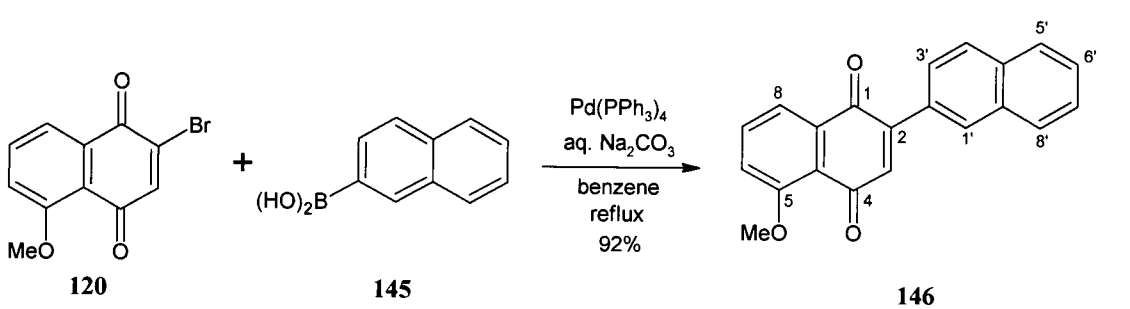


Scheme 5.12

Coupling to naphthalene-2-boronic acid

Coupling of the two regioisomeric naphthoquinones **120** and **125** to the commercially available naphthalene-2-boronic acid **145** using the Suzuki cross-coupling [95] methodology was next efficiently accomplished.

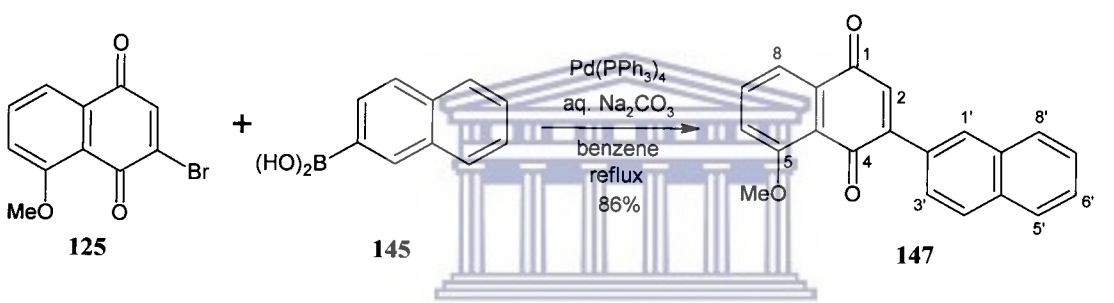
Thus, a mixture of naphthoquinone **120** and $Pd(PPh_3)_4$ in benzene was treated with an aqueous solution of sodium carbonate and naphthalene-2-boronic acid **145** in benzene at 24 °C, and then heated under reflux for 16 h to afford binaphthylquinone **146** in 92% yield **(Scheme 5.13)**.



Scheme 5.13

The 5-OCH_3 group appeared as a 3-proton singlet at δ 4.05 in the ^1H NMR spectrum and a 1-proton singlet at δ 7.13 was assigned to H-3. The doublet of a doublet at δ 7.35, with *J* 8.0 and 1.5 Hz was assigned to H-6 and the triplet assigned to H-7 appeared at δ 7.74 with *J* 8.0 Hz. The 4-proton multiplet at δ 7.86-7.90 was assigned to H-8, H-4', H-5', and H-8', whilst H-1' appeared as sharp double at δ 8.14 with *J* 1.4 Hz. The three downfield signals in the ^{13}C NMR spectrum at δ 159.4, 184.5, and 184.8 were assigned to C-5, C-1 and C-4. The HRMS ($M^+ + 1$) of 315.1006 supported a molecular formula of $C_{21}H_{15}O_3$ (requires: 315.1021).

Suzuki coupling [95] of the isomeric naphthoquinone **125** to naphthalene-2-boronic acid **145** gave the expected isomeric binaphthylquinone **147** in 86% yield **(Scheme 5.14)**.



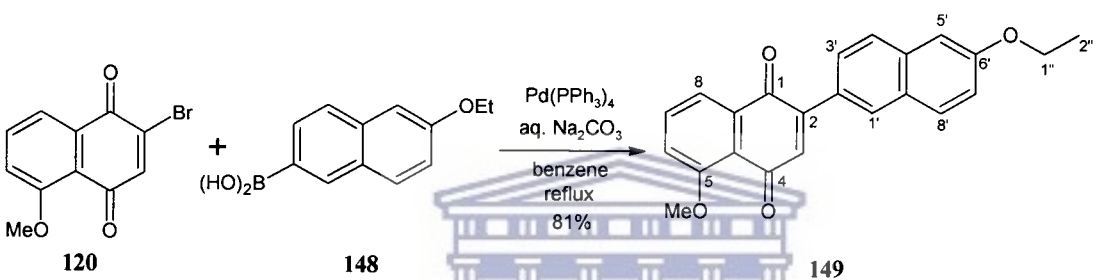
Scheme 5.14

A 3-proton signal at δ 4.03 is assignable to the 5-OMe group but the signals of the aromatic moiety were overlapping to an extent as to make any assignments ambiguous. The high resolution mass spectrum gave no molecular ion peak but showed a fragment ion $C_{20}H_{11}O_3$ at m/z 299.0705 (requires 299.0709) as a result of the loss of the CH_3 group of the 5-methoxy substituent.

At this stage of my research, preliminary evaluations of the prepared binaphthylquinones described above against various cancer cell lines that were performed by me on-site at our Biotechnology Department, it became apparent that the 5-methoxy-binaphthylquinone systems, with the linkage at C-2, exhibited higher anti-tumour activity than the C-3 isomers. We consequently therefore concentrated primarily on synthesizing more biaryl systems that were linked to C-2 of the quinonoid ring, by using naphthoquinone **120** as our naphthoquinone building block.

5.2.5 Coupling to 6-ethoxynaphthalene-2-boronic acid

The Suzuki cross-coupling [95] of naphthoquinone **120** to 6-ethoxynaphthalene-2-boronic acid **148** afforded the binaphthylquinone **149** in 81% yield **(Scheme 5.15)**. The rationale behind the coupling of naphthoquinone **120** to boronic acid **148** was that it had been reported that the diethyl ether derivative **20** of diospyrin **15** was more biologically active than diospyrin **15** against human cancer cell lines. [62] Hence, we wanted to determine whether substitution of the C-6' methoxy group by an ethoxy group would enhance the activity of the corresponding binaphthylquinone.



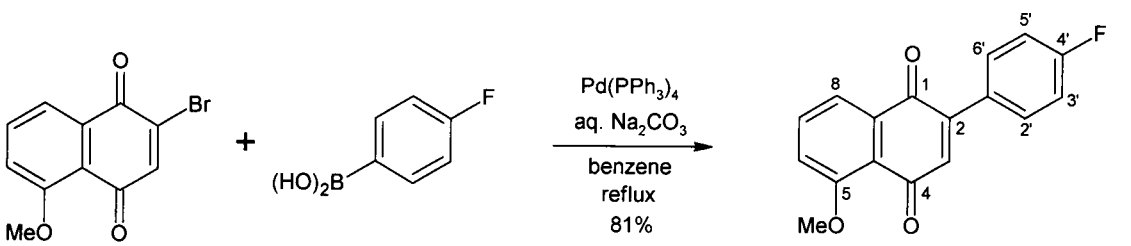
Scheme 5.15

Structural assignment of **149** was confirmed by its ^{1}H NMR spectrum, which had the following characteristics *inter alia*; a 3-proton triplet at δ 1.50 (*J* 7.0 Hz) assigned to 2''-CH_3 whilst the 1''-methene group appeared as a quartet at δ 4.18 (*J* 7.0 Hz). The 3-proton singlet at δ 4.05 was assigned to 5-OCH_3 and the 1-proton singlet at δ 7.10 to H-3. C-2'' and C-1'' resonated at δ 14.8 and 63.6 respectively in the ^{13}C NMR spectrum, while C-1 and C-4 appeared at δ 184.6 and 185.0. The HRMS (M^+ + 1) of 359.1267 confirmed a molecular formula of $C_{23}H_{19}O_4$ (requires: 359.1283).

5.3 Synthesis of naphthoquinone-phenyl biaryl systems

It was further decided to couple naphthoquinone **120** to phenyl boronic acids bearing different substituents, to investigate what effect these substituents have on the activity of these naphthoquinone-phenyl systems *in vitro* against *M. bacterium* and certain cancer cell lines. It would also give us an indication of what effect the size of the molecule has on the biological activity of these biaryl systems.

The first naphthoquinone-phenyl system prepared was the coupling product between naphthoquinone **120** and 4-fluorophenyl-1-boronic acid **150** using the Suzuki coupling [95] methodology **(Scheme 5.16)**. Flouride was chosen due to its known biological activity especially in streroids. Thus, a mixture of naphthoquinone **120** and $Pd(PPh_3)_4$ in benzene was treated with an aqueous solution of sodium carbonate and boronic acid **150** in benzene at 24 °C, and then heated under reflux for 16 h to afford quinone **151** in 81% yield.

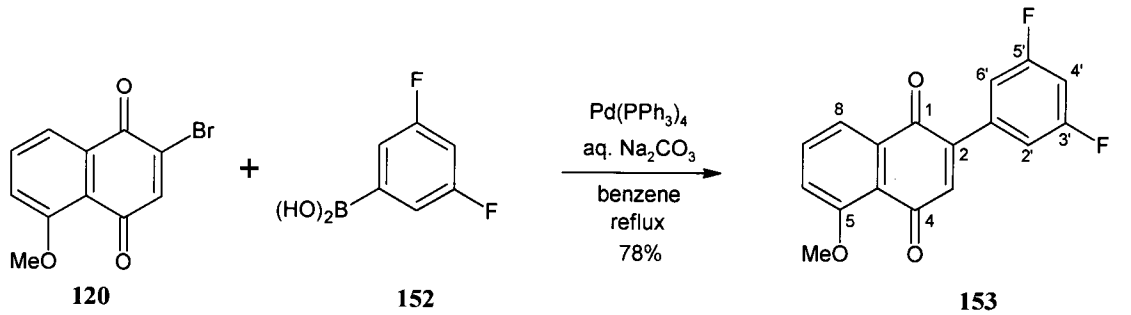


120 **150** **151**

Scheme 5.16

In the ^1H NMR spectrum of compound **151** the 3-proton singlet at δ 4.04 was assigned to 5-OCH_3 and the 1-proton singlet at δ 6.97 to H-3. The two 1-proton doublets at δ 7.13 and δ 7.17 (*J* 8.6 Hz) were assigned to H-2' and H-6', whilst the two doublets resonating at δ 7.57 and δ 7.59 (*J* 8.6 Hz) were assigned to H-3' and H-5'. The latter two hydrogens are more deshielded due to the neighbouring electron-withdrawing fluorine substituent. resultanting in their signals being downfield relative to H-2' and H-6'. The broad doublet at δ 163.8 (*J* 249.1) in the ^{13}C NMR spectrum, due to the coupling between the F-atom and C-4', confirmed the structure of compound **151**. The HRMS ($M^+ + 1$) of 283.0761 supported a molecular formula of $C_{17}H_{12}O_3F$ (requires: 283.0770).

Next naphthoquinone **120** was coupled with 3,5-difluorophenyl-1-boronic acid **152** using the Suzuki coupling [95] methodology, which afforded the desired product **153** in 78% yield **(Scheme 5.17)**.

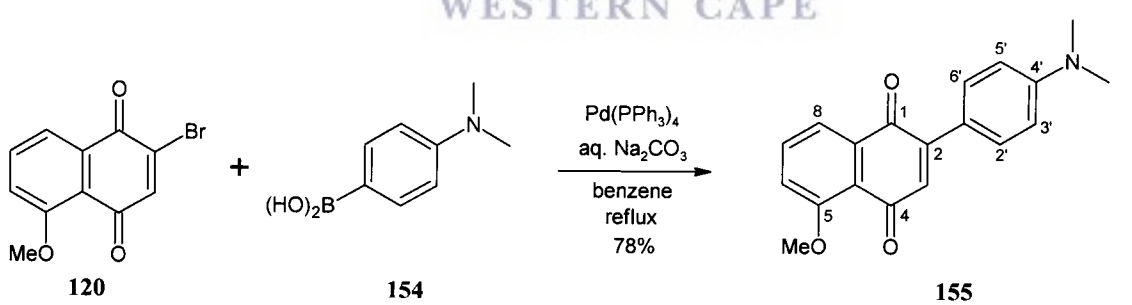


120 152 153

Scheme 5.17

The resulting material was assigned the structure **153** based on the following spectral evidence: the methoxy substituent at C-5 appeared as a 3-proton singlet at δ 4.04, whilst the 1-proton singlet at δ 6.99 was assigned to H-3. The 1-proton triplet of a triplet at δ 6.91 with *J* 8.8 and 2.2 Hz was assigned to H-4' clearly demonstrating coupling between H-4' and the F nucleus while the 2-proton multiplet at δ 7.13 was assigned to H-2' and H-6'. The (M^+ + 1) peak at *m/z* 301.0685 in the HRMS supported a molecular formula of $C_{17}H_{11}O_3F_2$ (requires: 301.0676).

Finally, naphthoquinone **120** was coupled to 4-(dimethylamino)-1-boronic acid **154** using the Suzuki coupling [95] methodology to afford naphthylquinone **155** in 78% yield **(Scheme 5.18)**.



120 154 155

Scheme 5.18

The structural assignment of **155** was confirmed by its ^{1}H NMR spectrum, which had *inter alia* the following characteristics; a 6-proton singlet at δ 3.04 assigned to the *N,N*-dimethyl groups of the 4'-amino substituent whilst the 3-proton singlet at δ 4.02 was assigned to 5-OCH_3. A 2-proton doublet at δ 6.75 (*J* 9.0 Hz) was assigned to H-3' and H-5', whilst the corresponding downfield 2-proton doublet at δ 7.59 was assigned to H-2' and H-6'. A 1-proton singlet at δ 6.94 was assigned to H-3. In the ^{13}C NMR

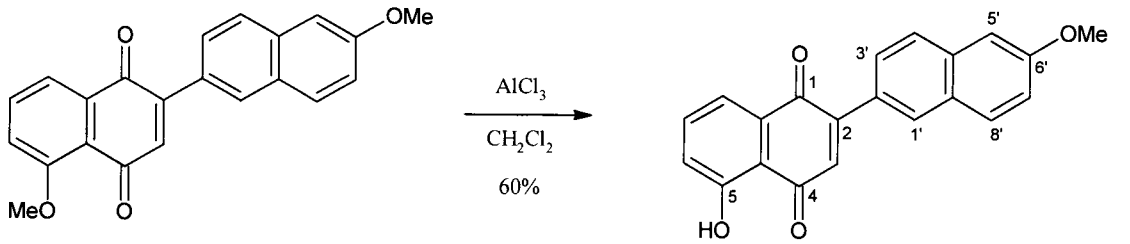
spectrum of compound **155** the three C-signals at δ 130.6, 111.8, and 40.1 were assigned to the three chemically equivalent pairs of C-atoms viz., C-3' and C-5', C-2' and C-6', and the two CH_3 groups of the dimethylamino substituent, respectively. The HRMS ($M^+ + 1$) of 308.1286 confirmed a molecular formula of $C_{19}H_{18}NO_3$ (requires: 308.1287).

5.4 Structural modification of biaryl systems

In previous studies by Lall *et al.* [32] and Mahapatra *et al.* [34] it was found that the synthetic alkoxy derivatives of diospyrin **15** and 7-methyljuglone **16** exhibited reduced activity against the drug-susceptible strain, H37Rv, of *M. tuberculosis*. However, Chakrabarty and co-workers [62] reported that the alkoxy derivatives of diospyrin **15** were more active than their hydroxy analogue when evaluated against four human cancer cell lines viz., acute myeloblastic leukaemia (HL-60), chronic myelogenic leukaemia (K-562), breast adenocarcinomia (MCF-7) and cervical epithelial carcinoma (HeLa). Hence, we decided to synthesize hydroxy analogues of two of our synthesized bi-aryl systems to investigate whether this will enhance or reduce their activity against *M. tuberculosis* or their apoptotic inducing potential against the cell lines used in the study.

5.4.1 Synthesis of hydroxy analogues

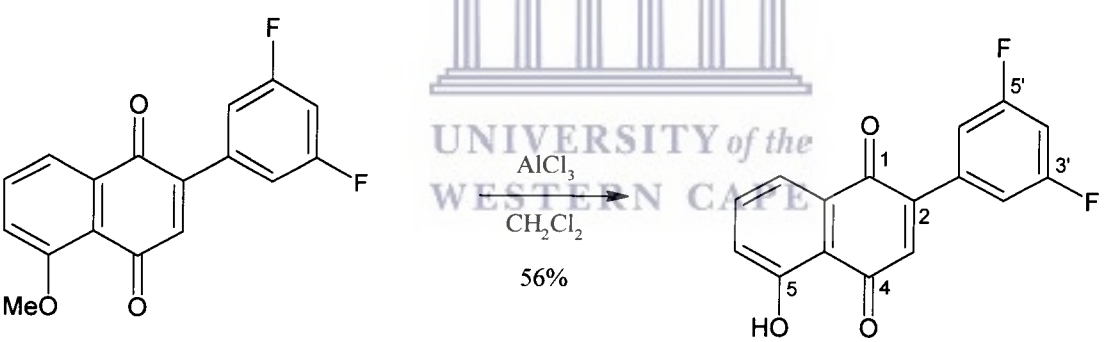
Demethylation of compound **133** was effected using the method of Syper *et al.* [84] Thus, a solution of methoxyquinone **133** in dry CH_2Cl_2 at 25 °C was treated with aluminium trichloride. The mixture was stirred at 25 °C for 24 h, poured into water, and then acidified with dilute HCl to afford the hydroxyquinone **156** in 60% yield **(Scheme 5.19)**. The disappearance of the 3-proton signal at δ 4.04, ascribed to the 5-OCH_3, and the presence of a strongly deshielded 1-proton singlet at δ 11.89 in the ^1H NMR spectrum were significant supporting evidence that demethylation of the 5-OCH_3 group had ocurred. The HRMS (M^+) of 330.0894 confirmed a molecular formula of $C_{21}H_{14}O_4$ (requires: 330.0892).



133 156

Scheme 5.19

Quinone **153** was subjected to the same reaction conditions as described above to afford the demethylated analogue **157** in 56% yield **(Scheme 5.20)**. The absence of the 3-proton methoxy signal and the presence of a strongly deshielded 1-proton signal at δ 11.92 in the ^{1}H NMR with a broad absorption peak at 3430 cm^{-1} in the IR spectrum confirmed the structure assigned to naphthol **157**. The (M^+ - 1) peak at *m/z* 285.0373 in the HRMS supported a molecular formula of $C_{16}H_7O_3F_2$ (requires: 285.0363).



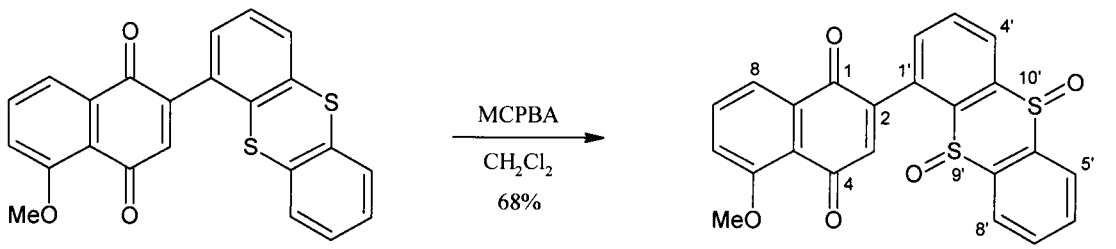
153 157

Scheme 5.20

5.4.2 Oxidation of thianthrenyl moieties

Preliminary studies [96] in our laboratory have shown that the apoptosis inducing ability of compounds containing thianthrenyl moieties are enhanced when the latter are oxidized to their sulphone analogues. Hence, utilizing the method reported by Nakayama *et al.* [97], compounds **139** and **140** were converted into their oxidized analogues to corroborate these findings.

A solution of **139** in DCM was treated with *m*-chloroperbenzoic acid to afford a 68% yield of the sulphone **158 (Scheme 5.21)**. The resulting material was assigned the structure **158** based on the following spectral evidence. In the ^{1}H NMR spectrum the 3-proton and 1-proton singlets at δ 4.08 and 6.98 were assigned to 5-OCH_3 and H-3, respectively.

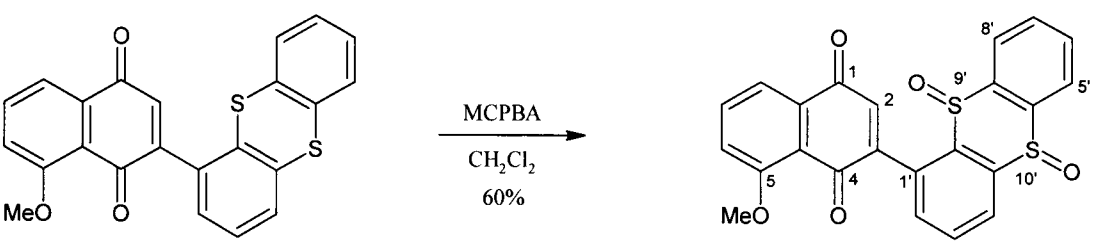


139 **158**

Scheme 5.21

The two 1-proton doublet of doublets at δ 7.41 and 7.85, with *J* 8.0 and 1.4 Hz, were assigned to H-6 and H-8, whilst the 1-proton triplet at δ 7.77 (*J* 8.0 Hz) was assigned to H-7. Further downfield, a 2-proton multiplet at δ 8.19 and a 1-proton doublet of a doublet at δ 8.31, with *J* 7.6 and 1.4 Hz, were assigned to H-5', H-8' and H-4', respectively. This significant chemical shift of the three latter protons, downfield from where they resonate in the ^{1}H NMR spectrum of compound **139**, is ascribed to the oxidized neighbouring sulphur groups. In the IR spectrum, peaks at 1164 and 1326 cm^{-1} were assigned to the two S=O groups and the HRMS (M^+ + 1) of 435.0355 confirmed a molecular formula of $C_{23}H_{15}O_5S_2$ (requires: 435.0361).

Treatment of compound **140** with *m*-chloroperbenzoic acid afforded the expected sulphone **159** in 60% isolated yield **(Scheme 5.22)**.



140 **159**

Scheme 5.22

The sulphone product **159** was again characterized by the downfield chemical shift of the 1-proton doublet of a doublet, assigned to H-4', to δ 8.37 in the ^{1}H NMR spectrum. Peaks at 1322 and 1163 cm^{-1}, in the IR spectrum, were assigned to the two S=O groups while the HRMS (M^+ + 1) of 435.0365 corresponds to a molecular formula of $C_{23}H_{15}O_5S_2$ (requires: 435.0361).

5.5 Conclusion

Coupling of triflate **107** to its naphthol precursor **106** via Michael addition using the method of Stagliano *et al.*, [76] afforded a yellowish powder. We were not able to identify the product using NMR spectroscopy and all our attempts to purify the product failed.

However, several naphthoquinone-naphthalene biaryl systems were successfully synthesized utilizing the Suzuki methodology. This was achieved by coupling commercially available boronic acids to three 1, 4-naphthoquinone regioisomers viz., **120**, **125**, and **128** synthesized in Chapter 4. The synthesis of three further naphthoquinone-phenyl biaryl compounds has also been achieved. Structural modification to a selected few compounds were also made to investigate whether it will have any effect on their biological activity.

CHAPTER 6

EXPERIMENTAL – GENERAL PROCEDURES

Purification of solvents

All solvents used for reactions and preparative chromatography, were distilled prior to use. Tetrahydrofuran and diethyl ether were dried using sodium wire and then distilled using the sodium benzophenone ketyl radical as indicator. Dimethylformamide, tetrahydrofuran, acetone and diethyl ether were stored over molecular sieves (4A). Other reagents obtained from commercial sources were used without further purification.

Chromatographic Separations

Preparative column chromatography was carried out on dry-packed columns using Merck silica gel (particle size 0.2 - 0.5 mm) as adsorbent and Merck silica gel 60 (0.063 - 0.2 mm) as the stationary phase. Mixtures of ethyl acetate and hexane were used as eluent.

Physical and Spectroscopic Data

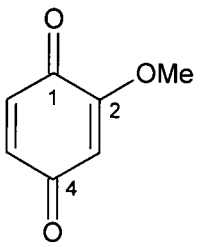
All melting points were obtained on a FISCHER-JOHNS melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded using a VARIAN 200 spectrometer (^{1}H, 200MHz; ^{13}C, 50MHz). The spectra were run at ambient temperature in deuterated chloroform ($CDCl_3$) solution, with $CHCl_3$ at δ 7.26 for ^{1}H NMR spectra and chloroform (δ 77.00) for ^{13}C-NMR spectra as internal standards. In the NMR spectra, assignments of signals with the same superscripts are interchangeable. Splitting patterns are designated as "s", "d", "t", "q", "m" and "bs". These symbols indicate "singlet", "doublet", "triplet", "quartet", "multiplet" and "broad singlet".

Infrared (IR) spectra were recorded as a nujol mull for solids and as thin films between sodium chloride plates for oils on a PERKIN ELMER FT-IR spectrometer PARAGON 2000. Mass spectra were performed on a Waters GCT Premier 70 eV High Resolution Mass Spectrometer at the University of Stellenbosch.

Other General Procedures

The term "residue obtained upon work-up" refers to the residue obtained when the organic layer was separated, dried over magnesium sulphate ($MgSO_4$) followed by filtration and the removal of solvent by evaporation.

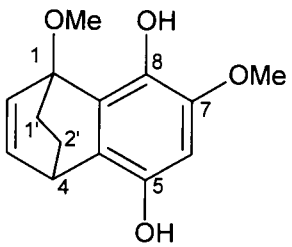
2-Methoxy-1,4-benzoquinone (101)



101

Vanillin, **99**, (3.00 g, 19.70 mmol) was dissolved in aqueous NaOH (45 ml of a 4% solution). Hydrogen peroxide (9 ml of a 30% solution in 45 ml water) was slowly dripped in with stirring. The solution gradually changed colour from light yellow to dark brown. The resulting solution was stirred for 1h and thereafter acidified with sulphuric acid (10.80 ml of a 20% solution). The acidic solution was cooled and extracted with ether (3 x 60 ml), which was evaporated to dryness and the resulting oil dissolved in water (30 ml). To this solution, H_2SO_4 (12 ml of a 20% solution) was added, and the resulting mixture then added drop wise to a stirred solution of sodium dichromate (4.80 g in 30 ml water) containing a little bit of ice. After the addition of more ice (60 g), stirring was continued for a further 25 min and the mixture then extracted with DCM (3 x 60ml). The residue obtained upon work-up afforded the benzoquinone **101** (2.19 g, 81%) as a brown solid, m.p. 140-143 °C (Lit. [82] m.p. 140-143 °C). ν_{max}/cm^{-1} 1695 and 1745 (C=O); δ_H 3.83 (3H, s, OCH_3), 5.94 (1H, s, H-3), 6.71 (2H, s, H-5 and H-6).

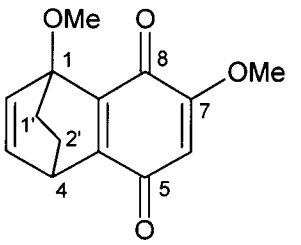
1,4-Dihydro-1,7-dimethoxy-1,4-ethanonaphthalene-5,8-diol (103)



103

1-Methoxycyclohex-1,3-diene **102** (4.29 g, 4.62 ml, 38.90 mmol) in benzene (25 ml) was added, under nitrogen, to a stirred solution of quinone **101** (2.00 g, 14.50 mmol) in benzene (25 ml) using a pressurized dropping funnel. The reaction mixture was then heated under reflux for 1.5 h, at which stage all the quinone was consumed. The residue was chromatographed using EtOAc:Hexane (3:7) as eluent to give the naphthol **103** as white crystals (3.42 g, 95%), m.p. 108-110 °C (Lit. [82] m.p. 108-110 °C). ν_{max}/cm^{-1} 3400-2500 (broad, O-H); δ_H 1.59 (4H, m, $-CH_2CH_2-$), 2.01 (1H, m, H-4), 3.67 (3H, s, 1-OCH_3), 3.77 (3H, s, 7-OCH_3), 4.48 (1H, s, 5-OH) 6.22 (1H, s, H-6), 6.49 (1H, dd, *J* 8.2 and 5.8, H-3), 6.65 (1H, dd, *J* 8.2 and 1.4, H-2) and 8.69 (1H, s, 8-OH); δ_C 26.0 (C-2')[a], 28.3 (C-1')[a], 32.3 (C-4), 52.2 (1-OCH_3), 56.5 (7-OCH_3), 86.3 (C-1), 99.2 (C-6), 120.4 (C-4a)[b], 127.7 (C-8a)[b], 134.3 (C-2)[c], 134.8 (C-3)[c], 136.8 (C-8)[d], 141.1 (C-5)[d] and 146.2 (C-7)[d]. (Found: HRMS (M^+ + 1) 249.1133. Calc. for $C_{14}H_{17}O_4$: 249.1127).

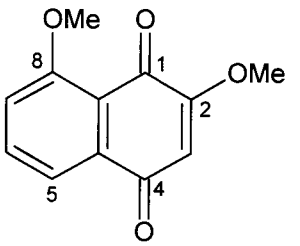
1,4-Dihydro-1,7-dimethoxy-1,4-ethanonaphthalene-5,8-dione (104)



104

To a stirred solution of phenol **103** (3.19 g, 12.85 mmol) in acetonitrile (80 ml) and water (15 ml), was added drop wise a solution of cerium(IV) ammonium nitrate (14.10 g, 25,70 mmol) in water (30 ml). Stirring was continued for an additional 30 min., followed by the addition of water (500 ml) and then extraction with dichloromethane (3 x 60 ml). The residue obtained upon work-up afforded quinone **104** (2.28 g, 72%), as an olive green solid, m.p. 115-118 °C (from ethanol) (Lit. [82] m.p. 117-119 °C). ν_{max}/cm^{-1} 1668 and 1745 (C=O); δ_H 1.65 (4H, m, $-CH_2CH_2-$), 3.61 (3H, s,1-OCH_3), 3.79 (3H, s, 7-OCH_3), 4.29 (1H, m, H-4), 5.76 (1H, s, H-6), 6.34 (1H, dd, *J* 7.6 and 6.2, H-3), 6.57 (1H, d, *J* 7.6, H-2); δ_C 25.0 (C-2')[a], 31.2 (C-1')[a], 33.4 (C-4), 55.7 (1-OCH_3), 56.4 (7-OCH_3), 84.9 (C-1), 104.9 (C-6), 131.2 (C-3)[a], 135.4 (C-2)[a], 143.3 (C-4a)[b], 149.4 (C-8a)[b], 158.8 (C-7), 177.4 (C-8)[c], 183.4 (C-5)[c]. (Found: HRMS (M^+) 246.0890. Calc. for $C_{14}H_{14}O_4$: 246.0892).

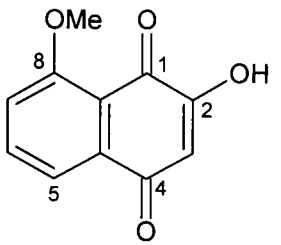
2,8-Dimethoxy-1,4-naphthoquinone (105)



105

The crude quinone **104** (3.15 g, 12.79 mmol) was pyrolysed a 140 °C, under nitrogen for 30 min to afford the naphthoquinone **105** (2.66 g, 95%) as green crystals, m.p. 199- 202 °C (from ethanol) (Lit. [82] m.p. 202-202.5 °C). ν_{max}/cm^{-1} 1670 and 1695 (C=O); δ_H 3.88 (3H, s, 2-OCH_3), 4.01 (3H, s, 8-OCH_3), 6.10 (1H, s, H-3), 7.25 (1H, m, H-7), 7.68-7.74 (2H, m, H-5 and H-6); δ_C 56.4 (2-OCH_3)[a], 56.5 (8-OCH_3)[a], 107.9 (C-3), 117.4 (C-7), 118.9 (C-5), 119.6 (C-8a), 134.4 (C-4a), 135.4 (C-6), 160.1 (C-2)[b], 161.1 (C-8)[b], 178.5 (C-1)[c], 184.8 (C-4)[c]. (Found: HRMS (M^+ + 1) 219.0654. Calc. for $C_{12}H_{11}O_4$: 219.0657).

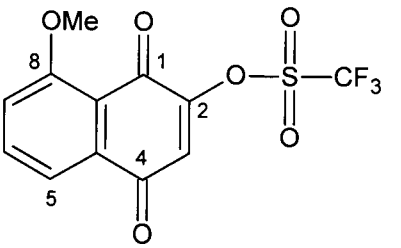
2-Hydroxy-8-methoxy-1,4-naphthoquinone (106)



106

The naphthoquinone **105** (2.66 g, 12.19 mmol) in aqueous 4% NaOH (55 ml) was stirred until it had dissolved. The solution was washed with ether (50 ml) and then acidified with 5M HCl. The resulting solution was extracted with dichloromethane (3 x 60 ml) and the residue afforded the quinone **106** (2.38 g, 96%) as a yellow solid, m.p. 208-211 °C (from ethanol) (Lit.[82] m.p. 209-211 °C, decomp.). ν_{max}/cm^{-1} 3200-2700 (broad, O-H) 1670 and 1687 (C=O); δ_H 4.05 (3H, s, 8-OCH_3), 6.29 (1H, s, H-3), 7.25-7.30 (1H, m, H-7), 7.70-7.81 (3H, m, H-5, H-6 and 2-OH); δ_C 56.5 (8-OCH_3), 108.5 (C-3), 116.9 (C-7), 119.5 (C-5), 127.8 (C-8a), 135.2 (C-4a), 136.8 (C-6), 156.8 (C-2), 160.4 (C-8), 180.1 (C-1)[a], 184.5 (C-4)[a]. (Found: HRMS (M^+ + 1) 205.0504. Calc. for $C_{11}H_9O_4$: 205.0501).

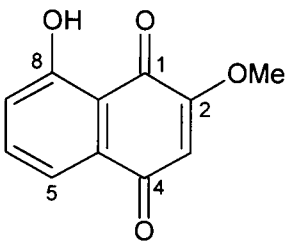
8-Methoxy-2-trifluoromethanesulphonyloxy-1,4-naphthoquinone (107)



107

Hydroquinone **106** (0.63 g, 3.09 mmol) was placed in a three-necked 50 ml round-bottom flask and flushed with N_2. DCM (25 ml) was added and the reaction mixture was degassed at least five times by a sequence of vacuum - N_2 replenishment, before being cooled to 0 °C. Triethyl amine (0.53 ml, 3.80 mmol) was added and the formally yellow solution mixture turned a dark red homogeneous solution. Triflic anhydride (0.64 ml, 3.80 mmol) was then added and the solution was stirred at 0 °C for 10 min and then at room temperature for 20 min. The residue obtained upon work-up was then chromatographed using EtOAc:Hexane (3:7) as eluent to afford triflate **107** (0.20 g, 20%) as a thick red solid. ν_{max}/cm^{-1} 1677 and 1690 (C=O); δ_H 4.05 (3H, s, 8-OCH_3), 6.84 (1H, s, H-3), 7.36-7.41 (1H, m, H-7), 7.76-7.80 (2H, m, H-5 and H-6); δ_C 56.6 (8-OCH_3), 117.8 (q, *J* 318.5, *C*F_3), 118.7 (C-3), 119.6(C-7), 121.8 (C-8a)[a], 124.7 (C-5), 133.8 (C-4a), 136.4 (C-6), 152.4 (C-2), 160.8 (C-8), 179.8 (C-1)[a], 183.4 (C-4)[a]. (Found: HRMS 335.9910. Calc. for $C_{12}H_7F_3O_6S$: 335.9915).

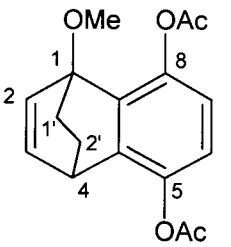
8-Hydroxy-2-methoxy-1,4-naphthoquinone (108)



108

To a solution of **105** (0.05 g, 0.23 mmol) in DCM (20 ml) at room temperature was added $AlCl_3$ (2.61 g, 19.6 mmol). The mixture was stirred at room temperature for 24 h, poured into water, then acidified with dilute HCl (0.1 M, 100 ml), and extracted with DCM (3 x 60 ml). The residue obtained upon work-up was chromatographed using EtOAc:Hexane (3:7) to afford quinone **108** (0.03 g, 61%) as a yellow solid, m.p. 219-222 °C. ν_{max}/cm^{-1} 3200-2700 (broad, O-H), 1676 and 1693 (C=O); δ_H 3.90 (3H, s, 2-OCH_3), 6.14 (1H, s, H-3), 7.23 (1H, m, H-7), 7.60-7.63 (2H, m, H-5 and H-6), 11.74 (1H, s, 8-OH); δ_C 56.6 (2-OCH_3), 110.5 (C-3), 114.3 (C-8a), 118.9 (C-5), 123.8 (C-7), 132.1 (C-4a), 137.1 (C-6), 160.1 (C-2), 162.0 (C-8), 183.9 (C-1)[a], 184.9 (C-4)[a]. (Found: HRMS (M^+) 204.0418. Calc. for $C_{11}H_8O_4$: 204.0422).

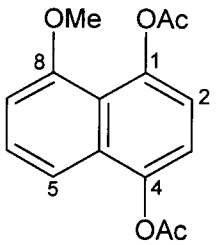
5,8-Diacetoxy-1,4-dihydro-1,4-ethano-1-methoxynaphthalene (112)



112

A solution of **102** (7.63 g, 8.21 ml, 0.045mol) and **109** (4.42 g, 0.041 mol) in dry benzene (50 ml) was heated under reflux in a N_2 atmosphere for 4h. The solvent was evaporated to give an oily residue to which was added dry acetone (100 ml) and K_2CO_3 (12 g). The mixture was vigorously stirred under reflux in a N_2 atmosphere for 2h, cooled, filtered and the solvent evaporated under reduced pressure. Acetic anhydride (18 g, 16.6 ml) and pyridine (14 g, 14.3 ml) were added and the solution was heated under reflux for 3h in a N_2 atmosphere after which it was poured into water. The solid was filtered off and chromatographed using EtOAc:Hexane (3:7) as eluent to afford compound **112** (9.42 g, 76%) as a off-white solid, m.p. 146-149 °C (from hexane) (Lit. [85] m.p. 147-148 °C). ν_{max}/cm^{-1} 1750 (C=O); δ_H 1.54-1.73 (4H, m, $-CH_2CH_2-$), 2.30 (3H, s, 5-OAc), 2.35 (3H, s, 8-OAc), 3.60 (3H, s, OCH_3), 3.90 (1H, m, H-4), 6.45 (1H, dd, *J* 8.4 and 6.2, H-3), 6.67 (1H, d, *J* 8.4, H-2), 6.74 (1H, dd, *J* 8.8 and 1.2, H-6), 6.85 (1H, dd, *J* 8.8 and 1.2, H-7); δ_C 20.8 (2 x $OCOCH_3$), 25.4 (C-1')[a], 28.8 (C-2')[a], 33.9 (C-4), 53.7 (OCH_3), 83.7 (C-1), 119.7 (C-6)[b], 120.7 (C-7)[b], 132.8 (C-2)[c], 135.1 (C-3)[c], 136.3 (C-4a)[d], 136.6 (C-8a)[d], 142.0 (C-8)[e], 142.1 (C-5)[e], 169.3 (5-$OCOCH_3$)[f], 170.0 (8-$OCOCH_3$)[f]. (Found: HRMS (M^+ + 1) 303.1220. Calc. for $C_{17}H_{19}O_5$: 303.1232).

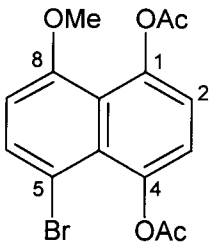
1,4-Diacetoxy-8-methoxynaphthalene (113)



113

Compound **112** (1.76 g, 5.82 mmol) was heated, in a N_2 atmosphere, at 210 °C for 40 min. The residue was chromatographed using EtOAc:Hexane (3:7) as eluent to afford compound **113** (1.35 g, 87%) as pale green crystals, m.p. 120-123 °C (from hexane) (Lit. [85] m.p. 122-123 °C). ν_{max}/cm^{-1} 1760 (C=O); δ_H 2.38 (3H, s, 4-OAc), 2.45 (3H, s, 1-OAc), 3.94 (3H, s, OCH_3), 6.89 (1H, dd, *J* 6.6 and 2.2, H-7), 7.06 (1H, d, *J* 8.0, H-3), 7.24 (1H, d, *J* 8.0, H-2), 7.39-7.50 (2H, m, H-5 and H-6); δ_C 21.0 (4-OCO*C*H_3)[a], 21.1 (1-OCO*C*H_3)[a], 56.3 (OCH_3), 106.9 (C-2), 114.2 (C-7), 118.5 (C-3), 118.7 (C-5), 120.1 (C-8a), 127.2 (C-6), 129.9 (C-4a), 144.3 (C-4), 144.4 (C-1), 155.6 (C-8), 169.3 (4-O*C*OCH$_3$), 170.2 (1-O*C*OCH$_3$). (Found: HRMS (M^+ + 1) 275.0914. Calc. for $C_{15}H_{15}O_5$: 275.0919).

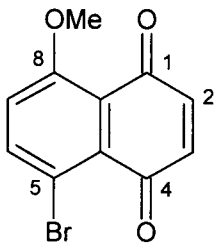
5-Bromo-1,4-diacetoxy-8-methoxynaphthalene (114)



114

Br_2 (0.62 g, 3.90 mmol, 0.20 ml) in AcOH (25 ml) was added to a stirred solution of **113** (1.07 g, 3.90 mmol) in AcOH (20 ml) at room temperature. After the addition of Br_2 the stirring was continued for another 15min before the reaction mixture was thrown into icy water (100 ml). The solid was filtered off to yield compound **114** (1.21 g, 88%) as yellow crystals, m.p. 171-174 °C (from hexane). ν_{max}/cm^{-1} 1758 (C=O); δ_H 2.36 (3H, s, 4-OAc), 2.43 (3H, s, 1-OAc), 3.90 (3H, s, OCH_3), 6.69 (1H, d, *J* 8.8, H-7), 7.09 (1H, d, *J* 8.0, H-3), 7.19 (1H, d, *J* 8.0, H-2), 7.72 (1H, d, *J* 8.8, H-6); δ_C 20.8 (4-OCO*C*H_3)[a], 22.0 (1-OCO*C*H_3)[a], 56.5 (OCH_3), 106.4 (C-5), 107.3 (C-7), 120.0 (C-2)[b], 122.1 (C-3)[b], 122.2 (C-8a), 127.1 (C-4a), 134.3 (C-6), 143.7 (C-4)[c], 144.8 (C-1)[c], 155.3 (C-8), 169.9 (4-O*C*OCH$_3$)[d], 170.0 (1-O*C*OCH$_3$)[d]. (Found: HRMS (M^+ + 1) 353.0040. Calc. for $C_{15}H_{14}O_5Br$: 353.0025).

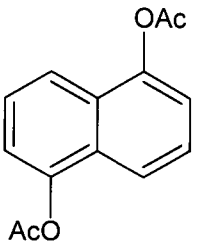
5-Bromo-8-methoxy-1,4-naphthoquinone (115)



115

To a stirred solution of **114** (0.71g, 2.01mmol) in acetonitrile (20ml) and water (10ml) at 0 °C, was added dropwise a solution of CAN (2.20g, 4.02mmol) in water (10ml). The reaction mixture was allowed to warm to room temperature and stirring was continued for an additional 30 min. Water (100 ml) was then added and the organic material extracted with dichloromethane (3 x 60 ml). The residue obtained upon work-up was then chromatographed using 30% EtOac/Hexane as eluent to afford naphthoquinone **115** (0.17 g, 32%) as yellow crystals, m.p. 162-165 °C (from hexane). ν_{max}/cm^{-1} 1674 and 1690 (C=O); δ_H 4.00 (3H, s, OCH_3), 6.82 (1H, d, *J* 10.4, H-2), 6.89 (1H, d, *J* 10.4, H-3), 7.16 (1H, d, *J* 8.8, H-7), 7.92 (1H, d, *J* 8.8, H-6); δ_C 56.8 (OCH_3), 112.5 (C-5), 118.7 (C-7), 130.9 (C-8a), 136.2 (C-4a), 137.3 (C-2)[a], 139.0 (C-3)[a], 141.8 (C-6), 159.4 (C-8), 183.5 (C-1)[b], 183.9 (C-4)[b]. (Found: HRMS (M^+ - 1) 265.9576. Calc. for $C_{11}H_7BrO_3$: 265.9578).

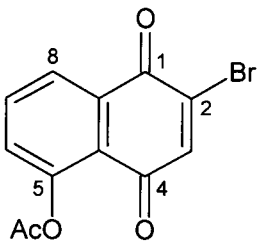
1,5-Diacetoxynaphthalene (117)



117

1,5 Naphthalenediol **116** (5.0 g, 31.2 mmol) was dissolved in pyridine (25 ml, 306.0 mmol) and Ac_2O (25 ml, 265.0 mmol). The solution was stirred for 12 hrs at room temperature under N_2, before the remaining Ac_2O was quenched by the addition of H_20 (100 ml). The solution was then filtered and the solid obtained was washed with ethanol:H_2O (1:19) and dried in the air to afford the product **117** (7.39 g, 97%) as a light brown solid, m.p. 158-161 °C (from benzene) (Lit.[86] m.p. 161 °C). δ_H 2.47 (6H, s, 2 x OAc), 7.29 (2H, d, *J* 8.4, H-4 and H-8), 7.51 (2H, t, *J* 8.4 and 8.0, H-3 and H-7), 7.79 (2H, d, *J* 8.0, H-2 and H-6).

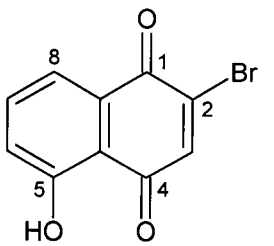
5-Acetoxy-2-bromonaphthalene (118)



118

A warm solution of substrate **117** (2.44 g, 10.0 mmol) dissolved in acetic acid (100 ml) was added over a period of 20 min., with stirring, to a solution of *N*-bromosuccinamide (7.2 g, 40.0 mmol) dissolved in acetic acid (100 ml) and water (200 ml). The solution of NBS was heated to 65 °C prior to the addition of the substrate and kept at 65 °C during the addition. The reaction mixture was stirred at 65 °C for 45 min following the addition and then poured into water (200 ml). The aqueous suspension was extracted with DCM (4 x 60 ml). The combined extracts were washed with water (4 x 200 ml) and saturated $NaHCO_3$ (2 x 100 ml) and then dried over $MgSO_4$. The solvent was evaporated off to afford the quinone **118** (2.83 g, 96%) as yellow needles, m.p. 154-156 °C (from ethanol) (Lit. [86] m.p. 154.5-156 °C). δ_H 2.44 (3H, s, 5-OAc), 7.39(1H, s, H-3), 7.42 (1H, dd, *J* 8.2 and 1.2, H-6), 7.77 (1H, t, *J* 8.2, H-7), 8.14 (1H, dd, *J* 8.2 and 1.2, H-8); δ_C 20.9 (5-OCO*C*H_3), 123.3 (C-4a), 126.4 (C-8), 130.4 (C-6), 132.7 (C-8a), 134.9 (C-7), 138.5 (C-2), 141.5 (C-3), 149.9 (C-5), 169.2 (5-O*C*OCH$_3$), 177.5 (C-1), 180.9 (C-4). (Found: HRMS (M^+) 293.9521. Calc. for $C_{12}H_7BrO_4$: 293.9528).

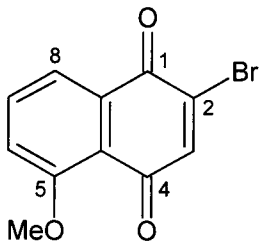
2-Bromo-5-hydroxynaphthalene (119)



119

A suspension of **118** (2.76 g, 9.35 mmol) in ethanol (100 ml) and H_2SO_4 (1.5M, 30 ml) was gently heated under reflux for 90 min. The solution was concentrated in vacuo to remove the ethanol and the residue extracted with DCM (3 x 60 ml). The combined extracts were washed with water (2 x 100 ml) and brine (1x 100 ml), and then dried over $MgSO_4$. The crude product was chromatographed using EtOAc:Hexane (3:7) to afford compound **119** (2.15 g, 8.51 mmol, 91%) as bright orange crystals, m.p. 133-135 °C (from hexane) (Lit. [86] m.p. 135-136 °C). ν_{max}/cm^{-1} 3200-2700 (broad, O-H), 1676 and 1693 (C=O); δ_H 7.32(1H, dd, *J* 8.2 and 1.6, H-6), 7.50 (1H, s, H-3), 7.64 (1H, t, *J* 8.2, H-7), 7.74 (1H, dd, *J* 8.2 and 1.6, H-8), 11.78 (1H, s, OH); δ_C 114.7 (C-4a), 121.0 (C-8), 125.1 (C-6), 130.7 (C-8a), 136.4 (C-7), 140.3 (C-3), 140.9 (C-2), 161.7 (C-5), 177.2 (C-1), 187.5 (C-4). (Found: HRMS (M^+ - 1) 250.9335. Calc. for $C_{10}H_4O_3Br$: 250.9344).

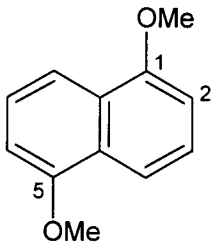
2-Bromo-5-methoxynaphthalene (120)



120

To a solution of quinone **119** (1.13 g, 4.47 mmol) in DCM (30 ml) was added silver(I) oxide (3.11 g, 13.40 mmol) and iodomethane (2.54 g, 1.11 ml, 17.88 mmol). The reaction mixture was stirred for 12 h at room temperature, before being filtered and then evaporated in vacuo to give the crude product. Purification was performed by flash chromatography using EtOAc:Hexane (3:7) as eluent to afford quinone **120** (1.09 g, 4.11 mmol, 92%) as brown orange needles, m.p. 130-132 °C (from hexane) (Lit. [87] m.p. 132-133 °C). ν_{max}/cm^{-1} 1678 and 1695 (C=O); δ_H 4.02 (3H, s, OCH_3), 7.34(1H, dd, *J* 8.4 and 1.0, H-6), 7.41 (1H, s, H-3), 7.70 (1H, t, *J* 8.4, H-7), 7.83 (1H, dd, *J* 7.8 and 1.0, H-8); δ_C 56.5 (OCH_3), 118.5 (C-6), 120.6 (C-8), 121.0 (C-4a), 133.0 (C-8a), 135.1 (C-7), 136.8 (C-2), 142.3 (C-3), 159.9 (C-5), 178.2 (C-1), 181.4 (C-4). (Found: HRMS (M^+ - 1) 265.9451. Calc. for $C_{11}H_6O_3Br$: 265.9578).

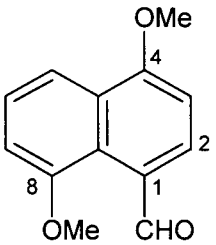
1,5-Dimethoxynaphthalene (121)



121

To a solution of 1,5-dihydroxynaphthalene **116** (4.00 g, 25.0 mmol) in acetone (20 ml), K_2CO_3 (4.15 g, 30.0 mmol) was added. Dimethyl sulphate (6.31 g, 4.73 ml, 50.0 mmol) was then added in one portion and the reaction mixture was heated under reflux for 12 h. After cooling to room temperature, the mixture was quenched by the addition of water (500 ml). The organic material was extracted by DCM (3 x 80 ml), washed with water (3 x 200 ml) and brine (1 x 100 ml), dried over $MgSO_4$, and the solvent evaporated to give the naphthalene **121** (4.54 g, 96%) as a light brown solid, m.p. 182-185 °C (from ethanol) (Lit. [98] m.p. 183-184 °C). δ_H 4.00 (6H, s, 2 x OCH_3), 6.85 (2H, d, *J* 8.0, H-2 and H-6), 7.39 (2H, t, *J* 8.0, H-3 and H-7), 7.84 (2H, d, *J* 8.0, H-4 and H-8); δ_C 55.5 (2 x OCH_3), 104.5 (C-2 and C-6), 114.2 (C-4 and C-8), 125.1 (C-3 and C-7), 126.6 (C-4a and C-8a), 155.2 (C-1 and C-5).

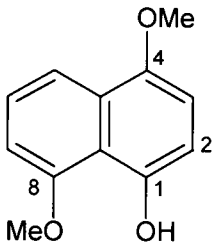
4,8-Dimethoxy-1-naphthalenecarboxaldehyde (122)



122

1,5 Dimethoxynaphthalene **121** (4.13 g, 21.94 mmol), DMF (2.57 g, 2.6 ml, 35.10 mmol), and toluene (3.64 g, 4.20 ml, 39.50 mmol) were slurried together and cooled in an ice bath, after which $POCl_3$ (4.03 g, 2.45 ml, 26.30 mmol) was added and the mixture was stirred in an ice bath for 30 min. and then heated to reflux. After 2 hrs, the reaction mixture was poured into aqueous NaOH (100 ml of 10% NaOH – 30 ml of ice) with stirring. The mixture was the extracted with DCM (3 x 60 ml), and the combined organic phases were washed sequentially with 5% aqueous HCl (2 x 60 ml), water (2 x 60 ml) and brine (60 ml), dried over $MgSO_4$ and evaporated to give naphthaldehyde **122** (4.31 g, 91%) as a brown solid, m.p. 124-126 °C (from hexane) (Lit. [88] m.p. 124-126 °C). δ_H 4.01 (3H, s, 4-OCH_3), 4.05 (3H, s, 8-OCH_3), 6.90 (1H, d, *J* 8.4, H-7), 7.04 (1H, d, *J* 7.8, H-3), 7.45 (1H, t, *J* 8.4, H-6), 7.96 (1H, d, *J* 7.8, H-2), 8.07 (1H, d, *J* 8.4, H-5), 11.05 (1H, s, CHO); δ_C 55.6 (4- OCH_3), 55.9 (8-OCH_3), 104.0 (C-3), 107.8 (C-7), 115.3 (C-5), 124.7 (C-8a), 125.8 (C-6), 127.1 (C-1), 127.8 (C-4a), 129.4 (C-2), 156.4 (C-8), 159.5 (C-4), 194.6 (CHO). (Found: HRMS (M^+ + 1) 217.0854. Calc. for $C_{13}H_{13}O_3$: 217.0865).

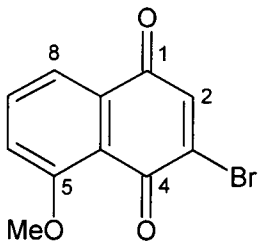
4,8-Dimethoxy-1-naphthol (124)



124

A solution of naphthalene **122** (6.48 g, 30.0 mmol) and mCPBA (12.24 g, 61.0 mmol, 86%) in DCM (200 ml) was rapidly stirred for 2 hrs and 20 min. Aqueous sodium thiosulphate (100 ml of 10% solution) was introduced, and after being stirred for 30 min the mixture was poured into an additional 250 ml of 10% aqueous sodium thiosulphate and vigorously shaken. The phases were separated, and the aqueous phase was extracted with DCM (2 x 100 ml). The combined organic phases were washed successively with aqueous thiosulphate (2 x 200 ml) and brine (200 ml), dried over $MgSO_4$, and then evaporated to give the crude formate **123** (28.20 g, 94%) as a dark brown solid. The crude formate ester was dissolved in degassed THF – CH_3OH (1:1, 200 ml) and cooled in an ice bath. KOH (4.25 g, 75.75 mmol) in ice-cold degassed CH_3OH (40 ml) was added, and after 15 min stirring 5% aqueous HCl was added to pH 1 and the reaction mixture was poured into water (1 L), and then extracted with DCM (3 x 150 ml). The organic phases were washed sequentially with water (2 x 200ml) and brine (200 ml), and dried over $MgSO_4$. The crude product was chromatographed using EtOAc:Hexane (3:7) to afford compound **124** (4.35 g, 71%) as a beige solid, m.p. 154-156 °C (from hexane) (Lit. [88] m.p. 155-156 °C). ν_{max}/cm^{-1} 3400-2800 (broad O-H); δ_H 3.94 (3H, s, 4-OCH_3), 4.06 (3H, s, 8-OCH_3), 6.78 (2H, s, H-2 and H-3), 6.85 (1H, dd, *J* 7.6 and 1.0, H-7), 7.34 (1H, dd, *J* 8.4 and 7.6, H-6), 7.85 (1H, dd, *J* 8.4 and 1.0, H-5), 8.95 (1H, s, OH); δ_C 56.0 (4-OCH_3)[a], 56.1 (8-OCH_3)[a], 105.0 (C-3), 106.3 (C-7), 109.0 (C-2), 115.6 (C-4a), 116.0 (C-5), 125.1 (C-6), 127.9 (C-8a), 148.0 (C-1)[b], 148.1 (C-4)[b], 156.0 (C-8). (Found: HRMS (M^+) 204.0785. Calc. for $C_{12}H_{12}O_3$: 204.0786).

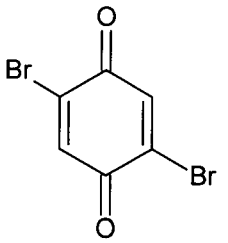
3-Bromo-5-methoxy-1,4-naphthoquinone (125)



125

Br_2 (1.25 g, 7.82 mmol, 0.40 ml) in AcOH (30 ml) was added to a stirring solution of naphthol **124** (1.59 g, 7.82 mmol) in AcOH (25 ml) at room temperature. After the addition of Br_2 stirring was continued for another 15 min before the reaction mixture was thrown into icy water (400 ml) and stirred for 30 min. The organic material was then extracted with DCM (3 x 60 ml) which was chromatographed using EtOAc: Hexane (3:7) as eluent to produce the bromonaphthol **125** (1.50 g, 72%) as yellow needles, m.p. 154-156 °C (from hexane) (Lit. [88] m.p. 154-155 °C). ν_{max}/cm^{-1} 1671 and 1693 (C=O); δ_H 4.02 (3H, s, 5-OCH_3), 7.32 (1H, dd, *J* 6.2 and 3.0, H-6), 7.45 (1H, s, H-2), 7.72 (2H, m, H-7 and H-8); δ_C 56.5 (5-OCH_3), 118.1 (C-6), 118.6 (C-4a), 119.5 (C-8), 133.9 (C-8a), 135.5 (C-7), 138.3 (C-2), 142.6 (C-3), 160.4 (C-5), 176.1 (C-4), 182.5 (C-1). (Found: HRMS (M^+ - 1) 265.9575. Calc. for $C_{11}H_6O_3Br$: 265.9578).

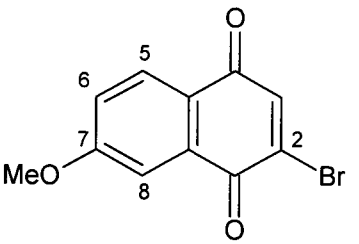
2,5-Dibromo-1,4-benzoquinone 127



127

To a solution of 1,4-dimethoxybenzene **129** (10.0 g, 72.5 mmol) in acetic acid (20 ml) was added drop-wise a solution of bromine (23.15 g, 7.44 ml, 145 mmol) in acetic acid (7 ml) at 24 °C. The reaction mixture was stirred for 2 h, then cooled down to 24 °C and filtered to afford 2,5-dibromo-1,4-dimethoxybenzene **130** (15.69 g). The filtrate was diluted with water (15 ml) and extracted with DCM, which was washed with a 10% aqueous solution of $NaHCO_3$, dried over $MgSO_4$ and evaporated, yielding an additional amount (2.86 g) of the product **130**. The combined fractions of **130** (18.55 g, 62.7 mmol) were dissolved in acetonitrile (150 ml) and heated to reflux temperature. A solution of cerium(IV) ammonium nitrate (75 g, 136.8 mmol) in 300 ml water was then added drop-wise to the boiling solution of the 2,5-dibromo-1,4-dimethoxybenzene **130** in acetonitrile. The reaction mixture was left to cool down to room temperature and stirred for an additional 30 min. The solid material was filtered and washed with water to afford benzoquinone **127** (15.21 g, 82%) as a yellow solid, m.p. 159-160 °C (from EtOAc/Hexane). (Lit. [89] m.p. 160-161 °C); δ_H 7.48 (2H, s, H-3 and H-6); δ_C 137.2 (C-2 and C-5)[a], 137.9 (C-3 and C-6)[a] and 177.0 (C-1 and C-4).

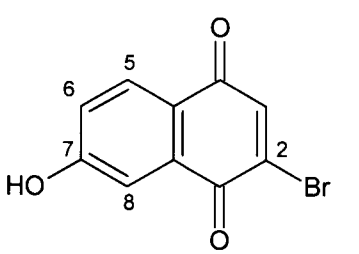
2-Bromo-7-methoxy-1,4-napthoquinone 128 and 2-bromo-7-hydroxy-1,4-naphthoquinone 131



128

To a stirred solution of 2,5-dibromobenzoquinone (6.38 g; 24 mmol) in dry benzene (60 ml) at 25 °C was dripped 1-methoxy-3-trimethylsilyloxybutadiene (5 g; 29.2 mmol) over a period of 30 min. The solution was then stirred at 50 °C until all quinone was consumed (TLC). The residue obtained after removal of solvent was chromatographed and eluted with EtOAc-hexane (3:7) as eluent to afford two products.

1. **2-Bromo-7-methoxy-1,4-naphthoquinone 128** (1.18 g, 18%) as a as yellow crystals (from ethyl alcohol) m.p. 134-135 °C. ν_{max}/cm^{-1} 1670 cm^{-1} (C=O); δ_H 3.96 (3H, s, 7-OCH_3), 7.23 (1H, dd, *J* 8.6 and 2.6 Hz, H-6), 7.26 (1H, s, H-3), 7.60 (1H, d, *J* 2.6 Hz, H-8), 8.12 (1H, d, *J* 8.6 Hz, H-5); δ_C 56.2 (OCH_3), 111.9 (C-8), 121.0 (C-6), 129.4 (C-4a), 130.8 (C-5), 133.0 (C-8a), 140.7 (C-2), 141.7 (C-3), 164.7 (C-7), 174.9 (C-1) and 176.1 (C-4). (Found: HRMS (M^+) 265.9571. Calc. for $C_{11}H_6O_3Br$: 265.9579).



131

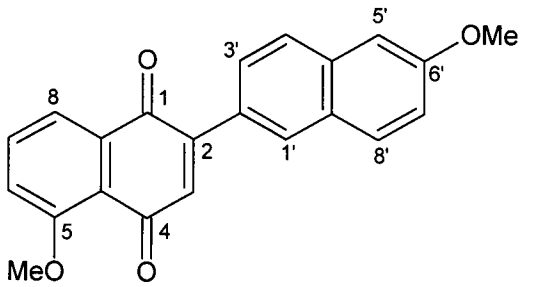
2. **2-Bromo-7-hydroxy-1,4-naphthoquinone 131** (3.06 g, 50%) as dark orange crystals (from EtOac-hexane). m.p. 233-235 °C. ν_{max}/cm^{-1} 3320 (OH) and 1690 cm^{-1} (C=O); δ_H (acetone-d_6) 7.16 (1H, s, H-3), 7.28 (1H, dd, *J* 8.8 and 2.4 Hz, H-6), 7.51 (1H, d, *J* 2.4 Hz, H-8), 8.03 (1H, d, *J* 8.8 Hz, H-5) and 10.0

(1H, bs, 7-OH); δ_C 114.6 (C-8), 122.2 (C-6), 124.3 (C-4a), 131.6 (C-5), 134.2 (C-8a), 142.1 (C-2), 143.7 (C-3), 163.9 (C-7), 175.4 (C-1) and 176.7 (C-4). Anal. calcd. for $C_{10}H_5BrO_3$: C47.5; H, 2.0%. Found: C, 47.8; H, 2.2%.

2-Bromo-7-methoxy-1,4-napthoquinone 128

The residue from an analogous condensation as described above and after removal of volatiles, was redissolved in dry benzene (100 ml) containing argentic oxide (10 g) and methyl iodide (13.63 g; 96 mmol) and stirred at 24 °C until methylation was complete (TLC). The mixture was filtered; the filter cake washed with benzene and the residue obtained was chromatographed using EtOAc:hexane (3:7) as eluent to give methoxyquinone **128** (5.13 g: 80%) identical in all respects to the product described earlier.

2-(6'-methoxynaphthalen-2'-yl)-5-methoxy-1,4-naphthoquinone 133

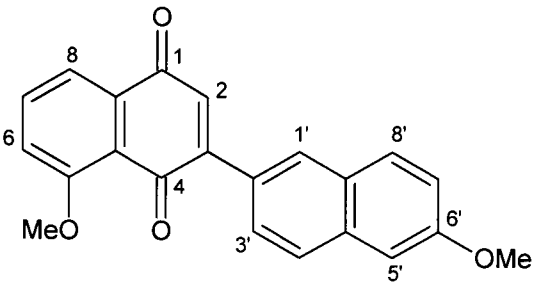


133

A mixture of **120** (0.33 g, 1.25 mmol) and $Pd(PPh_3)_4$ (0.15 g, 0.13 mmol) in benzene (20 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 6-methoxynaphthalene-2-boronic acid **132** (0.25 g, 1.25 mmol) in benzene (20 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **133** (0.34 g, 79%) as orange-brown crystals, m.p. 173-176 °C. ν_{max}/cm^{-1} 1676 and 1693 (C=O); δ_H 3.94 (3H, s, 6'-OCH_3), 4.04 (3H, s, 5-OCH_3), 7.10 (1H, s, H-3) 7.18 (2H, m, H-5' and H-7'), 7.34 (1H, dd, *J* 8.4 and 1.0, H-6), 7.62 (1H, dd, *J* 8.4 and 1.8, H-4'), 7.72 (1H, t, *J* 8.4, H-7), 7.80 (2H, bd, *J* 8.4, H-3' and H-8') 7.86, (1H, dd, *J* 7.8 and 1.0, H-8), 8.08 (1H, bs, H-1'); δ_C 55.4 (6'-OCH_3), 56.5 (5-OCH_3), 105.6 (C-5'), 117.7 (C-1'), 119.4 (C-6), 119.9 (C-7'), 120.0 (C-4a), 126.7 (C-3'), 126.9 (C-3), 128.2 (C-4a'), 128.5 (C-2'), 129.5 (C-4'), 130.3 (C-8), 134.8 (C-8'), 135.0 (C-8a'), 135.2 (C-8a), 136.9 (C-7), 145.6 (C-2), 158.8 (C-6'), 159.3 (C-5), 184.6 (C-4), 184.9 (C-1). (Found: HRMS (M^+ +1) 345.1125. Calc. for $C_{22}H_{17}O_4$: 345.1127).

3-(6'-Methoxynaphthalen-2'-yl)-5-methoxy-1,4-naphthoquinone 134 and

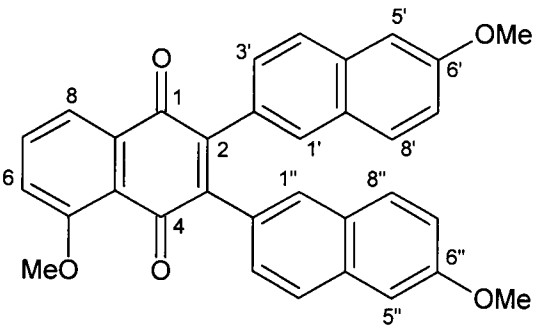
2,3-Di(6'-methoxynaphthalen-2'-yl)-5-methoxy-1,4-naphthoquinone 135



134

A mixture of **125** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 6-methoxynaphthalene-2-boronic acid **132** (0.20 g, 1.00 mmol) in benzene (15 ml) were added successively. The mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford two products

1. **3-(6'-Methoxynaphthalen-2'-yl)-5-methoxy-1,4-naphthoquinone 134** (0.10 g, 29%) as orange crystals (from EtOAc and Hexane), m.p. 159-162 °C. ν_{max}/cm^{-1} 1655 and 1663 (C=O); δ_H 3.95 (3H, s, 6'-OCH_3), 4.05 (3H, s, 5-OCH_3), 7.11 (1H, s, H-2), 7.16-7.21 (2H, m, H-5' and H-7'), 7.35 (1H, dd, *J* 8.0 and 1.0, H-6), 7.62 (1H, dd, *J* 8.6 and 1.8, H-4'), 7.71 (1H, t, *J* 8.0, H-7), 7.77-7.83 (2H, m, H-8' and H-8), 8.08 (1H, d, *J* 1.8, H-1'); δ_C 55.5 (6'-OCH_3), 56.7 (5-OCH_3), 105.8 (C-5'), 118.1 (C-1'), 118.8 (C-6), 119.5 (C-7'), 121.0 (C-4a), 126.8 (C-3'), 127.2 (C-2), 128.6 (C-2'), 129.1 (C-4a'), 130.0 (C-3), 130.5 (C-7), 132.5 (C-8), 134.6 (C-8a'), 135.0 (C-8'), 135.4 (C-8a), 149.9 (C-3), 159.0 (C-6'), 160.1 (C-5'), 184.3 (C-1), 185.3 (C-4). (Found: HRMS ($M^+ + 1$) 345.1122. Calc. for $C_{22}H_{17}O_4$: 345.1127).

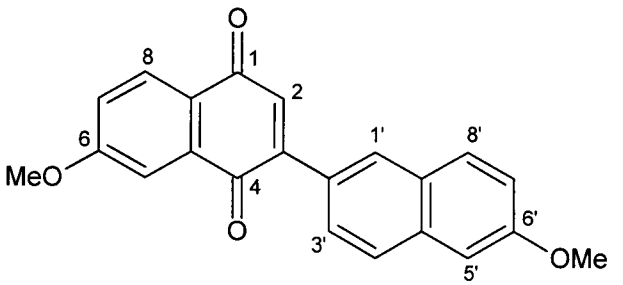


135

2. **2,3-Di(6'-methoxynaphthalen-2'-yl)-6-methoxy-1,4-naphthoquinone 135** (0.21 g, 42%) as a yellow solid, m.p. 233-236 °C. ν_{max}/cm^{-1} 1658 and 1668 (C=O); δ_H 3.86 (6H, s, 6'-OCH_3 and 6''-OCH_3), 4.03 (3H, s, 5-OCH_3), 6.99-7.12 (6H, m, H-3', H-3'', H-4', H-4'', H-5'' and H-5''), 7.36 (1H, dd, *J* 8.0 and 1.4, H-6), 7.43-7.48 (2H, m, H-7' and H-7''), 7.55-7.67 (4H, m, H-1', H-1'', H-8' and H-8''), 7.74 (1H, t, *J* 8.0, H-7), 7.88 (1H, dd, *J* 7.6 and 1.4, H-8); δ_C 55.4 (6'-OCH_3 and 6''-OCH_3), 56.6 (6-OCH_3), 105.7 (C-5' and C-5'')[a], 113.1 (C-1' and C-1'')[a], 118.0 (C-6)[b], 120.6 (C-7' and C-7'')[a], 122.6 (C-4a), 124.4 (C-3' and C-3'')[a], 124.6 (C-4' and C-4'')[a], 126.1 (C-8)[b], 127.1 (C-4a' and C-4a''), 130.5 (C-8' and C-8'')[a], 132.9 (C-2')[c], 134.9 (C-2'')[c], 135.0 (C-7)[b], 137.0 (C-8a), 137.1 (C-2)[c], 137.9 (C-3)[c], 138.0 (C-8a' and C-8a''), 158.4 (C-6' and C-6''), 161.3 (C-5'), 184.4 (C-1)[e], 185.1 (C-4)[e]. (Found: HRMS (M^+ + 1) 501.1681. Calc. for $C_{33}H_{25}O_5$: 501.1702).

3-(6'-Methoxynaphthalen-2'-yl)-6-methoxy-1,4-naphthoquinone 136 and

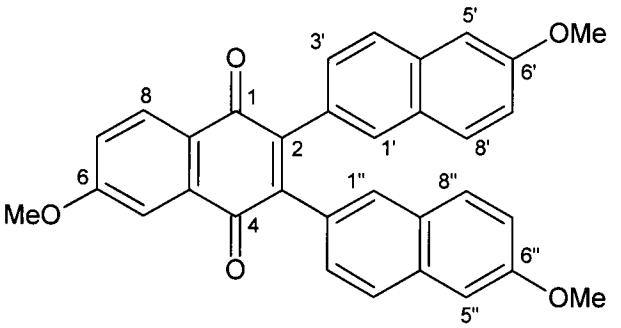
2,3-Di(6'-methoxynaphthalen-2'-yl)-6-methoxy-1,4-naphthoquinone 137



136

A mixture of **128** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 6-methoxynaphthalene-2-boronic acid **132** (0.20 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford two products

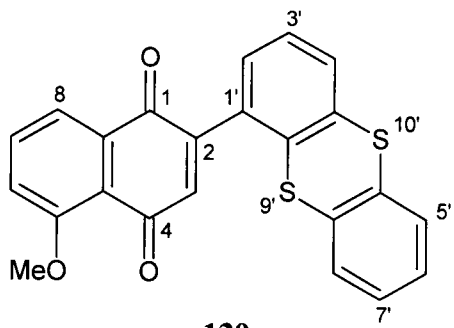
1. **3-(6'-Methoxynaphthalen-2'-yl)-6-methoxy-1,4-naphthoquinone 136** (0.21 g, 61%) as a yellow solid, m.p. 159-162 °C. ν_{max}/cm^{-1} 1655 and 1663 (C=O); δ_H 3.95 (3H, s, 6'-OCH_3), 3.98 (3H, s, 6-OCH_3), 7.12 (1H, s, H-2), 7.17-7.22 (2H, m, H-5' and H-7'), (1H, dd, *J* 8.4 and 2.6, H-7), 7.62 (1H, dd, *J* 8.4 and 1.4, H-3'), 7.64 (1H, d, *J* 2.6, H-5), 7.81 (2H, d, *J* 8.4, H-4' and H-8'), 8.06 (1H, d, *J* 1.4, H-1'), 8.08 (1H, d, *J* 8.4, H-8); δ_C 55.4 (6'-OCH_3), 55.9 (6-OCH_3), 105.6 (C-7')[a], 110.5 (C-2)[b], 119.5 (C-8')[a], 120.3 (C-7)[b], 125.9 (C-2')[c], 126.8 (C-3')[a], 126.9 (C-5')[a], 128.4 (C-5)[b], 128.5 (C-8)[b], 128.6 (C-3)[c], 129.6 (C-1')[a], 130.4 (C-4')[a], 135.1 (C-4a')[d], 135.3 (C-8a')[d], 147.5 (C-4a)[e], 147.7 (C-8a)[e], 158.9 (C-6), 164.2 (C-6'), 184.3 (C-1)[f], 184.9 (C-4)[f]. (Found: HRMS (M^+ + 1) 345.1121. Calc. for $C_{22}H_{17}O_4$: 345.1127).



137

2. **2,3-Di(6'-methoxynaphthalen-2'-yl)-6-methoxy-1,4-naphthoquinone 137** (0.14 g, 28%) as bright orange crystals, m.p. 178-181 °C. ν_{max}/cm^{-1} 1658 and 1668 (C=O); δ_H 3.87 (6H, s, 6'-OCH_3 and 6''-OCH_3), 3.98 (3H, s, 6-OCH_3), 7.00-7.19 (6H, m, H-3', H-3'', H-4', H-4'', H-5' and H-5''), 7.28 (1H, dd, *J* 8.8 and 2.6, H-7), 7.47 (2H, d, *J* 8.2, H-7' and H-7''), 7.56-7.62 (4H, m, H-1', H-1'', H-8' and H-8''), 7.65 (1H, d, *J* 2.6, H-5), 8.18 (1H, d, *J* 8.8, H-8); δ_C 55.4 (6'-OCH_3 and 6''-OCH_3), 56.1 (6-OCH_3), 105.7 (C-7' and C-7'')[a], 109.8 (C-7)[b], 118.9 (C-8' and C-8'')[a], 120.7 (C-5)[b], 126.0 (C-2')[a], 126.1 (C-2'')[a], 128.2 (C-2)[b], 128.3 (C-3)[b], 128.7 (C-3', C-3'', C-5' and C-5'')[a], 129.3 (C-8)[b], 130.0 (C-1')[a], 130.1 (C-1'')[a], 130.8 (C-4')[a], 130.9 (C-4'')[a], 134.2 (C-4a' and C-4a'')[c], 134.4 (C-8a' and C-8a'')[c], 145.3 (C-4a)[d], 145.7 (C-8a)[d], 158.4 (C-6' and C-6''), 164.3 (C-6), 184.2 (C-1)[e], 185.2 (C-4)[e]. (Found: HRMS (M^+ + 1) 501.1685. Calc. for $C_{33}H_{25}O_5$: 501.1702).

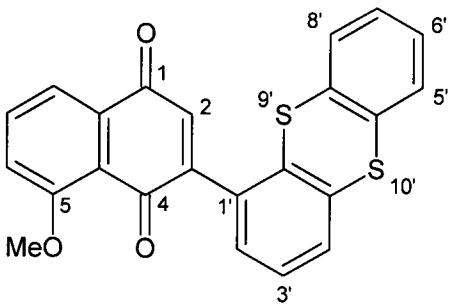
5-Methoxy-2-(thianthren-1'-yl)-1,4-naphthoquinone 139



139

A mixture of **120** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and thianthren-1-boronic acid **138** (0.26 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **139** (0.38 g, 94%) as a yellow powder, m.p. 214-217 °C. ν_{max}/cm^{-1} 1660 and 1671 (C=O); δ_H 4.07 (3H, s, 5-OCH_3), 6.87 (1H, s, H-3) 7.19-7.41 (6H, m, H-6, H-2', H-3', H-4', H-6, H-7), 7.51 (1H, dd, *J* 7.6 and 1.8, H-5'), 7.60 (1H, dd, *J* 7.6 and 1.8, H-8'), 7.75 (1H, t, *J* 8.4, H-7), 7.87 (1H, dd, *J* 8.4 and 1.8, H-8); δ_C 56.7 (5-OCH_3), 118.0 (C-6)[a], 120.1 (C-7)[a], 120.2 (C-4a)[b], 127.5 (C-6')[c], 127.8 (C-4')[d], 128.0 (C-7')[c], 128.7 (C-5')[d], 128.8 (C-8')[d], 129.1 (C-3')[c], 130.0 (C-2')[c], 134.5 (C-4a')[e], 134.6 (C-10a')[e], 135.2 (C-8)[a], 135.4 (C-1')[e], 135.9 (C-8a)[b], 136.3 (C-8a')[e], 137.1 (C-9a')[e], 139.0 (C-3)[a], 146.7 (C-2), 159.7 (C-5), 183.7 (C-4)[f], 184.3 (C-1)[f]. (Found: HRMS (M^+ + 1) 403.0446. Calc. for $C_{23}H_{15}O_3S_2$: 403.0463).

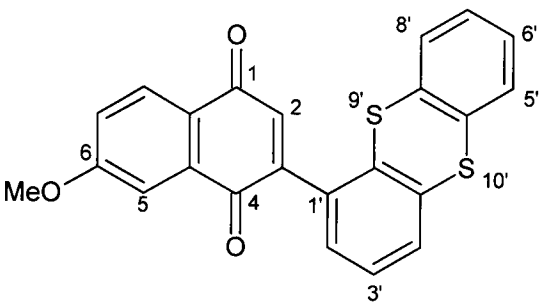
5-Methoxy-3-(thianthren-1'-yl)-1,4-naphthoquinone 140



140

A mixture of **125** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and thianthren-1-boronic acid **138** (0.26 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **140** (0.35 g, 86%) as a yellow powder, m.p. 195-198 °C. ν_{max}/cm^{-1} 1670 and 1688 (C=O); δ_H 4.02 (3H, s, 5-OCH_3), 6.88 (1H, s, H-2) 7.19-7.39 (6H, m, H-6, H-2', H-3', H-4', H-6', and H-7'), 7.50 (1H, dd, *J* 7.6 and 1.8, H-5'), 7.59 (1H, dd, *J* 7.6 and 1.8, H-8'), 7.74 (1H, t, *J* 8.0, H-7), 7.82 (1H, dd, *J* 8.0 and 1.6, H-8); δ_C 56.5 (5-OCH_3), 118.3 (C-6)[a], 118.9 (C-7)[a], 120.5 (C-4a)[b], 127.2 (C-6')[c], 127.7 (C-4')[d], 127.8 (C-8a)[b], 127.9 (C-7')[c], 128.6 (C-5')[d], 128.7 (C-8')[d], 129.0 (C-3')[c], 129.7 (C-2')[c], 134.4 (C-4a')[e], 134.5 (C-2)[a], 134.9 (C-8)[a], 135.2 (C-1'), 135.7 (C-8a')[e], 136.3 (C-9a')[e], 136.8 (C-10a')[e], 150.7 (C-3), 160.1 (C-5), 182.6 (C-4)[f], 185.1 (C-1)[f]. (Found: HRMS (M^+ + 1) 403.0470. Calc. for $C_{23}H_{15}O_3S_2$: 403.0463).

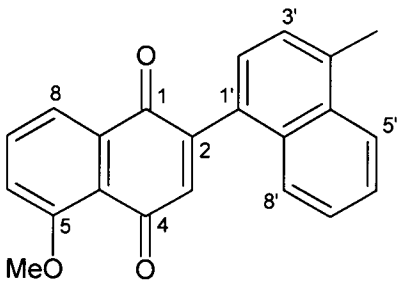
6-Methoxy-3-(thianthren-1'-yl)-1,4-naphthoquinone 141



141

A mixture of **128** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and thianthren-1-boronic acid **138** (0.26 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **141** (0.32 g, 79%) as a yellow powder, m.p. 184-187 °C. ν_{max}/cm^{-1} 1670 and 1688 (C=O); δ_H 3.99 (3H, s, 6-OCH_3), 6.90 (1H, s, H-2), 7.19-7.33 (6H, m, H-6, H-2', H-3', H-4', H-6', and H-7'), 7.50 (1H, dd, *J* 7.6 and 1.4, H-5'), 7.61 (1H, dd, *J* 7.6 and 1.4, H-8'), 7.64 (1H, d, *J* 2.6, H-5), 8.12 (1H, d, *J* 8.4, H-8); δ_C 56.0 (6-OCH_3), 110.6 (C-2)[a], 120.5 (C-7)[a], 127.4 (C-6')[b], 127.8 (C-4')[b], 128.0 (C-7')[b], 128.6 (C-5')[b], 128.8 (C-8')[b], 129.0 (C-3')[b], 129.9 (C-2')[b], 130.3 (C-1')[b], 134.3 (C-4a)[c], 134.7 (C-4a')[d], 134.8 (C-10a')[d], 135.0 (C-5)[a], 135.1 (C-8a')[d], 135.2 (C-9a')[d], 136.2 (C-8a)[c], 137.2 (C-8)[a], 148.6 (C-3), 164.4 (C-6), 183.5 (C-4)[e], 184.0 (C-1)[e]. (Found: HRMS (M^+ + 1) 403.0489. Calc. for $C_{23}H_{15}O_3S_2$: 403.0463).

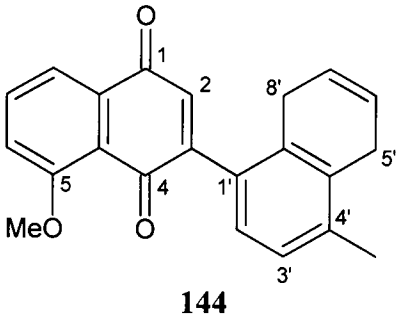
2-(4'-methylnaphthalen-1'-yl)-5-methoxy-1,4-naphthoquinone 143



143

A mixture of **120** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 4-methylnaphthalene-1-boronic acid **142** (0.19 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **143** (0.27 g, 81%) as an orange powder, m.p. 203-204 °C. ν_{max}/cm^{-1} 1676 and 1693 (C=O); δ_H 2.75 (3H, s, 4'-CH_3), 4.07 (3H, s, 5-OCH_3), 7.02 (1H, s, H-3) 7.30-7.58 (5H, m, H-6, H-2', H-3', H-6', and H-7'), 7.65 (1H, dd, *J* 7.8 and 1.4, H-5'), 7.70 (1H, t, *J* 8.0, H-7), 7.81 (1H, dd, *J* 8.0 and 1.6, H-8), 8.06 (1H, dd, *J* 7.8 and 1.4, H-8'); δ_C 19.7 (CH_3), 56.6 (5-OCH_3), 117.9 (C-6)[a], 120.0 (C-7)[a], 121.0 (C-8a)[b], 124.6 (C-3')[c], 125.8 (C-5')[c], 125.9 (C-8')[c], 126.0 (C-6')[c], 126.1 (C-7')[c], 127.0 (C-2')[c], 129.9 (C-4')[d], 131.4 (C-4a')[b], 132.6 (C-8a')[b], 134.7 (C-4a)[b], 135.0 (C-8)[a], 136.4 (C-1')[d], 139.9 (C-3)[a], 147.4 (C-2), 159.6 (C-5), 184.5 (C-1)[e], 184.6 (C-4)[e]. (Found: HRMS (M^+ + 1) 329.1180. Calc. for $C_{22}H_{17}O_3$: 329.1178).

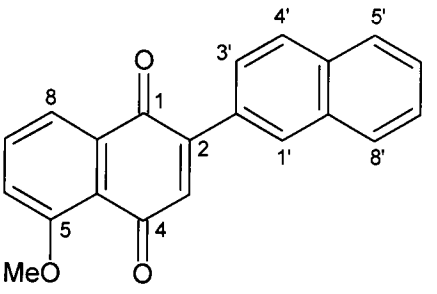
3-(4'-methylnaphthalen-1'-yl)-5-methoxy-1,4-naphthoquinone 144



144

A mixture of **125** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 4-methylnaphthalene-1-boronic acid **142** (0.19 g, 1.00 mmol) in benzene (15 ml) were added successively. The mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml). The residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **144** (0.29 g, 88%) as an orange solid, m.p. 157-160 °C. ν_{max}/cm^{-1} 1673 and 1695 (C=O); δ_H 2.75 (3H, s, 4'-CH_3), 3.97 (3H, s, 5-OCH_3), 7.02 (1H, s, H-2), 7.32-7.57 (5H, m, H-6, H-2', H-3', H-6', and H-7'), 7.68 (1H, dd, *J* 7.8 and 1.4, H-5'), 7.74 (1H, t, *J* 7.6, H-7), 7.84 (1H, dd, *J* 7.6 and 1.4, H-8), 8.05 (1H, dd, *J* 7.8 and 1.4, H-8'); δ_C 19.6 (CH_3), 56.5 (5-OCH_3), 118.1 (C-6)[a], 118.9 (C-7)[a], 120.4 (C-4a)[b], 124.5 (C-3')[c], 125.8 (C-6')[c], 125.9 (C-7')[c], 126.1 (C-5')[c], 126.1 (C-2')[c], 127.0 (C-8')[c], 130.7 (C-4'), 131.6 (C-4a')[b], 132.6 (C-8a')[b], 134.5 (C-8a)[b], 134.9 (C-2)[a], 135.6 (C-8)[a], 136.2 (C-1')[a], 151.6 (C-3), 160.1 (C-5), 183.6 (C-4)[d], 185.3 (C-1)[d]. (Found: HRMS (M^+ + 1) 329.1187. Calc. for $C_{22}H_{17}O_3$: 329.1178).

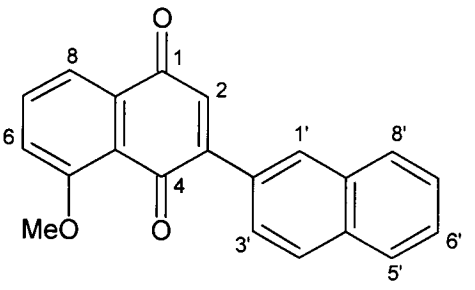
2-(2'-naphthyl)-5-methoxy-1,4-naphthoquinone 146



146

A mixture of **120** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and naphthalene-2-boronic acid **145** (0.17 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **146** (0.29 g, 92%) as a orange powder, m.p. 165-168 °C. ν_{max}/cm^{-1} 1672 and 1691 (C=O); δ_H 4.05 (3H, s, 5-OCH_3), 7.13 (1H, s, H-3), 7.35 (1H, dd, *J* 8.0 and 1.5, H-6), 7.54 (2H, m, H-6', H-7'), 7.65 (1H, dd, *J* 8.8 and 1.6, H-3'), 7.74 (1H, t, *J* 8.0, H-7), 7.86-7.90 (4H, m, H-4', H-5', H-8' and H-8), 8.14 (1H, d, *J* 1.4, H-1'); δ_C 56.6 (5-OCH_3), 117.8 (C-6)[a], 119.9 (C-7)[a], 120.0 (C-8a)[b], 126.2 (C-6')[c], 126.5 (C-5')[d], 127.2 (C-8')[d], 127.7 (C-7')[c], 128.0 (C-4')[d], 128.7 (C-1')[d], 129.6 (C-3')[c], 130.5 (C-4a')[b], 133.0 (C-8a')[b], 133.8 (C-4a)[b], 134.9 (C-8)[a], 135.0 (C-2')[a], 137.5 (C-3)[a], 145.7 (C-2), 159.4 (C-5), 184.5 (C-4)[d], 184.8 (C-1)[d]. (Found: HRMS ($M^+ + 1$) 315.1006. Calc. for $C_{21}H_{15}O_3$: 315.1021).

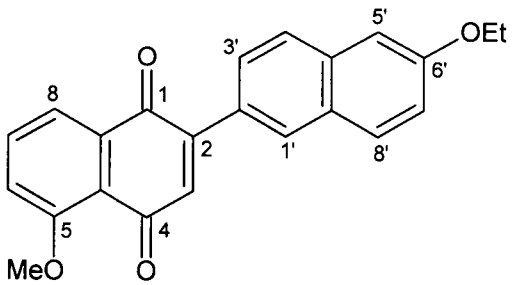
3-(2'-naphthyl)-5-methoxy-1,4-naphthoquinone 147



147

A mixture of **125** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and naphthalene-2-boronic acid **145** (0.17 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **147** (0.27 g, 86%) as a yellow powder, m.p. 226-229 °C. ν_{max}/cm^{-1} 1672 and 1691 (C=O); δ_H 4.03 (3H, s, 5-OCH_3), 7.17 (1H, s, H-2), 7.35-7.41 (2H, m, H-3' and H-6), 7.58 (2H, m, H-6', H-7'), 7.67-7.71 (4H, m, H-1', H-4', H-5', and H-8'), 7.75 (1H, t, *J* 7.6, H-7), 7.89 (1H, dd, *J* 7.6 and 1.4, H-8); δ_C 56.7 (5-OCH_3), 110.5 (C-1'), 118.3 (C-6), 121.7 (C-8), 123.4 (C-3'), 124.0 (C-4a), 123.4 (C-2), 124.5 (C-6')[d], 127.4 (C-4a'), 129.0 (C-7'), 130.0 (C-8'), 130.8 (C-5'), 131.6 (C-4'), 133.3 (C-2'), 134.8 (C-8a), 134.9 (C-7), 138.9 (C-8a'), 143.5 (C-3), 160.1 (C-5), 174.4 (C-1), 180.0 (C-4). (Found: HRMS (M^+ - CH_3) 299.0705. Calc. for $C_{20}H_{11}O_3$: 299.0708).

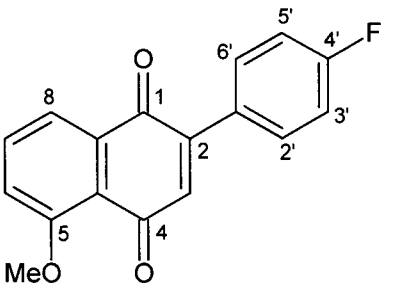
2-(6'-ethoxynaphthalen-2'-yl)-5-methoxy-1,4-naphthoquinone 149



149

A mixture of **120** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 6-ethylnaphthalene-2-boronic acid **148** (0.22 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hex (3:7) as eluent, to afford **149** (0.29 g, 81%) as a dark red powder, m.p. 144-147 °C. ν_{max}/cm^{-1} 1679 and 1694 (C=O); δ_H 1.50 (3H, t, *J* 7.0, $-OCH_2CH_3$, 4.05 (3H, s, 5-OCH_3), 4.18 (2H, q, *J* 7.0, $-OCH_2CH_3$,) 7.10 (1H, s, H-3), 7.18 (2H, m, H-5' and H-7'), 7.33 (1H, d, *J* 8.4, H-6), 7.61 (1H, dd, *J* 8.8 and 1.8, H-3'), 7.72 (1H, t, *J* 7.8, H-7), 7.78 (1H, d, *J* 8.4, H-8'), 7.80 (1H, d, *J* 8.8, H-4'), 7.86 (1H, d, *J* 7.8, H-8), 8.07 (1H, bs, H-1'); δ_C 14.8 ($-OCH_2CH_3$), 56.5 (5-OCH_3), 63.8 ($-OCH_2CH_3$), 106.4 (C-7')[a], 117.7 (C-6)[b], 119.7 (C-5')[a], 119.9 (C-7)[b], 120.1 (C-8a)[c], 126.6 (C-3')[b], 126.9 (C-8')[b], 128.1 (C-4a')[d], 128.5 (C-8a')[d], 129.5 (C-4')[a], 130.3 (C-1')[a], 134.8 (C-8)[b], 135.1 (C-4a)[c], 135.3 (C-2')[e], 136.8 (C-3)[b], 145.7 (C-2)[e], 158.2 (C-6')[f], 159.4 (C-5)[f], 184.6 (C-1)[g], 185.0 (C-4)[g] (Found: HRMS (M^+ + 1) 359.1267. Calc. for $C_{23}H_{19}O_4$: 359.1283).

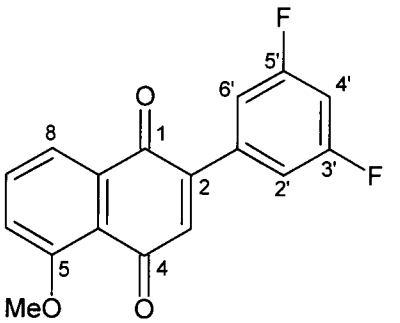
2-(4'-fluorobenzen-1'-yl)-5-methoxy-1,4-naphthoquinone 151



151

A mixture of **120** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 4-fluorophenyl-1-boronic acid **150** (0.14 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hexane (3:7) as eluent, to afford **151** (0.23 g, 81%) as orange needles (from EtOAc and Hexane), m.p. 183-186 °C. ν_{max}/cm^{-1} 1663 and 1670 (C=O); δ_H 4.04 (3H, s, 5-OCH_3), 6.97 (1H, s, H-3), 7.13 (1H, d, *J* 8.6, H-2'), 7.17 (1H, d, *J* 8.6, H-6'), 7.34 (1H, dd, *J* 8.4 and 1.2, H-6), 7.57 (1H, d, *J* 8.6, H-3'), 7.59 (1H, d, *J* 8.6, H-5'), 7.71 (1H, t, *J* 8.4 and 7.6, H-7), 7.83 (1H, dd, *J* 7.6 and 1.2, H-8); δ_C 56.6 (5-OCH_3), 115.4 (C-2')[a], 115.8 (C-6')[a] 117.9 (C-6)[b], 119.9 (C-7)[b], 129.0 (C-4a)[c], 129.1 (C-8a)[c], 131.2 (C-3')[a], 131.4 (C-5')[a], 134.8 (C-1')[a], 134.9 (C-8)[b], 137.1 (C-3)[b], 144.6 (C-2), 159.4 (C-5), 163.8 (d, *J* 249.1, C-4'), 184.3 (C-1)[d], 185.0 (C-4)[d]. (Found: HRMS (M^+ + 1) 283.0761. Calc. for $C_{17}H_{12}O_3F$: 283.0770).

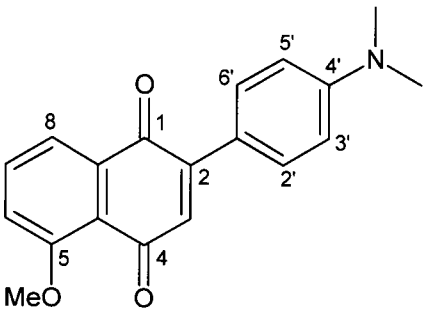
2-(3',5'-difluorobenzen-1'-yl)-5-methoxy-1,4-naphthoquinone 153



153

A mixture of **120** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 3,5-difluorophenyl-1-boronic acid **152** (0.16 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hexane (3:7) as eluent, to afford **153** (0.23 g, 78%) as yellow needles (from EtOAc and Hexane), m.p. 221-224 °C. ν_{max}/cm^{-1} 1667 and 1676 (C=O); δ_H 4.04 (3H, s, 5-OCH_3), 6.91 (1H, tt, *J* 8.8 and 2.2, H-4'), 6.99 (1H, s, H-3) 7.13 (2H, m, H-2' and H-6'), 7.35 (1H, dd, *J* 8.4 and 1.2, H-6), 7.74 (1H, t, *J* 8.4, H-7), 7.84 (1H, dd, *J* 7.6 and 1.2, H-8); δ_C 56.6 (5-OCH_3), 105.1 (t, *J* 25.1, C-4'), 112.2 (C-6'), 112.7 (C-2'), 118.0 (C-6), 119.9 (C-3), 120.0 (C-4a), 134.5 (C-2), 135.2 (C-8 and C-8a), 138.1 (C-7), 143.5 (C-1'), 159.6 (C-5), 162.9 (d, *J* 247.6, C-3' and C-5'), 183.7 (C-4), 183.8 (C-1). (Found: HRMS ($M^+ + 1$) 301.0685. Calc. for $C_{17}H_{11}O_3F_2$: 301.0676).

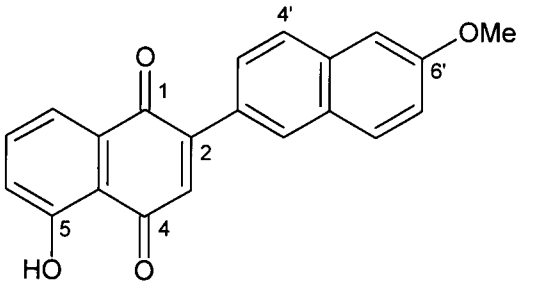
2-[(4'-dimethylamino)benzen-1'-yl]-5-methoxy-1,4-naphthoquinone 155



155

A mixture of **120** (0.27 g, 1.00 mmol) and $Pd(PPh_3)_4$ (115 mg, 0.10 mmol) in benzene (10 ml) was stirred for 30 min at room temperature, under nitrogen. An aqueous solution of Na_2CO_3 (2 M, 1.0 ml) and 4-(dimethylamino)phenyl-1-boronic acid **154** (0.17 g, 1.00 mmol) in benzene (15 ml) were added successively and the mixture was heated under reflux for 16 h with vigorous stirring, allowed to cool to room temperature and then quenched with water (50 ml). The resulting mixture was extracted with DCM (3 x 60 ml) and the residue obtained upon work-up was chromatographed using EtOAc:Hexane (3:7) as eluent, to afford **155** (0.24 g, 78%) as purple needles (from EtOAc and Hexane), m.p. 189-192 °C. ν_{max}/cm^{-1} 1660 and 1671 (C=O); δ_H 3.04 (6H, s, 2 x CH_3), 4.02 (3H, s, 5-OCH_3), 6.75 (2H, d, *J* 9.0, H-3' and H-5'), 6.94 (1H, s, H-3), 7.30 (1H, dd, *J* 8.4 and 1.0, H-6), 7.59 (2H, d, *J* 9.0, H-2' and H-6'), 7.67 (1H, t, *J* 7.6, H-7), 7.82 (1H, dd, *J* 7.6 and 1.0, H-8); δ_C 40.1 (2 x CH_3) 56.5 (5-OCH_3), 111.8 (C-2' and C-6')[a], 117.5 (C-6)[b], 119.8 (C-7)[b], 120.2 (C-8a)[c], 130.6 (C-3' and C-5')[a], 133.5 (C-8)[b], 134.4 (C-3)[b], 134.4 (C-1'), 135.4 (C-4a)[c], 145.1 (C-2), 151.6 (C-4'), 159.2 (C-5), 184.8 (C-1)[d], 185.6 (C-4)[d]. (Found: HRMS ($M^+ + 1$) 308.1286. Calc. for $C_{19}H_{18}NO_3$: 308.1287).

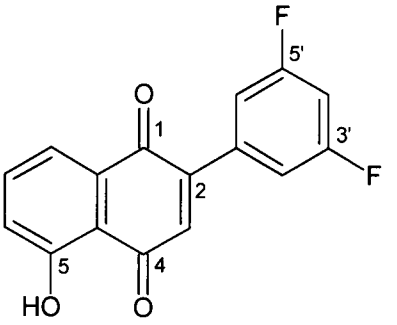
2-(6'-Methoxynaphthalen-2'-yl)-5-hydroxy-1,4-naphthoquinone 156



156

To a solution of **133** (0.17 g, 0.50 mmol) in DCM (20 ml) at room temperature was added $AlCl_3$ (2.61 g, 19.6 mmol). The mixture was stirred at room temperature for 24 h, poured into water, acidified with dilute HCl (0.1 M, 100 ml), and extracted with DCM (3 x 60 ml). The residue obtained upon work-up was chromatographed using EtOAc:Hexane (3:7) to afford binaphthol **156** (0.10 g, 60%) as orange-brown crystals (from EtOAc and Hexane), m.p. 211-214 °C. ν_{max}/cm^{-1} 3300-2700 (broad, O-H), 1668 and 1687 (C=O); δ_H 3.96 (3H, s, 6'-OCH_3), 7.14 (1H, s, H-3), 7.20 (2H, m, H-5' and H-7'), 7.30 (1H, dd, *J* 8.0 and 1.0, H-6), 7.62 (1H, dd, *J* 8.4 and 1.8, H-3'), 7.66 (1H, t, *J* 8.0, H-7), 7.74 (1H, dd, *J* 8.0 and 1.0, H-8), 7.81 (2H, d, *J* 8.4, H-4' and H-8'), 8.09 (1H, bs, H-1'), 12.09 (1H, s, 5-OH); δ_C 55.5 (6'-OCH_3), 105.6 (C-7')[a], 115.4 (C-6)[b], 119.7 (C-8')[a], 119.9 (C-7)[b], 124.3 (C-2'), 126.8 (C-3')[a], 127.1 (C-5')[a], 128.3 (C-4a')[c], 128.6 (C-8a')[c], 130.1 (C-1')[a], 130.6 (C-4')[a], 132.7 (C-4a)[d], 134.5 (C-8)[b], 135.7 (C-8a)[d], 136.4 (C-3)[b], 149.2 (C-2), 159.2 (C-6')[e], 161.2 (C-5)[e], 184.2 (C-1)[f], 190.3 (C-4)[f]. (Found: HRMS (M^+) 330.0894. Calc. for $C_{21}H_{14}O_4$: 330.0892).

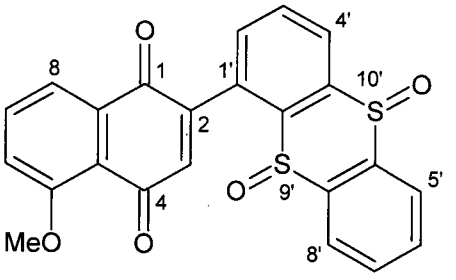
2-(3',5'-Difluorobenzen-1'-yl)-5-hydroxy-1,4-naphthoquinone 157



157

To a solution of **153** (0.17 g, 0.50 mmol) in DCM (20 ml) at room temperature was added $AlCl_3$ (2.61 g, 19.6 mmol). The mixture was stirred at room temperature for 24 h, poured into water, acidified with dilute HCl (0.1 M, 100 ml), and extracted with DCM (3 x 60 ml). The residue obtained upon work-up was chromatographed using EtOAc:Hexane (3:7) to afford binaphthol **157** (0.08 g, 56%) as yellow needles (from EtOAc and Hexane), m.p. 219-222 °C. ν_{max}/cm^{-1} 3430 (broad, O-H), 1668 and 1687 (C=O); δ_H 6.95 (1H, tt, *J* 8.8 and 2.6, H-4'), 7.04 (1H, s, H-3), 7.13 (2H, m, H-2' and H-5'), 7.32 (1H, dd, *J* 7.2 and 1.0, H-6), 7.70 (2H, m, H-7 and H-8), 11.92 (1H, s, 5-OH); δ_C 105.7 (t, *J* 22.4, C-4'), 112.4 (C-6'), 113.0 (C-2'), 119.9 (C-6), 120.1 (C-3), 124.7 (C-8), 125.1 (C-4a), 128.3 (C-4a'), 132.3 (C-8a'), 136.1 (C-2), 136.8 (C-7), 137.3 (C-1'), 161.4 (C-5), 163.7 (d, *J* 178.0, C-3' and C-5'), 189.7 (C-1), 191.4 (C-4). (Found: HRMS (M^+) 330.0894. Calc. for $C_{21}H_{14}O_4$: 330.0892).

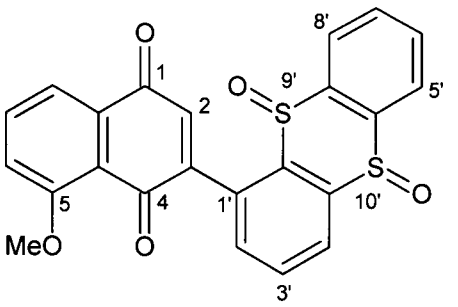
5-Methoxy-2-(9', 10'-dioxythianthren-1'-yl)-1,4-naphthoquinone 158



158

A mixture of 5-methoxy-2-(thianthren-1'-yl)-1,4-naphthoquinone **139** (0.20 g, 0.50 mmol) and m-chloroperbenzoic acid (2 mol equiv., 0.17 g) in DCM (20 ml) was stirred for 2 h at 25 °C. The reaction mixture was then washed with aqueous sodium hydrogen carbonate and the organic material extracted with DCM (3 x 60 ml). The residue obtained upon workup was chromatographed using EtOAc:Hexane (3:7) to afford sulphone **158** (0.15 g, 68%) as yellow needles, m.p. 141-144 °C (from EtOAc:Hexane). ν_{max}/cm^{-1} 1662 (C=O), 1672 (C=O), 1326 (S=O) and 1164 (S=O); δ_H 4.08 (3H, s, 5-OCH_3), 6.98 (1H, s, H-3), 7.41 (1H, dd, *J* 8.0 and 1.4, H-6), 7.48-7.68 (4H, H-2', H-3', H-6' and H-7'), 7.77 (1H, t, *J* 8.0, H-7), 7.85 (1H, dd, *J* 8.0 and 1.4, H-8), 8.19 (2H, m, H-5' and H-8'), 8.31 (1H, dd, *J* 7.6 and 1.4, H-4'); δ_C 56.7 (5-OCH_3), 118.4 (C-6), 120.1 (C-8), 121.7 (C-4a), 125.6 (C-5')[a], 126.5 (C-3)[a], 127.4 (C-2')[a], 128.2 (C-4')[a], 129.1 (C-1' and C-7')[a], 132.2 (C-6')[a], 133.3 (C-3')[c], 133.4 (C-8')[a], 135.5 (C-7)[a], 140.7 (C-9a' and C8a), 144.2 (C-4'), 147.5 (C-8'), 147.8 (C-10a'), 149.5 (C-2), 159.9 (C-5), 183.2 (C-4), 183.6 (C-1). (Found: HRMS (M^+ + 1) 435.0355. Calc. for $C_{23}H_{15}O_5S_2$: 435.0361).

5-Methoxy-3-(9', 10'-dioxythianthren-1'-yl)-1,4-naphthoquinone 159



159

A mixture of 5-methoxy-3-(thianthren-1'-yl)-1,4-naphthoquinone **140** (0.20 g, 0.50 mmol) and m-chloroperbenzoic acid (2 mol equiv., 0.17 g) in DCM (20 ml) was stirred for 2 h at 25 °C. The reaction mixture was then washed with aqueous sodium hydrogen carbonate and the organic material extracted with DCM (3 x 60 ml). The residue obtained upon workup was chromatographed using EtOAc:Hexane (3:7) to afford sulphone **159** (0.13 g, 60%) as orange needles, m.p. 134-135 °C (from EtOAc:Hexane). ν_{max}/cm^{-1} 1669 (C=O), 1678 (C=O), 1322 (S=O) and 1163 (S=O); δ_H 3.99 (3H, s, 5-OCH_3), 7.00 (1H, s, H-2), 7.40 (1H, dd, *J* 8.0 and 1.4, H-6), 7.45-7.68 (4H, H-2', H-3', H-6' and H-7'), 7.71 (1H, t, *J* 8.0, H-7), 7.83 (1H, dd, *J* 8.0 and 1.4, H-8), 8.11 (1H, dd, *J* 7.0 and 2.0, H-5'), 8.25 (1H, *J* 7.0 and 2.0, H-8'), 8.37 (1H, dd, *J* 7.6 and 1.4, H-4'); δ_C 56.6 (5-OCH_3), 118.5 (C-7)[a], 119.4 (C-8)[a], 125.9 (C-4a), 126.6 (C-2), 128.5 (C-5')[b], 128.7 (C-2')[b], 131.6 (C-1'), 132.0 (C-4' and C-7')[c], 132.1 (C-3' and C-6')[c], 132.2 (C-8')[c], 132.7 (C-8a), 133.9 (C-7), 134.7 (C-9a')[d], 135.5 (C-4a')[d], 136.1 (C-8a')[d], 138.7 (C-10a')[d], 140.1 (C-3), 160.3 (C-5), 184.1 (C-4)[e], 184.2 (C-1)[e]. (Found: HRMS (M^+ + 1) 435.0365. Calc. for $C_{23}H_{15}O_5S_2$: 435.0361).

CHAPTER 7

EVALUATION OF THE QUINOIDAL COMPOUNDS AS ANTI-MYCOBACTERIAL AGENTS

It has been reported that diospyrin **15** and 7-methyljuglone **16** exhibited remarkable activity against the H37Rv strain, [32, 34] and a possible mechanism for their toxicity has been suggested (Chapter 3). [33] Quinonoid compounds, by virtue of their facile redox cycle capacity, are known to possess wide-ranging anti-mycobacterial activities. However, the underlying mechanism of action is still not fully understood. In the literature, two theories seem to persist viz., redox cycling and reductive alkylation (Chapter 9).

Redox cycling is the coined term for the concept in which compounds catalytically partake in a cycle and generate oxidative radicals or reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, which in turn damage the cell. NADPH oxidases, also known as NOX proteins, produce ROS in response to exogenous stimuli. NADPH oxidases catalyse the univalent reduction of O_2 to produce O_2^-, which is then rapidly converted to H_2O_2 **(Fig 7.1)**. [99]

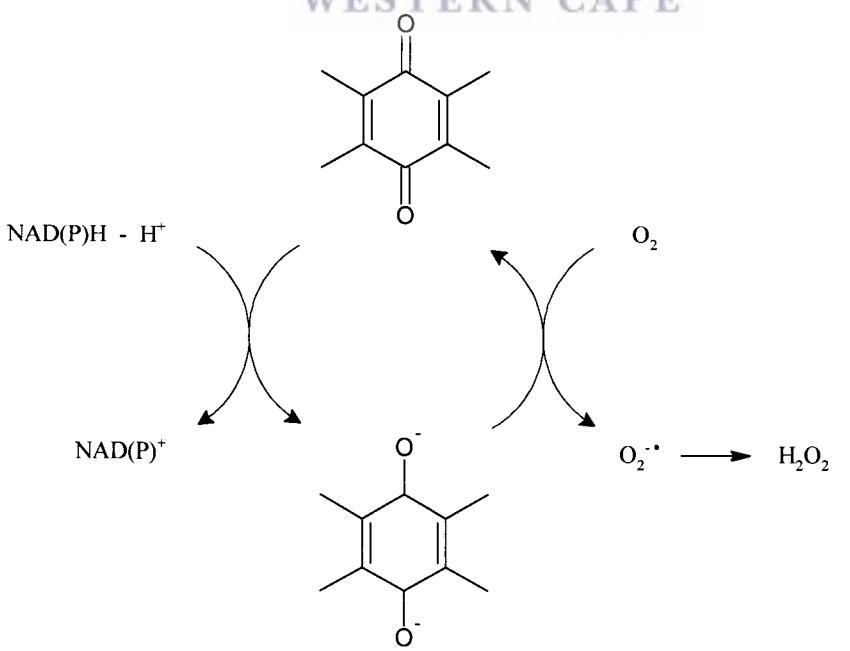


Fig. 7.1 Redox cycling of NADH or NAD(P)H and a quinone

Eight of the total number of synthesized compounds was selected for evaluation of their anti-mycobacterial activity against a drug-susceptible strain, H37Rv, of *Mycobacterium tuberculosis* using the radiometric BACTEC method. In addition to determining the mycobacterial viability against the synthesized methyl ether and hydroxyl quinone analogues, we also evaluated bi-aryl compounds bearing different phenyl moieties to ascertain what effect, if any, these groups have on the activity of the compounds.

7.1 Materials and Methods

All work was carried out in the Biosafety Level 3 laboratory of the Division of Molecular Biology and Human Genetics at the University of Stellenbosch. A *Mycobacterium tuberculosis* reference strain (H37Rv) was used and a clinical isolate of *M. tuberculosis* was selected from a bank of genetically well-characterized (according to their IS6110 fingerprint profile) strains located in the Division of Molecular Biology and Human Genetics at the University of Stellenbosch for the evaluations. [100] The clinical strain used was drug sensitive to Isoniazid and Rifampicin.

The 7H9 middlebrook mycobacterial growth medium, which contains the ^{14}C-labeled substrate (palmitic acid) as a source of carbon, was used for the testing. Growth of the organisms leads to the consumption of the substrate, with subsequent release of $^{14}CO_2$ into the atmosphere above the medium in the sealed vial. As a result the BACTEC TB-460 instrument (Becton Dickinson and Company, USA) developed, detects the amount of $^{14}CO_2$ and records it as a growth index (GI) on a scale of 0-999.

Compounds to be tested for the antimycobacterial activity were dissolved in DMSO (Merck) and filter sterilized through 0.22 micron syringe filters (millex LG). The *M. tuberculosis* clinical isolate was grown on Lowenstein-Jensen culture medium for 4 weeks. [101] After incubation at 37°C, some of the bacteria were collected with a sterile inoculation loop, suspended in 7H9 mycobacterial growth medium and the bacterial suspension allowed to settle for five minutes. A volume of 0.1 ml of the upper suspension was added to a BACTEC vial containing BACTEC 12B growth medium (Becton Dickinson, USA). Growth was monitored every 24 h until a growth

index (GI) value of 500 was reached. A volume of 0.1 ml of this culture was again added to a new BACTEC vial and the growth monitored every 24 h until a GI value of 500 was reached. [102] This culture was the primary culture and was used for testing bacterial viability against a variety of inhibitors. A volume of 0.1 ml of inhibitor (compound to be tested) was added to a BACTEC vial to give a final concentration of 125 µg/ml. A 1:11 dilution with DMSO was made of the compounds to give a final concentration of 12 µg/ml and 0.1 ml of this solution was added to a BACTEC vial. BACTEC vials were incubated at 37°C and the growth index was monitored every 24 h.

7.2 Results

A growth index (GI) value below 10 was considered as negative growth and that above 10 as positive growth. The GI of the treated samples was compared to a control containing sterile DMSO. The control experiments clearly showed that the final amount of DMSO (1%) in the media had no effect on the growth of *M. tuberculosis*. The GI of the bacteria was measured over a period of 5 days. **Fig 7.2** represents the effect of the compounds tested, at a final concentration of 10 µg/ml, on the growth of the H37Rv strain of *M. tuberculosis*.

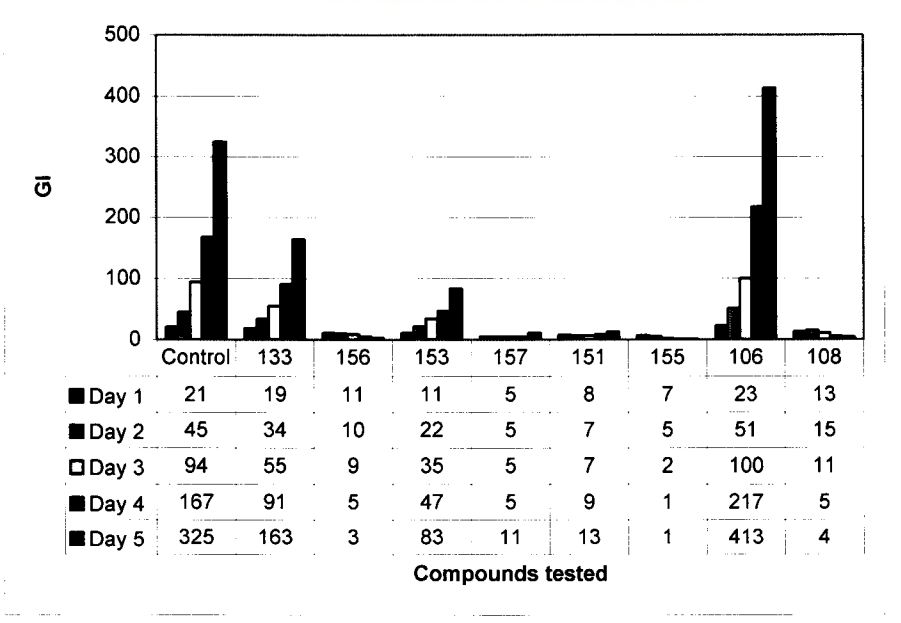
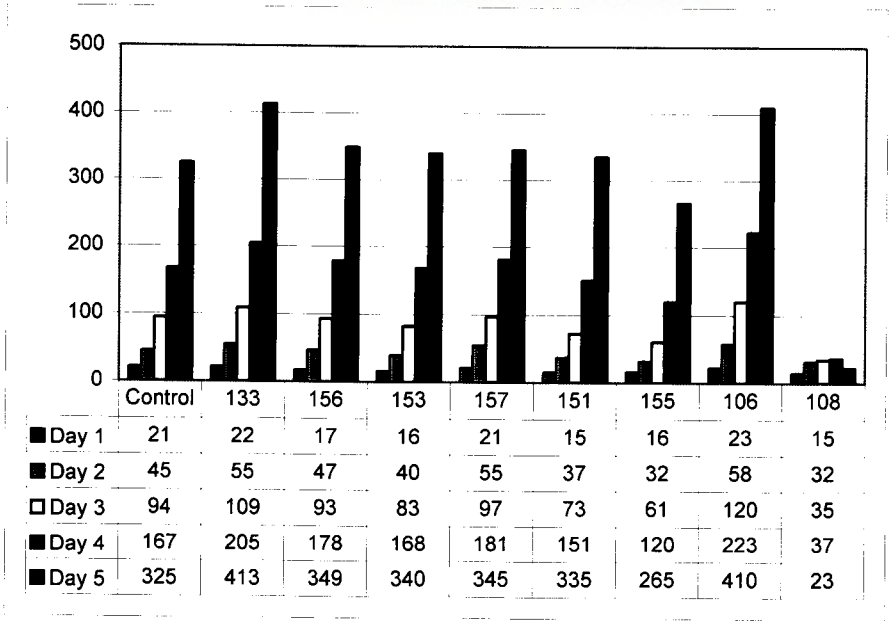


Fig. 7.2 The effect of the test compounds, on the H37Rv strain, at 10 µg/ml.

The control has a normal doubling rate every 24 hrs. The graph shows that the growth rates (Δ GI), of H37Rv, induced with compounds **108**, **151**, **155**, **156**, and **157** are zero or less than zero, indicating bactericidal activity. At day 5 of incubation **151** and **157** start losing their influence and the GI for these compounds are becoming just positive (>10) viz., **151** = 13 and **157** = 11. **106**, on the other hand, showed no effect on the growth rate of the bacteria, whilst both **133** and **153** appeared to be a bit more active than the former. The GI for these two compounds are relatively low compared to the control and this could be attributed to two things viz., quite a few bacteria may have been killed at the beginning of the experiment and the remainder that survived have continued to double in the normal way from day to day, or a percentage of the drug has been metabolised to a level where the bacteria can continue to grow normally but lagging far behind the controls.

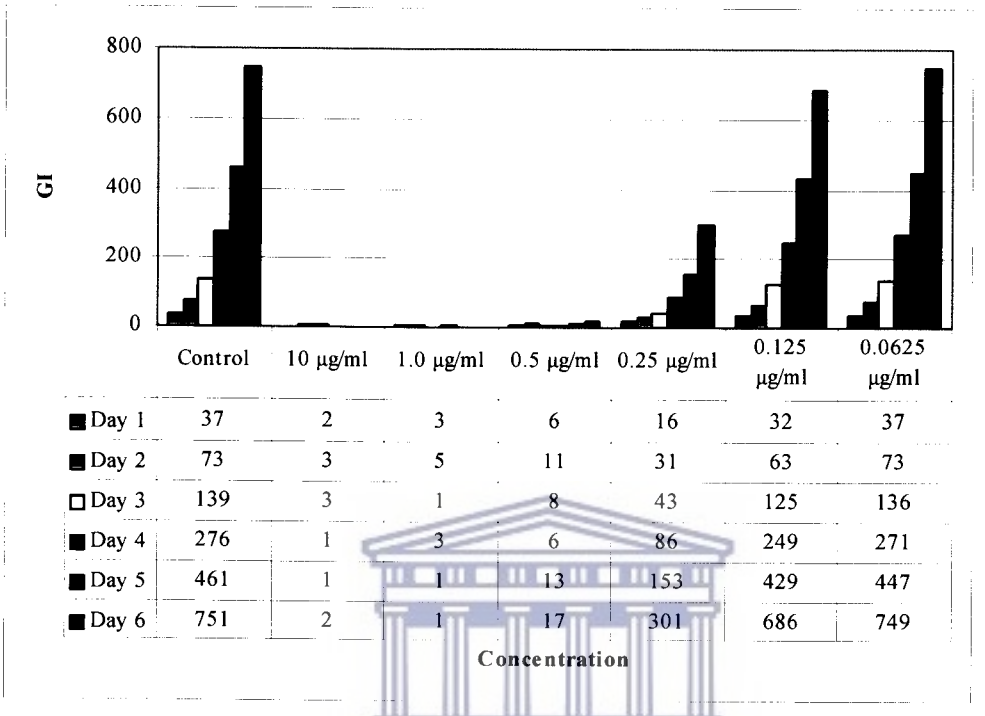
At 1.0 µg/ml final concentration, the drug that exhibited good biological activity was **108 (Fig. 7.3)**. Although **108** shows positive growth, the GI does not increase with time. This implies that the bacterial cells metabolize and produce CO_2 but do not multiply. At day 5 the GI is in a decreasing mode, indicating that the cells cannot sustain this static metabolism under the drug's influence. The growth rate is also becoming negative (Δ GI = -14).



	Control	133	156	153	157	151	155	106	108
Day 1	21	22	17	16	21	15	16	23	15
Day 2	45	55	47	40	55	37	32	58	32
Day 3	94	109	93	83	97	73	61	120	35
Day 4	167	205	178	168	181	151	120	223	37
Day 5	325	413	349	340	345	335	265	410	23

Fig. 7.3 The effect of the test compounds, on the H37Rv strain, at 1.0 µg/ml.

Compound **108** was then separately tested at lower concentrations to determine the minimum inhibitory concentration (MIC) against the H37Rv strain of *M. tuberculosis* **(Fig. 7.4)**. The GI of the bacteria was measured over a period of 6 days.



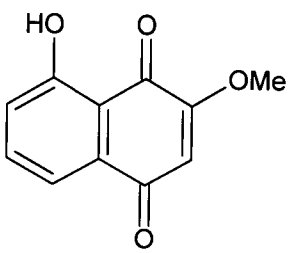
	Control	10 µg/ml	1.0 µg/ml	0.5 µg/ml	0.25 µg/ml	0.125 µg/ml	0.0625 µg/ml
Day 1	37	2	3	6	16	32	37
Day 2	73	3	5	11	31	63	73
Day 3	139	3	1	8	43	125	136
Day 4	276	1	3	6	86	249	271
Day 5	461	1	1	13	153	429	447
Day 6	751	2	1	17	301	686	749

Fig. 7.4 The effect of T8 on H37Rv at various concentrations

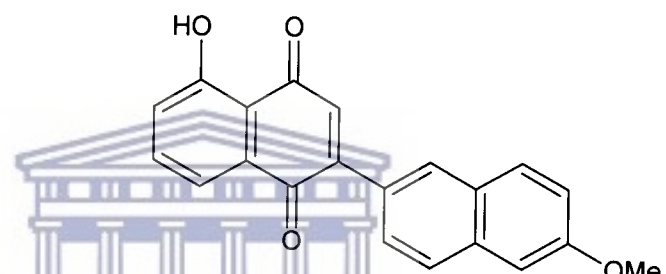
The above histogramme shows that **108** has no effect on the growth rate of the bacteria at the lowest concentrations tested viz., 0.125 µg/ml and 0.0625 µg/ml. At 0.25 µg/ml the GI is low relative to the control. The lower GI is due to a reduced CO_2 production which in turn is due to fewer bacteria being present but may also be attributed to that observed in the cases of **133** and **153** at 10 µg/ml, which has been discussed earlier. From the graph it can be concluded that **108** is bactericidal up to 0.5 µg/ml. Although the GI at this concentration is becoming positive (GI>10) the GI has not doubled from day 5 to day 6. After 6 days of incubation the MIC for **108** lies just below 0.5ug/ml, but is definitely higher than 0.25ug/ml.

These results clearly demonstrate that by replacing a *peri* methoxy group to the carbonyl with a hydroxyl group, the activity of the molecule is enhanced as observed in the cases of **108**, **156** and **157** compared to their methyl ether analogues **106, 133** and **153**. Previous studies have shown that the hydroxyl analogues of diospyrin **15** and

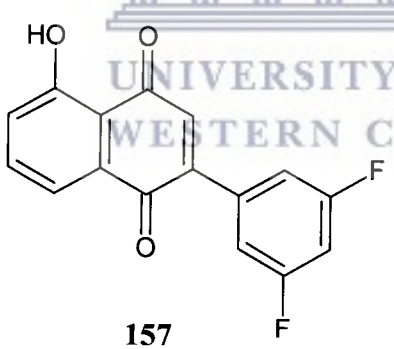
7-methyljuglone **16** are more active than their corresponding alkoxy derivatives against the H37Rv strain. [32, 34] It was suggested that the activity of 7-methyljuglone **16** against *M. tuberculosis* could be attributed to the structural similarities between itself and the natural redox cycler, viz., menaquinone **97** [33] which mediates electron transfer between different membrane-bound enzymes of the respiratory chain in *Mycobacterium*. [77] Hence, it is feasible to postulate that those compounds with an hydroxyl group *peri* to the carbonyl group can interact with enzymes in the mycobacterial electron chain, through a hydrogen bonding mechanism, whereas in the case of the *peri* methoxy groups, such an interactions are no longer possible and hence a decrease in their activity is observed.



108



156

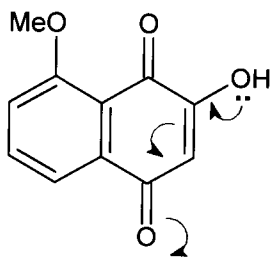


157

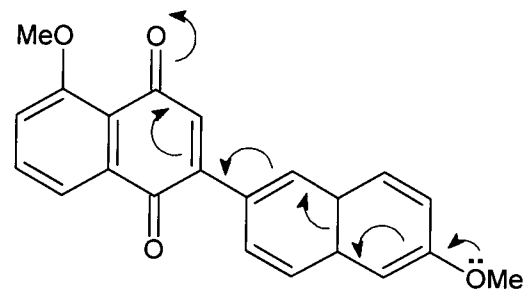
Scheme 7.1

Despite having a hydroxyl group *ortho* to the carbonyl moiety, **106** exhibited no effect on the growth of the bacteria, whereas its structural isomer **108** exhibited anti-mycobacterial activity at a concentration as low as 0.5 μg/ml. The obvious major structural difference between **106**, having the 3-OH group conjugated to the 1-C=O as shown in **Scheme 7.2**, and **108** which has the chelatable *peri* 5-OH to the carbonyl, clearly plays a significant role in the activity. Thus it appears that the mere fact of having an OH group present is not as significant as that this OH must be *peri* to a C=O of a quinone system. This must favour both chelation to active sites in the

receptors thereby blocking them to be available for alternative interactions or hydrogen bonding with donor sites, thus altering the tertiary structures of the proteins and their consequent activities.



106

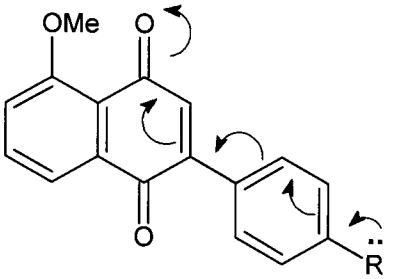


133

Scheme 7.2

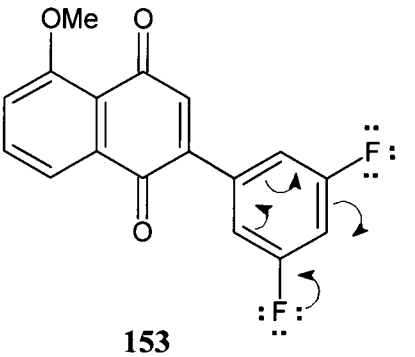
The higher activity of **133** and 153 compared to that of naphthoquinone **106** could be ascribed to the fact that these two compounds have a naphthyl and a phenyl substituent on C-2 respectively, enabling the delocalisation of electrons through the resonance electron effect to the carbonyl group *peri* to the methoxy group. It also appears that by having a *meta* difluoroaryl substituent compared to a methoxy naphthyl substituent, the activity of the former compound is higher than the latter. Whether this is due to the difference in electron-donating ability or the fluorine atoms is a matter of conjecture.

Naphthoquinones **151** and **155** on the other hand are very active at 10 μg/ml compared to naphthoquinone methyl ether **153** having the *meta* difluoroaryl substituent . This could only be ascribed to the fact that both these compounds have a *para* electron-donating substituent on the benzene ring, which is capable of donating electrons into the carbonyl group, *peri* to the methoxy group in the naphthoquinone system, via the resonance electron-donating effect. This would thus enhance the chelating abilities of the two *peri* oxygens.



151 : R = F

155 : R = $N(CH_3)_2$



153

Scheme 7.3

7.3 Conclusion

Eight compounds were evaluated against a drug-susceptible strain, H37Rv, of Mycobaterium tuberculosis using the BACTEC method. At 10 ug.ml the 5-hydroxyl analogues proved to be more active than their 5-methoxy counterparts, suggesting the presence of an hydroxyl group peri to the carbonyl is essential for these compounds to be effective as anti-TB agents. These results correlate with previous studies done by Lall et. al. [32] and Mahapatra [34] which showed that alkoxy derivatives were less active than their hydroxyl analogues. My results also show that substituents capable of donating electrons through the resonance electron effect enhance the activity of these quinoidal systems.

CHAPTER 8

APOPTOSIS

8.1 Introduction

Cell death is a vital part of life in multicellular organisms. Over the years a number of alternative expressions have been used to describe cell death, viz., degeneration, necrosis, autolysis, chromatolysis, and cell suicide. However, the most commonly found names in today's literature are necrosis, apoptosis and sometimes programmed cell death. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis refers to the process of programmed cell death, where the cell itself is actively involved in its own demise. Cells die in response to a variety of stimuli and, irrespective of the cell death-inducing signal; during apoptosis they do so in a controlled and regulated sequence.

Apoptosis is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms, which include a reduction in cell volume, membrane blebbing, chromatin condensation and nuclear DNA fragmentation. These processes, whose result does not damage the organism, differentiate apoptosis from necrosis, in which uncontrolled cell death leads to the rupturing of cells, inflammatory responses and, potentially, to serious health problems.

Apoptotic cell death is a fundamental feature of virtually all animal cells. It is an indispensable process during normal development, tissue homeostasis, development of the nervous system as well as the regulation of the immune system. [103, 104] Many diseases are associated with either too much or too little apoptosis, such as AIDS, cancer, and autoimmunity. [103]

Development of the term "apoptosis"

In 1951, Glücksmann [105] described morphological similarities and differences between various forms of cell death occurring during vertebrate ontogeny, but it wasn't until 1964 that Lockshin *et al.*, [106] suggested the term "programmed cell death"

to describe an apparent program-driven regularity with which specific cells die in sequence during insect development. The term "apoptosis" was first introduced in a publication, in 1972, by Kerr et al., [107] in order to describe the morphological processes leading to controlled cellular self-destruction as an instrument for the disposal of unwanted cells during embryonic development, during normal cell turnover in proliferating tissues and in pathological situations. *Apoptosis* is of Greek origin and refers to the "dropping off" or "falling off" of petals from flowers, or leaves from trees. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms.

The terms "programmed cell death" and "apoptosis" have often been used interchangeably on the basis that the death of a cell by apoptosis follows an intracellular 'program'. However, accumulating evidence suggests that it is important to distinguish between these two terms, even though there is an overlap in their biochemical underpinnings. [108]

8.2 Morphology of apoptotic cells

Cells dying by apoptosis can be recognized by stereotypical morphological changes, which can often be easily detected under the microscope. Some changes can be seen by light microscopy, sometimes with the aid of specific dyes, and some changes can be detected only by electron microscopy. There are three distinct phases of apoptosis as illustrated in **(Fig. 8.1)**. [109] The first phase involves condensation of chromatin along the perimeter of the nucleus. The cell shrinks, shows deformation and looses contact with its neighbouring cells. Subsequently, fragmentation of the nucleus occurs. In the second phase, the plasma membrane starts to protrude and retract, commonly referred to as blebbing or budding, and the cell has the appearance of boiling. The cell is then fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles. In the third phase, the apoptotic bodies are then phagocytosed by macrophages or neighbouring cells. Phagocytosis is initiated by the apoptotic cell through an alteration in the normally asymmetric distribution of phosphatidylserine in the plasma membrane, leading to the appearance of phosphatidylserine on the outer surface of the plasma membrane. [110]

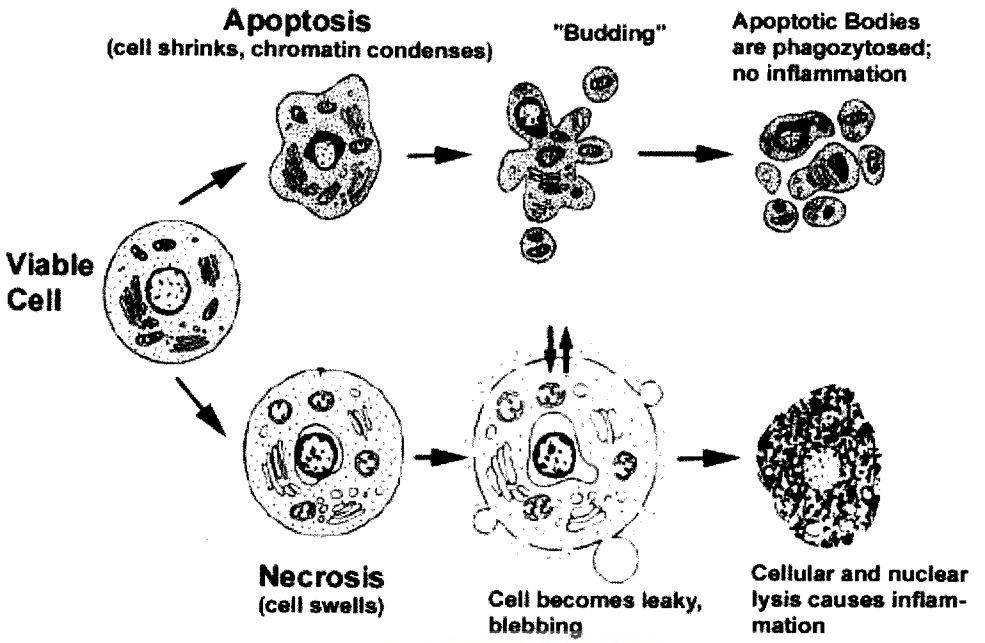


Fig. 8.1 Hallmarks of the apoptotic and necrotic cell death process. [109]

During necrosis, in contrast to apoptosis, the cellular contents are released in an uncontrolled manner into the cell's environment, which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue.

8.3 Caspases – the executioners of apoptosis

Apoptosis is orchestrated by the action of a group of intracellular proteases, called caspases (derived from cysteine-dependent aspartate-specific proteases). Unlike many other proteases, which appear to function predominantly as degradative enzymes, caspases are tightly regulated signaling proteases. [111, 112] All caspases are synthesized in cells as relatively inactive zymogens and must undergo proteolytic activation during apoptosis. When caspases are activated, they cleave specific substrates, either activating or inactivating them, which produce the morphologic and phagocytic changes associated with apoptosis. At least 14 distinct mammalian caspases have been identified. [113] There are two general categories of caspases involved in apoptosis, and they are distinguished by their specific chemistry and their particular functions viz., the initiators, which include caspases-2, -8, -9, and -10, and the effectors, which include caspases-3, -6, and -7. Caspase zymogens are composed of three domains and

include an amino terminal prodomain, followed by a region that will become, upon cleavage and activation, a large subunit (typically 17-20 kilodaltons), and then a region that will become a smaller subunit (typically 10-12 kilodaltons). [108] The prodomain is typically shorter in the case of the effector caspases such as caspase-3 and caspase-7, and longer for the other caspases. The longer prodomains include recognizable signaling motifs in some cases, such as the CARD (caspase activation and recruitment domain) in caspase-1, -2, -4, -9, and Ced-3, and the DED (death effector domain) in caspases-8 and -10 **(Fig. 8.2)**. [114]

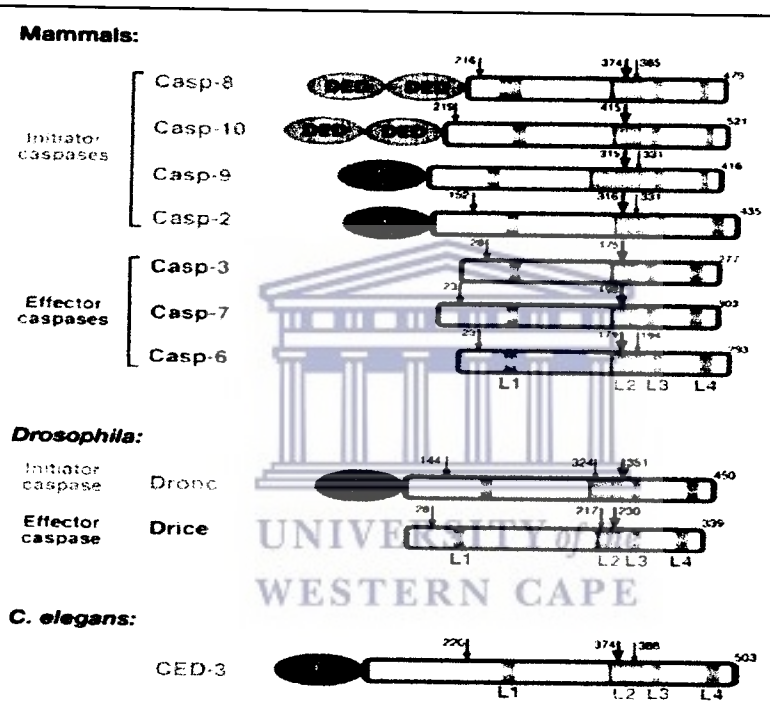


Fig. 8.2 Representative caspases in mammals, *Drosophila*, and *C. elegans*. [114]

The initiator caspases exist in their proforms as monomers and, under apoptotic conditions, are activated by their enforced dimerization. This activation occurs when adapter molecules bind to protein-interaction domains near the N-terminal prodomains of the initiator caspases. The activation of an effector caspase, such as caspase-3 or -7, is performed by an initiator caspase, such as caspase-9, through internal cleavages to separate the large and small subunits, and it appears that this is the only way they can be activated. [115]

There are at least two broad pathways that lead to apoptotic activation and cell death and are defined by the initiator caspases and the adapter molecules that bind and oligomerize them. [116] These are the death receptor pathway and the mitochondrial pathway, which are also known as the 'extrinsic' and 'intrinsic' pathways, respectively.

8.4 Extrinsic (Receptor) Pathway

Extrinsic apoptosis signalling is mediated by the activation of so called "death receptors" that transmit apoptotic signals after ligation with specific ligands. Death receptors, located on the surface of the cell and characterized by extracellular cysteine-rich domains, belong to the tumour necrosis factor (TNF) receptor family. [117] The best-characterized death receptors include Fas and TNFR1 (Tumour Necrosis Factor Receptor-1). These trimeric death receptors feature a single, intracellular protein-interaction domain called a death domain (DD), which is thought to be responsible for the recruitment of adapter proteins. Upon binding to extracellular apoptosis-inducing ligands, the DDs of these receptors bind to a similar DD on an adapter molecule, such as Fas-associated DD (FADD). An exception is TNFR1, which binds to a different adapter, TRADD (Toll receptor activated death domain) that in turn binds to FADD.

In addition to a DD, FADD has another protein-interaction domain called a death effector domain (DED). The DED of FADD binds to a DED in the prodomains of the initiator caspases, caspase-8 and caspase-10. The complex formed by the ligated death receptors, FADD, and the caspases is called a death-inducing signaling complex (DISC) and results in the dimerization and activation of the initiator caspases **(Fig. 8.3)**. [116] Once activated, either of these initiator caspases can cleave and activate the effector caspases, principally caspase-3, caspase-6 and caspase-7, which in turn cleave a variety of substrates, to give rise to internucleosomal DNA degradation and characteristic morphological changes.[118, 119]

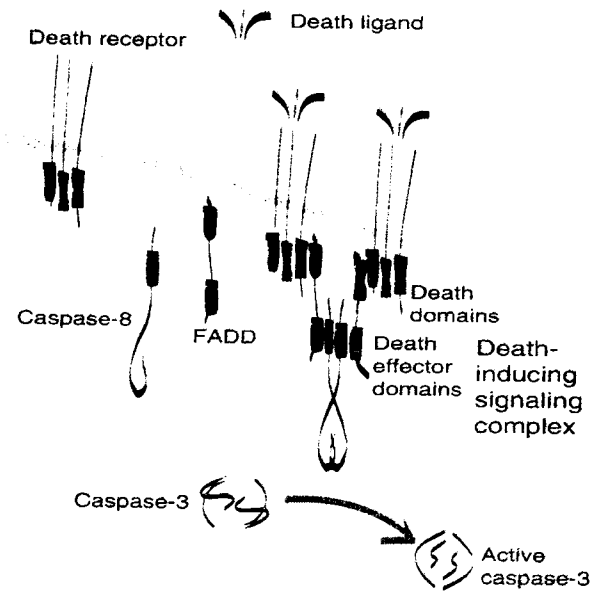


Fig. 8.3 The death receptor pathway of caspase activation. [116]

8.5 The Intrinsic (Mitochondrial) Pathway

The mitochondrial or intrinsic pathway represents the other well-characterized mechanism of initiator caspase activation and, as its name suggests, the intrinsic pathway is initiated from within the cell. This is usually in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress. [120, 121] Protein 53 (hereafter referred to as p53), a transcription factor, is a sensor of cellular stress and is a critical activator of this apoptotic pathway that also hinges on the balance of activity between pro- and anti-apoptotic members of the Bcl-2 family of proteins, which act to regulate the permeability of the mitochondrial membrane. [122]

p53 initiates apoptosis by transcriptionally activating pro-apoptotic Bcl-2 family members, which move to the mitochondrial membrane and disrupt the function of the anti-apoptotic Bcl-2 proteins thereby allowing permeabilization of the mitochondrial membrane. This mitochondrial outer membrane permeabilization (MOMP) allows the release of proteins of the intermembrane space, including cytochrome c and

SMAC/DIABLO [second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI] into the cytosol. [123, 124] SMAC/DIABLO binds to *inhibitor of apoptosis proteins* (IAPs) and deactivates them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. Cytochrome c binds the adaptor apoptotic protease activating factor-1 (APAF-1), forming a large multi-protein structure known as the apoptosome **(Fig. 8.4)**. [116] The exposed death-fold caspase recruitment domain (CARD) domains of the apoptosome now bind the CARD domains of monomeric caspase-9, and activates it. The now active caspase-9, in turn, activates the downstream effector caspases, including caspase-3, -6, and -7, leading to apoptosis. [123]

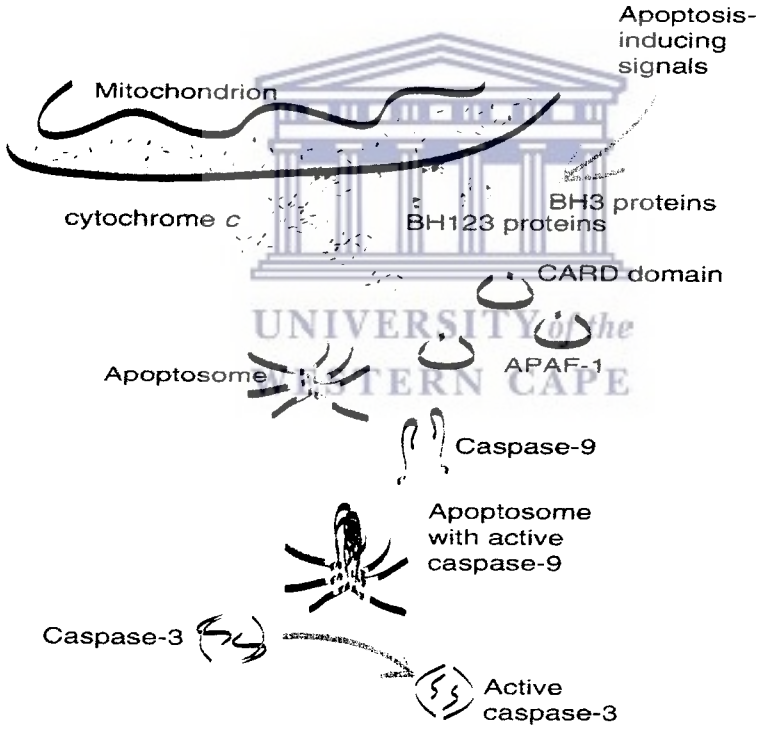


Fig. 8.4 The mitochondrial pathway of caspase activation. [116]

8.6 Regulators of Apoptosis

Apoptosis is fundamental to the maintenance of cellular homeostasis. However, if not regulated, apoptosis can cause many diseases ranging from neurodegenerative disorders to cancer. Cell death is continuously repressed by survival signals viz.,

growth factors, hormones, and nutrients provided by other cells of the organism. Those survival signals enhance the expression and/or activity of anti-apoptotic regulatory molecules thereby keeping in check the activation of pro-apoptotic factors. [125, 126] Several proteins have been discovered that regulate apoptosis and it is the fine control of these proteins, which induces or prevents apoptosis in cells.

8.6.1 Bcl-2 Family of Proteins

The Bcl-2 family of proteins plays a central role in the regulation of apoptosis. As mentioned before, they are the gatekeepers in the mitochondria-initiated intrinsic apoptosis. On the basis of function and sequence similarity, Bcl-2 proteins are grouped into three subfamilies. The anti-apoptosis subfamily, represented by Bcl-2 and Bcl-x_L in mammals, prevents MOMP, and thus prevents the release of cytochrome c and APAF-1 activation. The pro-apoptotic members of the Bcl-2 family are of two types. Some share three of the Bcl-2 homology domains (BH1, BH2, and BH3) and are therefore 'multi-domain' or 'BH123' proteins. [127] These proteins include Bax, Bak, and Bok. The BH123 proteins are activated by another set of pro-apoptotic Bcl-2 family members that share the short BH-3 domain and are therefore 'BH3-only' proteins. There are several of these, including Bim, Bid, Bad, Bmf, BNIP-3, Puma, Noxa, and others. [128]

In summary, a current model of how the Bcl-2 family members regulate apoptosis can be described as follows (**Fig. 8.5**) [129]: specific apoptotic stress signals trigger the activation of particular BH3-only proteins which then interact with anti-apoptotic members on the outer mitochondrial membrane, resulting in the release of the pro-apoptotic BH123 proteins. The BH123 proteins undergo a conformational change (possibly assisted by some BH3-only proteins), and then insert themselves into the outer mitochondrial membrane to provoke the release of apoptogenic proteins, cytochrome c and SMAC/DIABLO. [123]

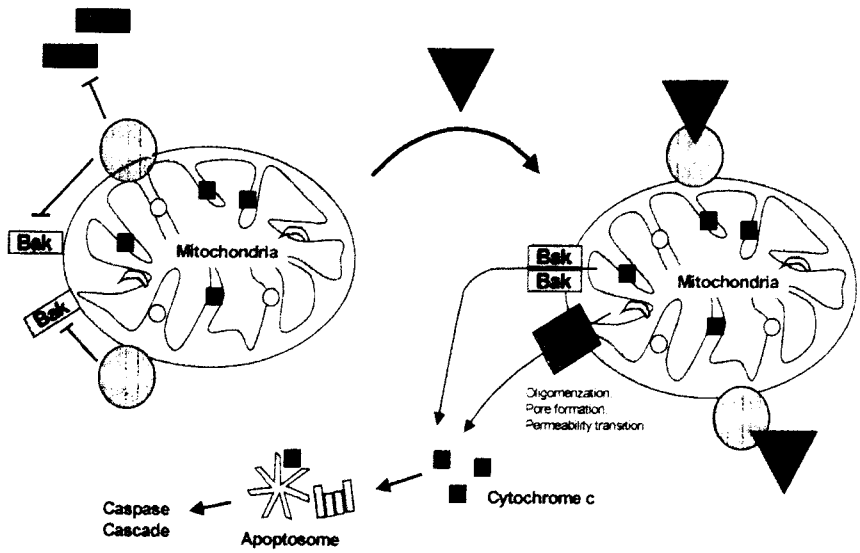


Fig. 8.5 Regulation of apoptosis by the Bcl-2 family. [129]

8.6.2 Inhibitors of Apoptosis

The inhibitor of apoptosis (IAP) family of proteins represents an important class of regulators of programmed cell death, by negatively regulating caspases. They were initially discovered in baculoviruses where they were found to inhibit apoptosis in host cells during viral infection. [130] Cellular proteins have been identified that inhibit specific 'upstream' or initiator caspases, but the IAPs are the only known endogenous proteins that regulate the activity of both initiator (caspase-9) and effector caspases (caspase-3 and -7). [131] So far, eight human IAP homologues have been identified, among others NAIP, c-IAP1, c-IAP2, XIAP and survivin.

The hallmark of an IAP is the presence of a least one conserved zinc-binding BIR (baculovirus IAP repeat) domain, thought to directly or indirectly inhibit caspases. [132] Structural and functional studies have provided important insights into the molecular mechanism by which IAPs inhibit caspase-3, -7, and -9. [133-136] In XIAP, the third BIR domain (BIR3) potently inhibits caspase-9 [137] whereas the linker region between BIR1 and BIR2 specifically targets caspase-3 and –7. [135, 136]

A flexible linker N-terminal to the BIR2 domain binds the substrate groove of caspase-3 and -7 adopting a reverse orientation as compared to that of classic caspase substrates, thus blocking the substrate's access to the enzyme.

8.7 DNA Fragmentation

Apoptosis is characterized morphologically by condensation and fragmentation of nuclei and cells and biochemically by fragmentation of chromosomal DNA into nucleosomal units. [138] The fragmentation of the genomic DNA is a late event during apoptosis. It occurs in response to various apoptotic stimuli in a wide variety of cell types. Three types of DNA fragmentation have been reported during apoptosis viz., internucleosomal DNA cleavage; fragmentation into large, 50-300 kbp lengths; and single-strand cleavage events. [139]

In mammalian cells, caspase-independent apoptotic DNA degradation has been attributed to two mitochondrial proteins endonuclease G and apoptosis-inducing factor (AIF) that translocate to the nucleus upon release. [140, 141] AIF induces nuclear condensation and large-scale DNA fragmentation and is required for apoptosis during embryonic body cavitation. [142]

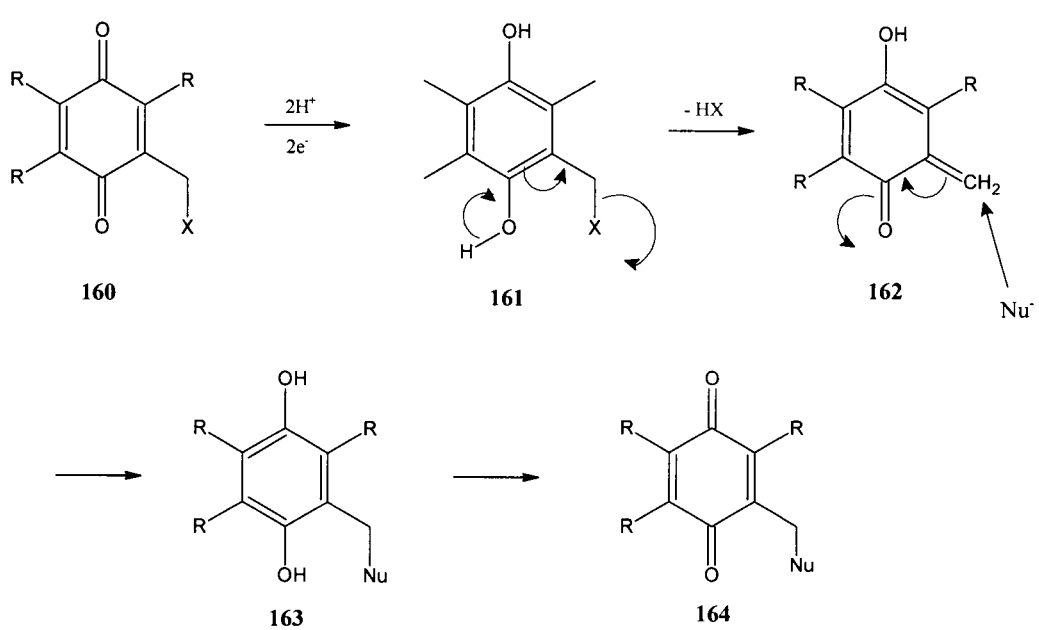
Molecular characterization of this process identified a specific DNase (CAD, caspase-activated deoxyribonuclease) that cleaves chromosomal DNA in a caspase-dependent manner. [119, 143-146] CAD, which is also known as the DNA fragmentation factor (DFF40), is a heterodimeric protein complex that is normally complexed with its inhibitor, ICAD, in growing, non-apoptotic cells. [118, 119] During apoptosis, activated caspases, viz., caspase-7 and especially caspase-3 cleave the DFF40/CAD: DFF45/ICAD complex and CAD is released to carry out DNA fragmentation in the nucleus. [118, 119, 147]

CHAPTER 9

SCREENING OF THE SYNTHESIZED COMPOUNDS FOR PRO-APOPTOTIC ACTIVITY

The objective of this aspect of the research was to perform a preliminary screening of the apoptotic potential of the synthesized analogues of the lead molecule viz., diospyrin **15** against various cancer cell lines at our disposal. Diospyrin **15**, a naturally occurring bisnaphthoquinone, and some of its synthetic analogues have been shown to exhibit encouraging anti-tumour activity. [55, 62] The mechanism of toxicity is still under investigation, but two theories viz., redox cycling (see Chapter 7) and reductive alkylation *vide infra,* dominate the literature, with some quinones proposed to exhibit one or both mechanisms.

The term bioreductive alkylating agents refers to those types of compounds that become potent alkylating agents after they undergo reduction *in vivo*. Such reactive species may then alkylate DNA/RNA and/or other biomolecules resulting in potentially effective cancer-inhibitory drugs. From the work done by Sartorelli *et al.* [148, 149], Moore [150] proposed a mechanism for the biological activity of several natural products as depicted by **Scheme 9.1**.



Scheme 9.1

According to Moore the quinone **160** undergoes initial *in vivo* bioreduction to the quinol **161**. Mesomerically assisted cleavage of a benzylic substituent by formal loss of HX results in the formation of the highly active quinone methide **162**. This in turn is able to react with biological nucleophiles such as DNA, proteins and carbohydrates to yield **163** effectively altering the activities of the nucleophilic species due to their interaction with intermediate **162**. Further oxidation of the quinol product **163** results in a biologically inactive quinone **164**, which is now bonded to the biological target. Because of this, the cell is no longer able to carry out its normal functions or to replicate, thus leading to cell death.

In the present study, a number of novel biaryl analogues of diospyrin **15** were synthesized and subjected to anti-tumour evaluations which were conducted at the Biotechnology Department at the University of the Western Cape, which has in-house facilities available to undertake apoptosis analysis. The choice of the cell lines was restricted to the routinely used cell lines in the department. This chapter reports on the potential that these compounds have, to induce apoptosis in five different human cancer cell lines viz., H157, Hek239T, HeLa, Jurkat, MCF-7 and two non cancerous cell lines viz., CHO (non-human) and KMST-6 (human cell line) using the APOP*ercentage*™ assay. The pro-apoptotic activity was also conclusively demonstrated with DNA fragmentation, a late event in the apoptotic process, utilizing the APO-DIRECT™ Kit.

9.1 Materials used

9.1.1 General chemicals and assay kits

Active Caspase-3 FITC Mab Apoptosis Kit	BD Biosciences
APOP*ercentage*™ apoptosis assay	Biocolor Ltd
Crystal Violet	Sigma
DMSO	Sigma
Ampicillin	Roche

APO-DIRECT™ Kit	Promega
Propidium Iodide (1 mg/ml in 3.8 mM sodium citrate)	Sigma
Rnase	Roche
Foetal calf serum (FCS)	Roche

9.1.2. Tissue culture medium and cell lines

Invitrogen supplied tissue culture media:

Dulbecco's modified eagle medium (DMEM)	with 4500 mg/l glucose and Glutamax™
Ham's F12	
RPMI 1640 medium	
Phosphate Buffer Saline (PBS)	Ca^{++}, Mg^{++} free
100x penicillin streptomycin	GIBCO

Table 9.1 The cells used during apoptosis analysis

Species	Cell lines	Medium	Serum
Hamster	CHO	Hams F12	10% FCS
Human	Jurkat	DMEM	10% FCS
Human	HeLa	DMEM	10% FCS
Human	H157	DMEM	10% FCS
Human	KMST-6	DMEM	10% FCS
Human	MCF-7	RPMI 1640	10% FCS
Human	Hek 239 T	DMEM	10% FCS

9.1.3. Preparation of culture media

Complete Ham's F12 = Ham's F12 + 0.2% penicillin streptomycin + 10% FCS
Complete DMEM = DMEM + 0.2% penicillin streptomycin + 10% FCS
Complete RPMI = RPMI + 0.2% penicillin streptomycin + 10% FCS

9.2 Culturing of cells

9.2.1 Thawing of cells

The frozen cell filled vials were removed from storage at -150°C and immediately thawed in a 37°C water bath. The contents of the vials were then transferred into a 15 ml tube containing 5 ml of media. The 15 ml tube was then centrifuged for 2 min. @ 2000 rpm. The supernatant was discarded and the pellet was re-dissolved in 5 ml of media that was transferred to a 25cm^3 tissue culture flask for incubation. All the cell lines used, except Jurkat, were adherent and were incubated @ 37°C in an atmosphere of 5% CO_2.

9.2.2 Trypsinization of cells

Once cells reach confluency, they require to be trypsinized (or potentially die of overcrowding). Thus the media in the flask was then discarded and the remaining cells were washed with trypsin, and then allowed to trypsinize with 3 ml of 0.0625% trypsin in an incubator. After 3 min, 12 ml of media was added to the flask to stop trypsinization. The cells were then devided into three 25cm^3 tissue culture flasks for incubation (media in flasks are topped up to a minimum of 5 ml).

9.2.3 Freezing of cells

To ensure continuity of cell lines, cells were grown to confluency and then stored at -150°C. To store cells they were trypsinized after which the pellet was re-dissolved in a 10% DMSO and 90% FCS. The suspensions were aliquoted into 2 ml cryo-vials, then stored at -150°C.

9.2.4 Seeding of cells

Once cells have been thawed and incubated in a 25cm^3 tissue culture flask, they were left for 2 - 3 days to grow to confluency. Once confluent, the cells are ready to be seeded in the appropriate well plates for testing. That is, cells are trypsinized, centrifuged to remove supernatant and re-suspended in 5 ml of media. Cells are then

counted using a Hausser Scientific Fuchs Rosenthal Ultra Plane Hemocytometer and seeded at a concentration of $2.5x10^4$ cells per well. When 6 or 24 well plates were used 2 ml or 500 µl of cells were seeded, respectively. Cells are incubated overnight at 37°C and were ready for testing.

9.3 Apoptosis evaluation techniques used

9.3.1 APOP*ercentage*™ apoptosis assay

The APOP*ercentage* dye was prepared by adding 15.9 ml media to 0.1 ml APOP*ercentage* dye. The cells were seeded in 24 well tissue culture plates and treated with varying concentrations of the test compounds. All evaluations were performed in triplicate and the error bars represent standard deviations. The cells induced with test compounds were then incubated for 24 h before the media were removed and placed in 15 ml tubes. Cells were washed with 1 ml PBS and trypsinized with 300 µl trypsin and added to the contents of the tube. Cells were then centrifuged @ 3000 rpm for 5 min and the supernatant discarded. The cells were re-suspended in 200 µl of the prepared APOP*ercentage* dye and then incubated for 30 min at 37°C. The cells were washed with 2 ml PBS to remove the excess dye. The cells were then re-suspended in 200 µl PBS and analysed by flow cytometry within the hour.

9.3.2 APO-DIRECT™ Kit (DNA fragmentation)

The cells were seeded at a density of 2.0 x 10^6 per ml in 6 well tissue culture plates and allowed to grow to 80% confluence; incubated at 37°C in humidified CO_2 incubator. When the cells had reached the required confluency, the culture media was replaced with fresh media and induced with 50 µM of the test compounds for 24 h at 37°C in a humidified CO_2 incubator. After the 24 h incubation period, the cells were trypsinized and re-suspended in 5 ml of 1% (w/v) paraformaldehyde in PBS and placed on ice for 15 min. The cells were centrifuged @ 3000 rpm for 5 min and the supernatant was discarded. The cells were washed twice in 5 ml of PBS followed by centrifugation @ 3000 rpm for 5 min. The cells were re-suspended in 0.5 ml of PBS and 5 ml of ice-cold 70% (v/v) ethanol in order to permeabilize the cells. For proper permeabilization, the cells were stored at -20°C for 48 h prior to staining. The cells

were then centrifuged @ 3000 rpm for 5 min; the supernatant discarded, and the cells were re-suspended in 1 ml of wash buffer. The cells were again centrifuged; the supernatant removed and the washing process with the wash buffer repeated. The cell pellet was suspended in 50 ml of the staining solution for 60 min at 37°C. At the end of the incubation, 1 ml of rinse buffer was added to each of tube and centrifuged as before. The supernatant was removed by aspiration. The treatment with rinse buffer was repeated and the cells were re-suspended in 0.5 ml of the Propidium Iodide (PI)/Rnase A solution. The cells were incubated in the dark for 30 min at room temperature and then analysed by flow-cytometry.

Table 9.2 APO-DIRECT™ kit staining solution

Staining Solution	1 Assay (µl)	4 Assays (µl)
TdT reaction	10	40
Tdt enzyme	0.75	3
Fluorescein-dUTP	8	32
dH_2O	32	128
Total volume	50.75	203

9.4 Analysis of the apoptotic potential of a selection of compounds

9.4.1 Evaluation using the APOP*ercentage*™ assay.

In viable cells, the distribution of phospholipids between the inner and outer leaflet of the plasma membrane is asymmetric. Phosphatidylcholine and sphingomyelin are predominantly located in the outer leaflet, while phosphatidylethanolamine and phosphatidylserine (PS) are found in the inner plasma membrane leaflet. [151] During apoptosis PS is externalised to the outer-leaflet of the plasma membrane and can thus be used as a biomarker to identify apoptotic cells. It was demonstrated that annexin-V has a strong affinity for PS, and this prompted the development of flow cytometry–based bioassays that use fluorescein isothiocyanate (FITC)-labelled or Phycoerythrin-lablled annexin-V to detect and quantify apoptosis.

The APOP*ercentage*™ assay has been shown to compare well with the standard, more expensive, annexin-V–based apoptosis assay. [152] The APOP*ercentage*™ is also a dye uptake assay that makes use of an anionic halogenated fluorescein dye, the disodium salt of 3, 4, 5, 6 -tetrachloro-2', 4', 5', 7'-tetraiodofluorescein (TCTF), which stains apoptotic cells with a red colour. The exposure of the PS allows the unidirectional uptake of the APOP*ercentage*™ dye, hence only apoptotic cells will be dye labelled, whereas the normal and necrotic cells that are present remain unlabelled. [153]

All evaluations were performed in triplicate and the error bars represent standard deviations. The cells were plated in 24 well tissue culture plates and treated with the test compounds for 24 h at concentrations ranging between 10 μM and 50 μM. After the cells were treated, permealized and stained with APOP*ercentage*™ dye, they were subsequently analysed on a (Fluorescent Activated Cell Sorting) FACScan instrument using CELLQuest PRO software (BD Biosciences). The cell fluorescence was measured by flow cytometry using the FL2 channel and a minimum of 10 000 events was acquired per sample. Results were acquired and analyzed in the form of a histogram where normal cells will fluoresce in the first decade (10^1), whilst a horizontal fluorescent shift along the X-axis from the first decade (10^1) to the second (10^2) or third decade (10^3) is expected for apoptotic cells as illustrated in **Fig. 9.1**. Shown here are overlayed histograms of the relative fluorescence of the treated and untreated cells after staining.

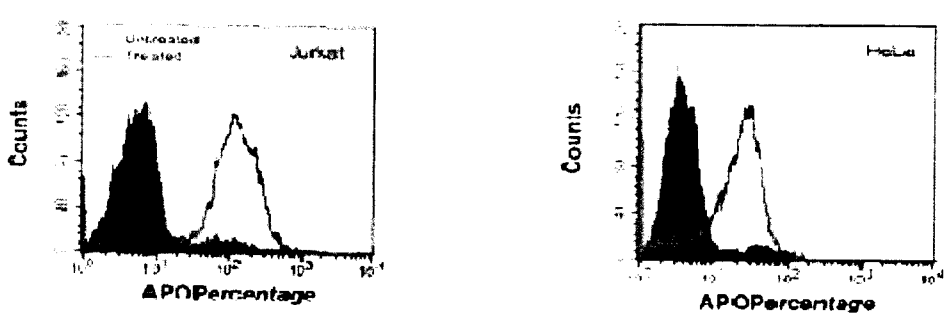


Fig. 9.1 The quantification of apoptosis in Jurkat T and HeLa cells by flow cytometry

9.4.1.1 H157 cell line

Several of the synthesized compounds were screened for their activity against the human carcinoma H157 cell line. This cell line represents a large cell lung cancer and is so called since the cells appear large and rounded when viewed under a microscope. **Fig. 9.2** indicates that the test compounds displayed different apoptotic abilities towards the H157 cells. The results also show that synthetic functional group and structural modification, on either naphthyl moiety of the binaphthyl system, has a remarkable and encouraging influence on the apoptotic potential of the test compounds.

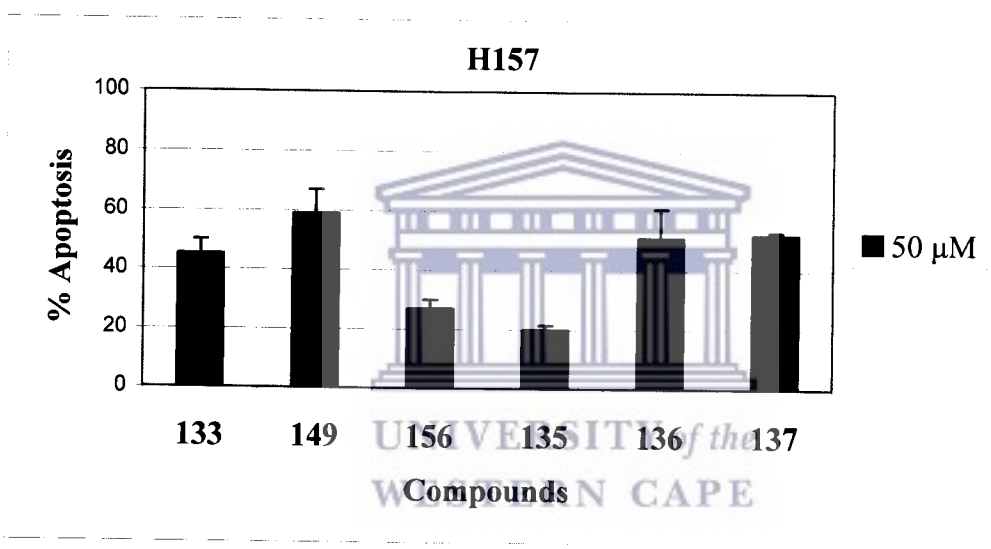
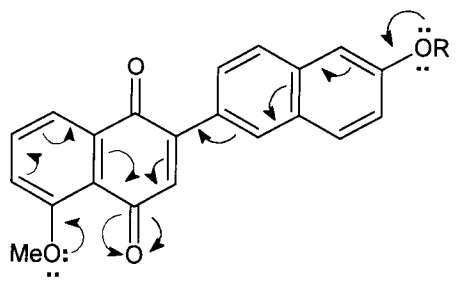


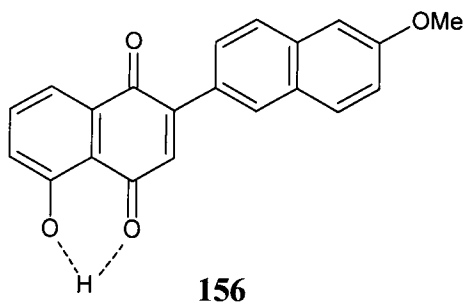
Fig. 9.2 Results for compound 133, 149, 156, 135, 136, and 137 for testing on H157 cell line.

The methyl ether analogue **133** appears to be more active than the hydroxyl analogue **156**, whereas by the simple substitution of the methyl group of the naphthyl ether ring of compound **133** with an ethyl group (compound **149**) an increase in its activity becomes apparent. The lower activity of compound **156** could be ascribed to H-bonding between the *peri* 5-OH group and the oxygen at the C4-carbonyl group as depicted in **Scheme 9.2**. Replacing the *peri* OH group by an OR effectively eliminates H-bonding at position 5. The resonance electron-donating effect of both the alkoxy substituents donating electrons to the carbonyl group, *peri* to position 5, may in large part explain the increased activity of compounds **133** and **149** (**Scheme 9.2**).



133 : R = CH_3

149 : R = CH_2CH_3



156

Scheme 9.2

On the other hand compound **136**, a structural isomer of compound **133**, is more active than **133** and this is most likely due to the positional change of the methoxy group from C5 to C6. In so doing any *peri* influence is removed leaving an electronic effect as the only one that may operate. What was of notable interest to the project was that the bisnaphthyl analogue of **133**, viz., compound **135**, showed a significant drop in activity on the one hand compared to compound **133**, whilst on the other hand bisnaphthyl compound **137** the structural isomer of compound **135** was as active as its mono-naphthyl analogue **136**.

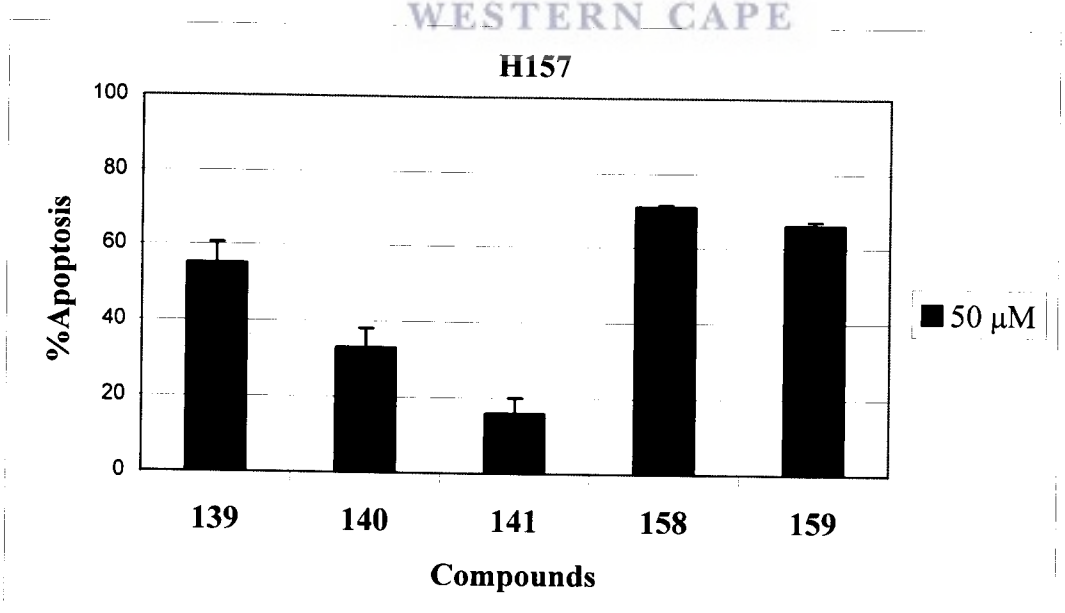


Fig. 9.3 Results for compounds 139, 140, 141, 158, and 159 for testing on H157 cell line.

In the sulphur series of binaphthyl-type compounds evaluated **(Fig. 9.3)**, the 5-methoxynaphthoquinone derivative **139** demonstrated a higher activity compared to its 8-methoxy isomer (compound **140**) while the 6-methoxy isomer (compound **141**) appears not to be as active except at medium concentrations. Introduction of the S=O groups, by oxidizing compounds **139** and **140** using MCPBA as oxidant produced the desired disulphones **158** and **159** respectively, which both demonstrated our hypothesized and anticipated increase in their activities compared to the unoxidized starting materials. We believe the sulphones mimic the anthraquinone moiety to provide the increased activity.

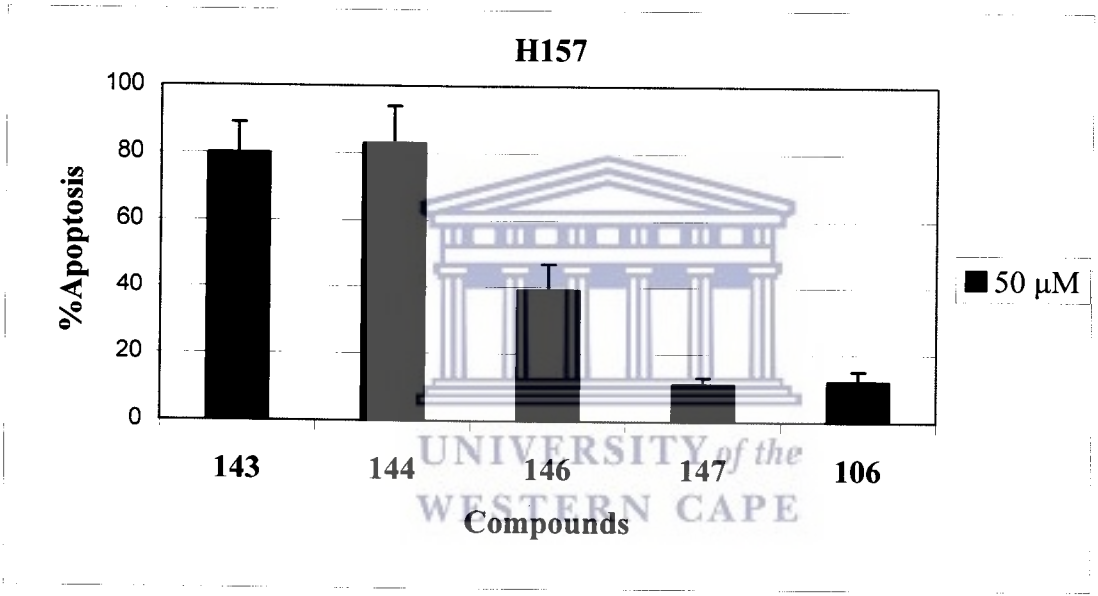
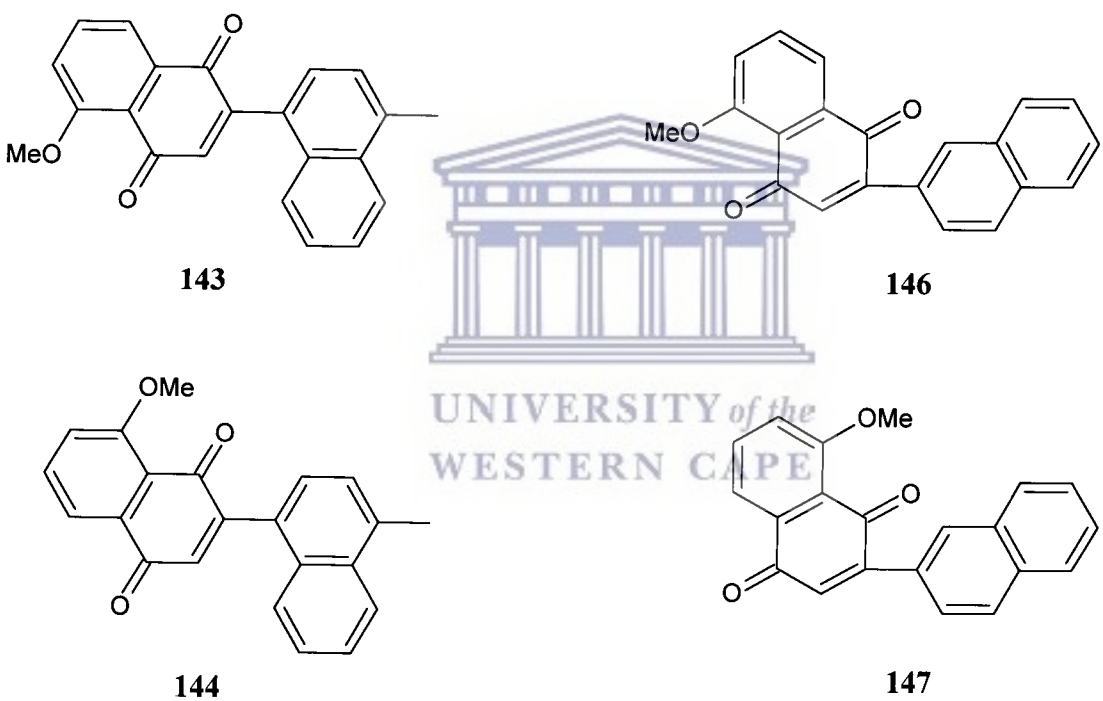


Fig. 9.4 Results for compound 143, 144, 146, 147, and 106 for testing on H157 cell line.

The structural isomers **143** and **144** demonstrated the highest percentage apoptosis of all the compounds tested for activity against the H157 cell line. Interestingly, there was no similar trend in the activities between the structural isomeric pair **146** and **147** in which a marked difference was apparent **(Fig.9.4)**. The difference between the pairs **143/144** and **146/147** is in a methyl group. The question that immediately begs itself is: to what could this observation be attributed? If one were to assume that the depicted structures of **143**, **144**, **146** and **147** are as in **Scheme 9.3**, than one could argue that the naphthyl ring in compound **147** is blocking the one side of the naphthoquinone ring but that this argument would equally apply to the **146** isomer.

However, the side blocked in the case of compound **147** is the one with the *peri* methoxy substituent, whereas this is not the case in compound **146**. It thus seems that, besides the carbonyl moieties, the methoxy substituent does indeed play a role in the apoptotic potential of these types of compounds. In the case of compounds **143** and **144**, the side bearing the *peri* methoxy substituent is apparently not obstructed to the same extent and hence the similarity in their activity. In part, this could be due to secondary van der Waal interactions by the naphthyl methyl group and the tertiary structure of the environment into which the molecule needs to enter to perform the apoptosis. The extent to which the role played by electronic factors to address the differences between the activities **143/144** and **146/147** cannot be ruled out either.



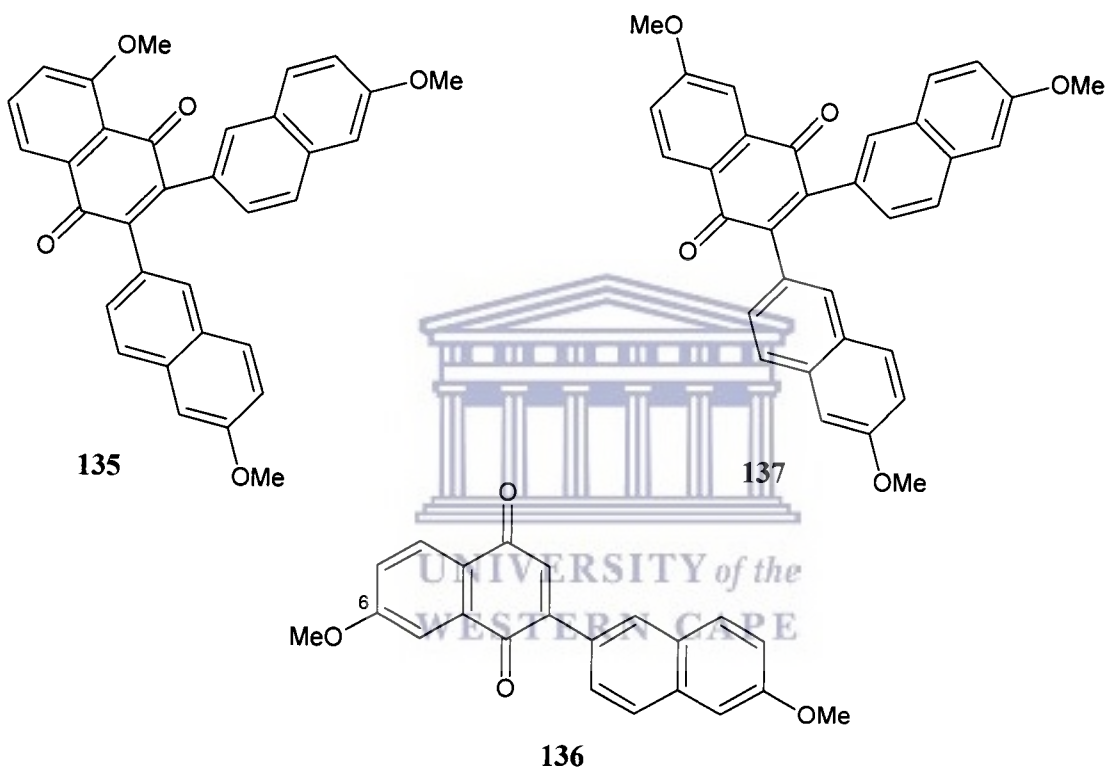
143 **146**

144 **147**

Scheme 9.3

The naphthyl compound, 2-hydroxy-8-methyl juglone **106**, included in **Fig. 9.4** proved not to be as active as their binaphthyl counterparts suggesting that for the particular mechanism of activity in the cases studied there is a requirement for a second naphthyl ring to be present in order for these compounds to be effective as apoptosis inducing drugs.

Steric hindrance in the vicinities of the carbonyl groups could also in a sense, explain the trend observed in **Fig. 9.2**, in which case introduction of a second naphthyl system viz., the bisnaphthyl compound **135 (Scheme 9.4)** results in a slight reduction in its activity compared to the mononaphthyl compound **133**. However, in compound **137** the methoxy substituent of the quinone moiety is on C6, and is thus further removed from any steric effect of the naphthalene ring resulting in the negligible difference in activity between compounds **136** and **137 (Scheme 9.4)**.



Scheme 9.4

The naphthoquinone-phenyl systems viz., **151**, **153**, **157**, and **155** were not as active as the bi-naphthyl systems against the H157 cell line **(Fig. 9.5)**. However, the results clearly demonstrate that substituents on the benzyl ring capable of donating electrons to the carbonyl group, *peri* to the methoxy group in the naphthoquinone moiety by the resonance electron-donating effect, increase the activity of the compound.

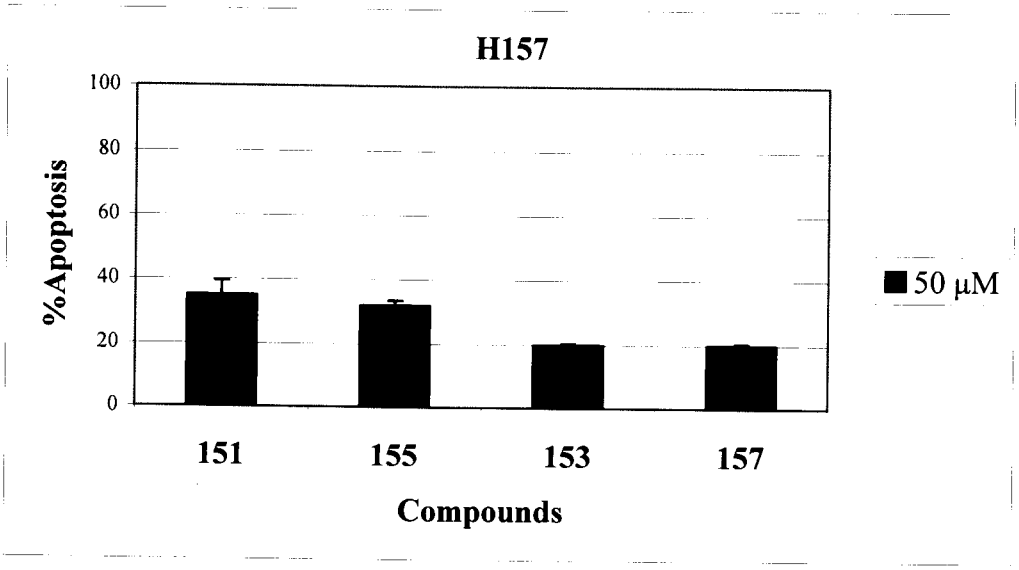
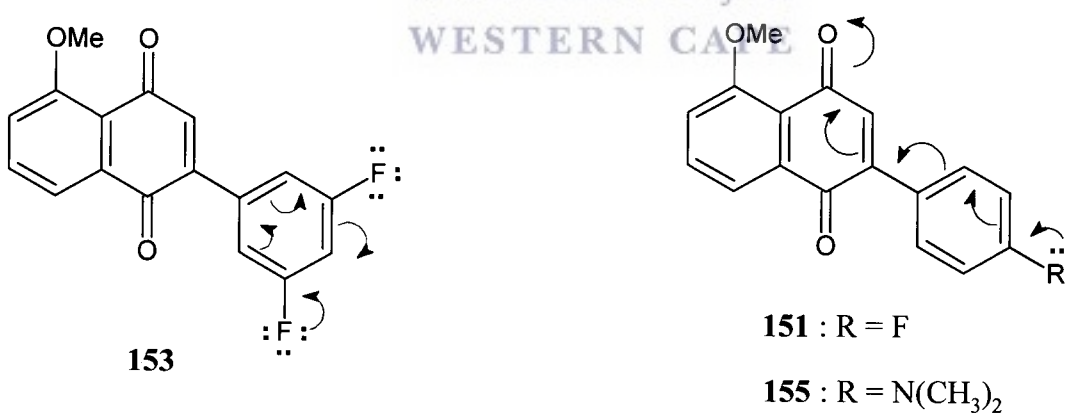


Fig. 9.5 Results for compound 151, 153, 157, and 155 for testing on H157 cell line.

In compound **153**, there are two *meta* fluorine atoms present on the benzyl ring, neither of which is capable of donating electrons through the resonance effect to the naphthoquinone moiety. On the other hand compounds **151** and **155** both possessing an electron-donating *para* substituent exhibit an increase in activity compared to the former and the electronic effects are depicted in **Scheme 9.5**.



Scheme 9.5

9.4.1.2 MCF 7 cell line

Several of the synthesized compounds were screened for their activity against the breast adenocarcinoma, MCF 7. The resulting pattern of activity for the respective compounds **(Fig. 9.6)** is more or less similar to what we observed in the screening against the H157 cell line. The two methyl ether compounds **133** and **149** exhibited enhanced activity compared to the naphthol **156**, again illustrating that the latter's influence on the MCF 7 cell line might be reduced due to hydrogen bonding. The bisnaphthyl analogue **135** of compound **133** had no effect on the MCF 7 cell line whereas the bisnaphthyl compound **137** appeared to be as active as its bi-naphthyl analogue **136**.

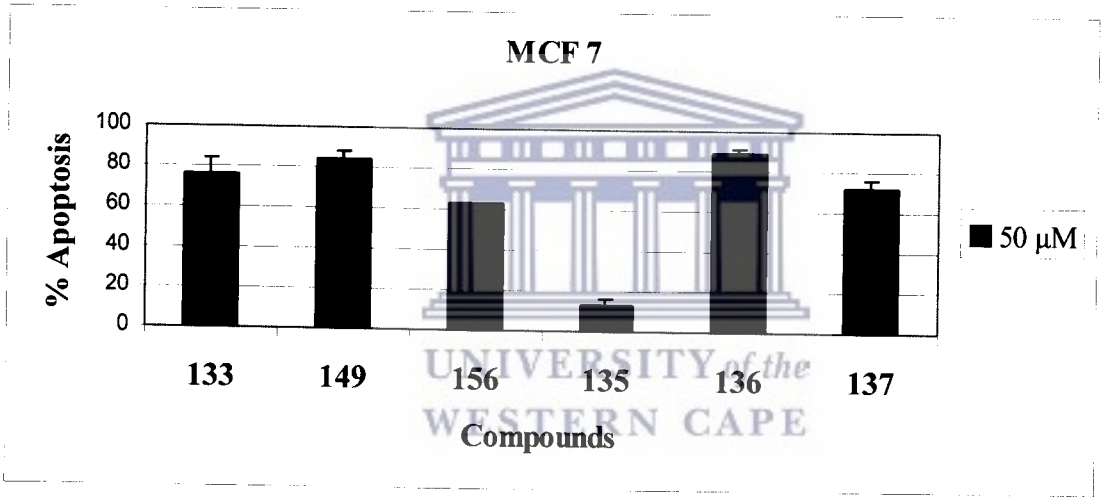


Fig. 9.6 Results for compound 133, 149, 156, 135, 136, and 137 for testing on MCF 7 cell line.

The same trend can be observed in the activity against the MCF 7 cell line of the sulphur series of compounds **(Fig. 9.7)** as in the evaluation against the H157 cell line. Again a higher activity was observed when compounds **139** and **140** were converted into their sulphone analogues **158** and **159**, respectively.

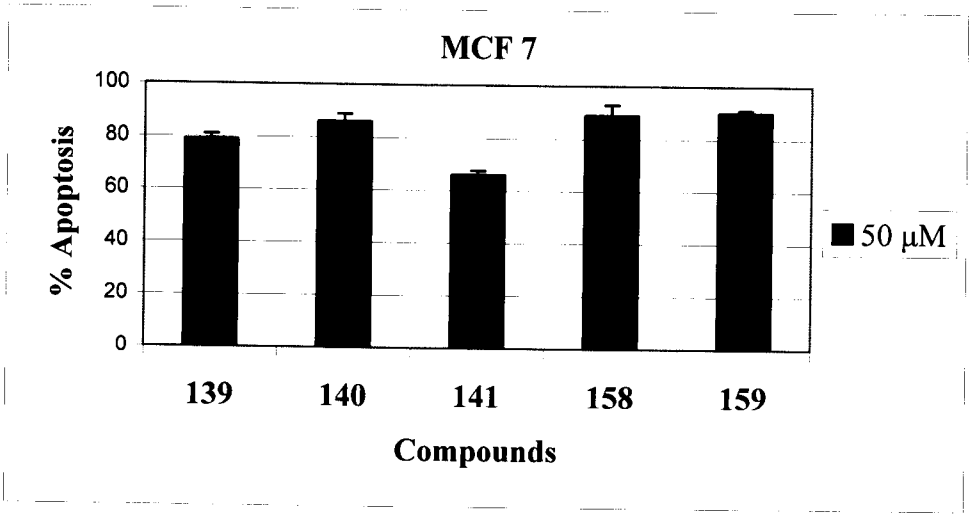


Fig. 9.7 Results for compound 139, 140, 141, 158 and 159 for testing on MCF 7 cell line.

The structural isomers **143** and **144** exhibited the highest activity against the MCF 7 cell line, as was the case against the H157 cell line whereas a huge contrast in activity was again observed between the isomeric pair **146** and **147 (Fig. 9.8)**. The naphthoquinone monomer **106** displayed a similar trend in its activity against the MCF 7 cell line as was observed against the H157 cell line.

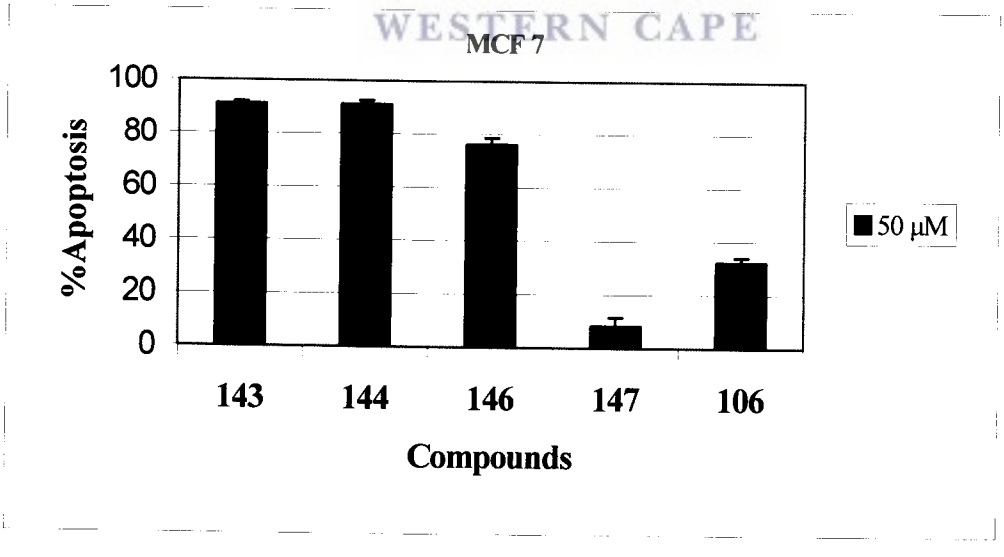


Fig. 9.8 Results for compound 143, 144, 146, 147, and 106 for testing on MCF 7 cell line.

The naphthoquinone-phenyl systems viz., **151**, **153**, **157**, and **155** showed an encouraging increase in activity against the MCF 7 cell line compared to their activity against the H157 cell line **(Fig. 9.9)**.

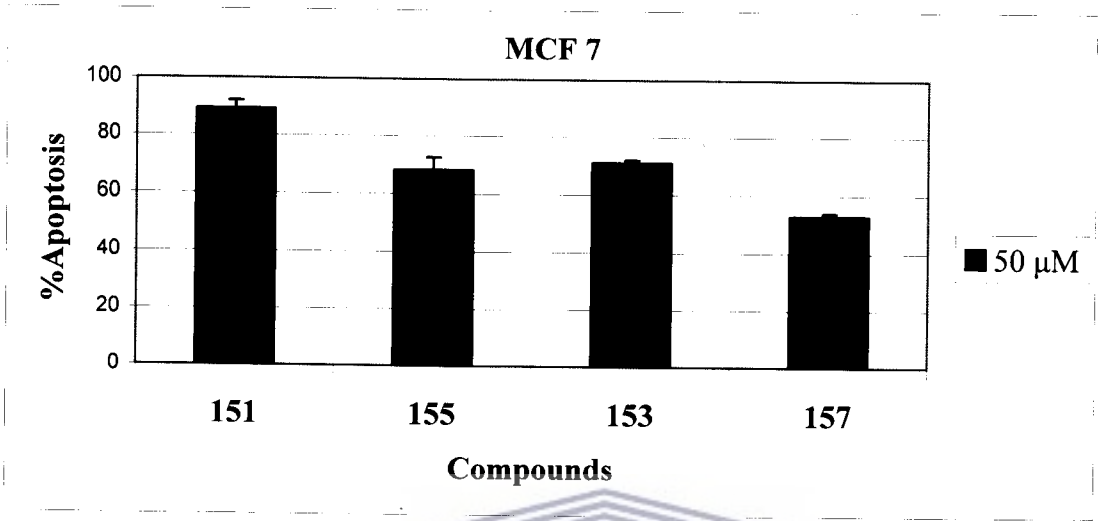


Fig. 9.9 Results for compound 151, 153, 157, and 155 for testing on MCF 7 cell line.

MCF 7 has been characterized with caspase-3 gene mutation [154] rendering it "deficient" in caspase-3. From the extensive coverage of the most common extrinsic and intrinsic pathways involved in apoptosis (Chapter 8) it can be inferred that these pathways commonly converge at caspase-3 and thus play a pivotal role in the mechanism of most apoptosis inducers. It can thus be deduced that these systems, compounds **151**, **153**, **157**, and **155** induce an apoptotic pathway independent of caspase-3. It was again observed that the naphthol **157** was less active than its methyl ether analogue **153**. Compound **151** was the most active analogue in this series which illustrates the fact that by having a substituent capable of donating electrons via the electron resonance effect the activity of the compound demonstrates an enhancement.

9.4.1.3 HeLa cell line

Several of the synthesized compounds were screened for their activity against the HeLa cell line, which is derived from human cervical carcinoma **(Fig. 9.10)**. Although there is a slight reduction, a similar trend in the activity can be observed for these compounds towards the HeLa cell line as compared to the cell lines discussed earlier. However, the tri-aryl compound **137** are more active than its bi-aryl analogue **136**. Although borh compounds' C-6 mehtoxy group is further away from any steric effects of the naphthyl substituents, the higher activity shown by tri-aryl **137** could be ascribed to the fact that the two naphthalene moieties can donate electrons to both carbonyl groups.

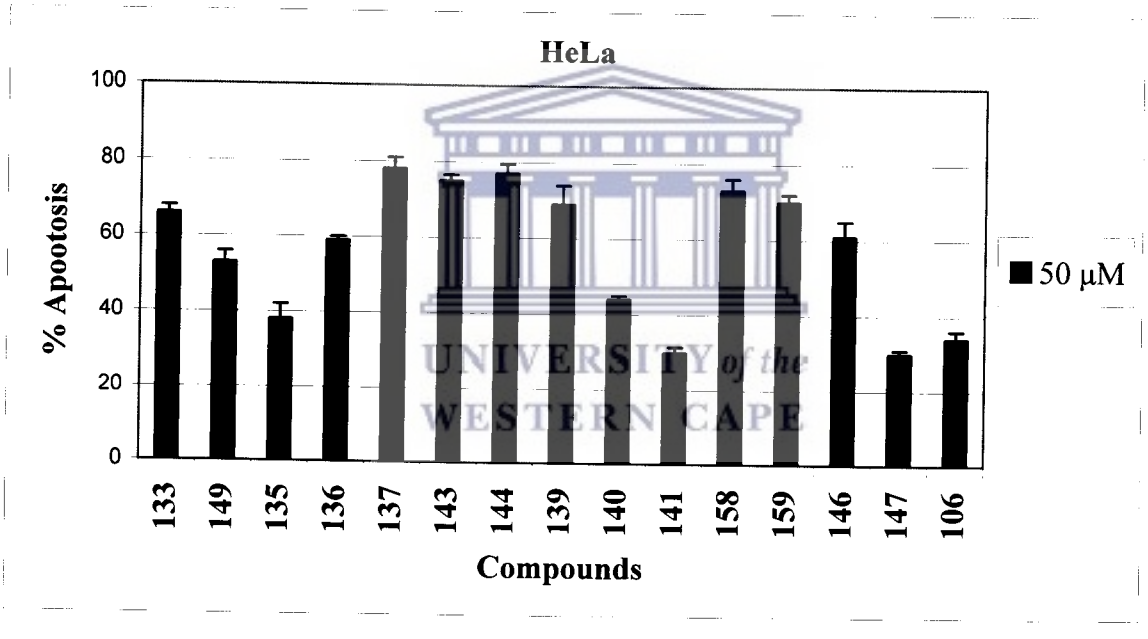


Fig. 9.10 Results for compounds screened against the HeLa cell line.

The regioisomers **143** and **144** were almost identical in activity, whilst the oxidation of compounds **139** and **140**, to their respective sulphone derivatives **158** and **159**, resulted in an increase in their activity. Compound **146** was again more active than its regioisomer **147**, whereas the naphthalene monomer **106** was only able to induce apoptosis in 34% of the cell population.

9.4.1.4. Hek239T cell line

A similar activity pattern was observed for the test compounds in the screening against the Hek239T cell line; a carcinogenic human cell line derived from embroynal kidney cells **(Fig. 9.11)**. Mononaphthyl **133** was more active than its bisnaphthyl analogue **135**, whereas the opposite was again observed for the **136/137** pairing. Compounds **140** and **141** were not as active as their structural isomer **139**. However, an expected increase was observed when compound **140** was converted to its oxidized analogue **159**.

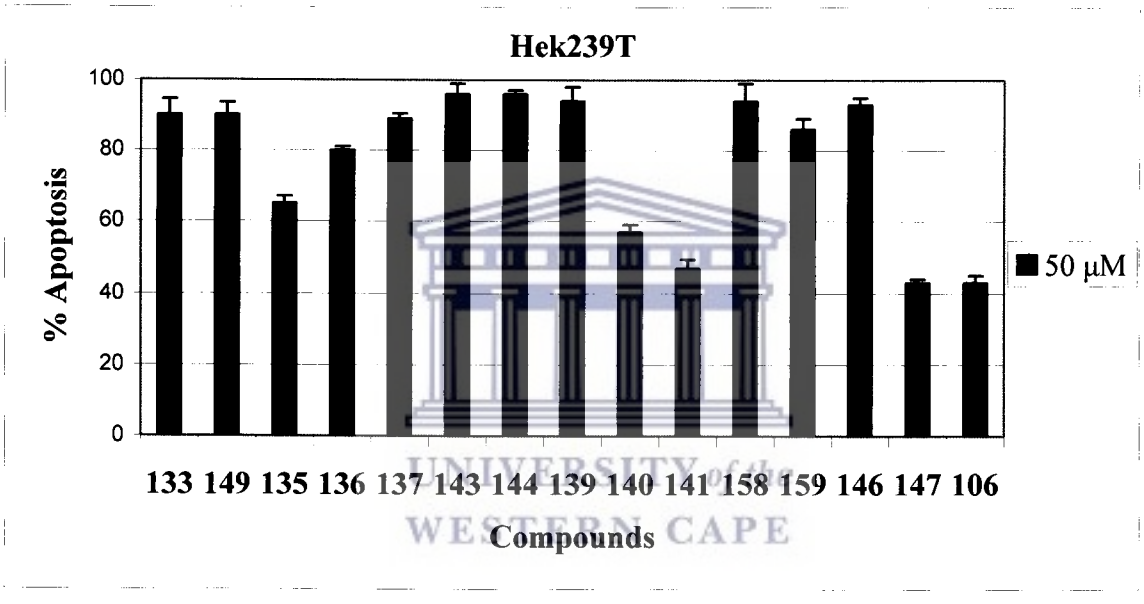


Fig. 9.11 Results for compounds screened against the Hek239T cell line.

Compounds **143** and **144**, both inducing apoptosis in 96% of the cell population, were the most active of all the compounds tested. A remarkable difference was again observed in the activity of the two regioisomers **146** and **147**.

9.4.1.5 Jurkat T cell line

Several of the synthesized compounds were screened for their activity against the Jurkat T cell line **(Fig. 9.12)**. Jurkat cells are an immortalized leukaemia cell line of T lymphocyte cells that are used to study acute, amongst others, T cell leukaemia and T cell signalling. However, their primary use is to determine the mechanism of differential susceptibility of cancers to drugs and radiation.

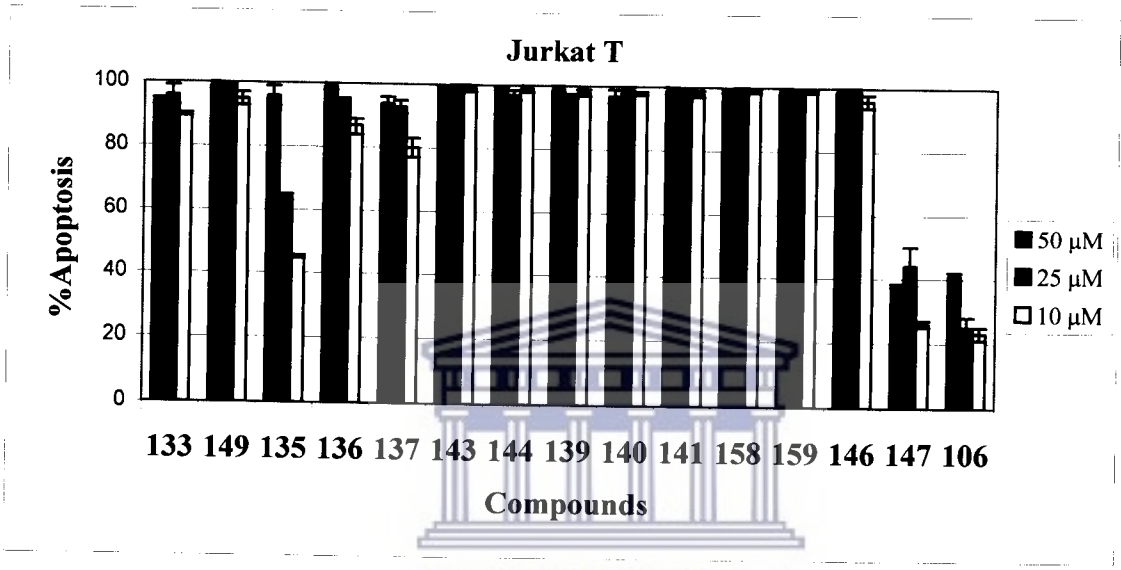


Fig. 9.12 Results for compounds screened against the Jurkat T cell line.

Most of the compounds exhibited remarkable activity (> 90%) against the cell line at all the concentrations tested viz., 50 µM, 25 µM and 10 µM. This could be ascribed to a larger surface area being exposed to the drugs, due to the fact that the Jurkat T cell line is in suspension, whilst the rest of the cell lines used in the study are of the adherent type. However, tri-aryl compound **135** showed a significant drop in activity at the lower concentrations, whereas bi-aryl **147** and quinone **106** demonstrated a similar trend in activity as they did against the other adherent cell lines discussed earlier. This again illustrate the importance of the side of the molecule bearing the carbonyl and methoxy group, peri to each other, not being too sterically crowded making the "active site" easy accessible. The indolence of quinone **106** highlights the need for a second ring for these compounds to be effective as apoptotic inducing drugs.

9.4.1.6 KMST-6

Our next objective was to evaluate the synthesized compounds against two non-cancerous cell lines viz., the human KMST-6 cell and the non-human CHO cell line. The KMST-6 cell line is derived from normal human embroynal fibroblasts that were transformed, in culture, into immortal, non-tumorogenic cells.

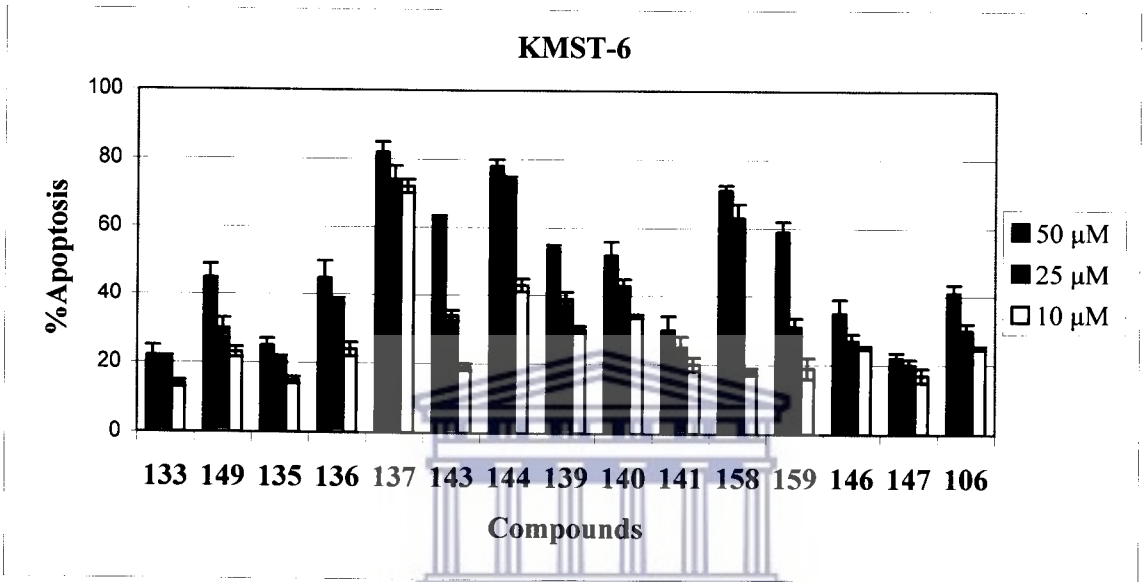


Fig. 9.13 Results for compounds screened against the KMST-6 cell line.

The activity of the compounds is concentration dependent and most of the compounds showed a notable reduction in their activity against the normal cell line **(Fig. 9.13)** compared to the other carcinogenic cell lines. These results are encouraging and illustrate that these compounds are capable of the selective killing of cancer cells. Especially compounds **133** and **146**, which exhibited activity against all the cancer cell lines used in the study, showed little activity (below 30%) against the normal cell line KMST-6. Although somewhat subdued, a similar trend in the activity of the sulphur series of compounds can be observed against the normal cell line compared to the cancer lines with the sulphone compounds **158** and **159** being more active than their unoxidized analogues **139** and **140**.

9.4.1.7 CHO cell line

Several of the synthesized compounds were screened against the normal mammalian cell line viz., the CHO (Chinese Hamster Ovaries) cell line **(Fig. 9.14)**. The majority of the compounds were efficient in inducing apoptosis at a concentration of 50 μM and appeared to be similar in activity when compared to the other cancer cell lines as apposed to the normal human cell line, KMST-6. This selective killing might be due to the different underlying mechanisms of apoptosis.

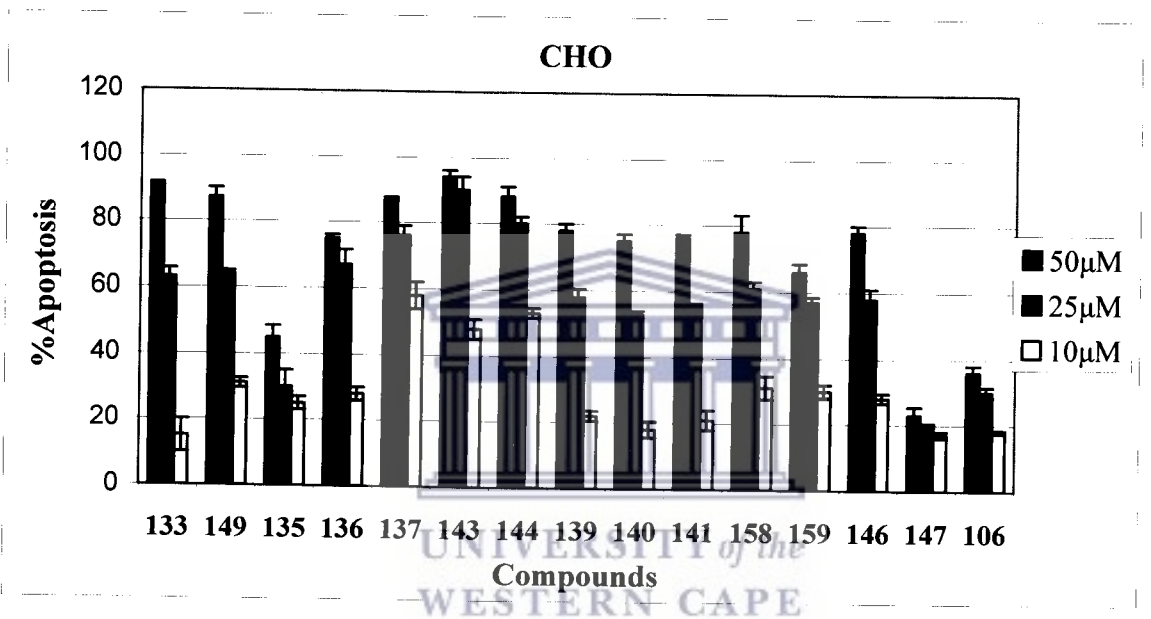


Fig. 9.14 Results for compounds A-O for testing on the CHO cell line.

The purpose of the above analyses was to identify a lead compound, which could be subjected to further testing for identifying downstream apoptotic signals. Upon deciding which cell lines and compounds to use we looked at the apoptotic potentials of the test compounds against the different cancer cell lines in conjunction with its activity against the normal human cell line KMST-6. Although, the regioisomeric pair **143** and **144**, and the sulphone pair **158** and **159**, had shown outstanding results against all the cancer cell lines they showed to be quite active against the normal human cell line KMST-6 as well. Since compounds **133** and **146** showed remarkable activity against all the cancer cell lines and were less active against the KMST-6 cell line, whereas their respective analogues **135** and **147** were always contrasting in activity, we decided to perform further analyses using these four compounds. And in

addition to the non-carcinogenic KMST-6 cell line we chose the H157 cancer cell line due to the difference in activity of compounds **133** and **146** against the latter, at a concentration of 50 μM and an incubation period of 24 h.

9.4.2 Screening for specific markers of apoptosis: DNA fragmentation

DNA fragmentation is a late event and one of the hallmarks of apoptosis. The APO-DIRECT™ Kit allows for the detection and quantification of DNA breaks by FACS analysis. During apoptosis, DNA fragmentation exposes 3'-hydroxyl groups in terminal positions. This characteristic is then used to differentiate apoptotic cells from viable cells by labeling the DNA breaks with fluorescein-tagged deoxyuridine triphosphate nucleotides (F-dUTP). The enzyme, terminal deoxynucleotidyl trransferase (TdT), catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl terminal groups of double- or single-stranded DNA [155]. Consequently, the apoptotic cells, that contain DNA breaks will fluoresce.

The cells were plated in 6 well tissue culture plates and induced with 50 μM of the test compounds for 24h. After the cells were treated, permealized and labelled with F-dUTP using the APO-DIRECT™ staining kit, they were subsequently analysed on a (Fluorescent Activated Cell Sorting) FACScan instrument using CELLQuest PRO software (BD Biosciences). Results can be acquired and analyzed in the form of a histogram or dot plot. For the results obtained in histogram form, normal cells will fluoresce in the first decade (10^1), whilst a horizontal fluorescent shift along the X-axis from the first decade (10^1) to the second (10^2) or third decade (10^3) is expected for apoptotic cells. In the case of results obtained as a dot plot, the normal convention of this display is to put DNA (Linear Red Fluorescence) on the X-axis and the F-dUTP (Log Green Fluorescence) on the Y-axis, apoptotic cells will display an increase in fluorescence of fluorescein by shifting vertically up along the Y-axis. The graph is devided into two regions, e.g. R2 and R1, corresponding to the percentage of apoptotic and normal cells, respectively.

9.4.2.1 H157 cell line

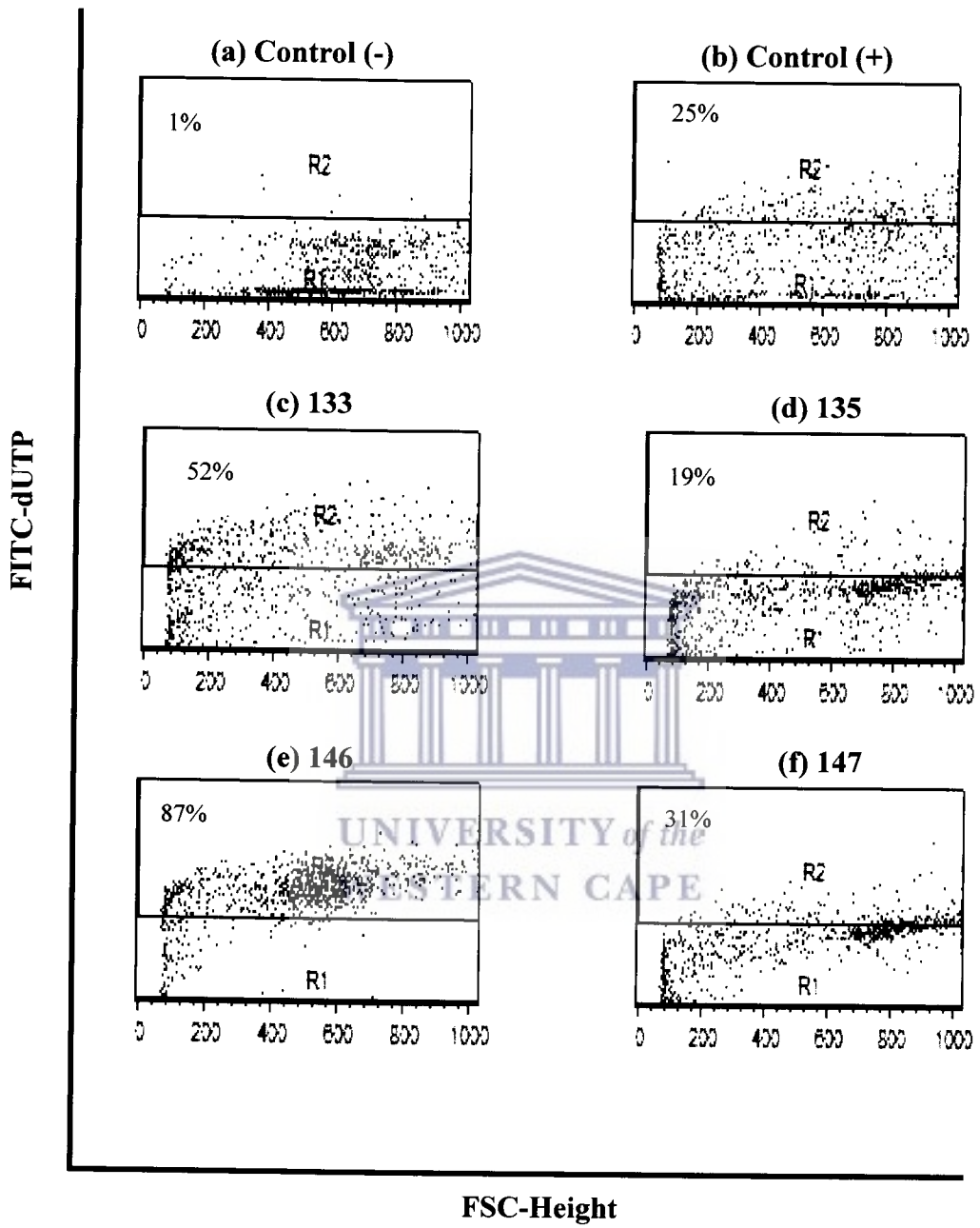


Fig. 9.15 DNA fragmentation in H157 cells after 24 h

In **Fig. 9.15 (a)** it can be seen that the cells in the normal state, i.e. without any apoptosis inducing agents, do undergo apoptosis and that only a small percentage of cells die (1%) during normal growth cycles. This is the negative control index to compare all apoptosis percentage levels obtained after the induction of the of the test compounds against the H157 cell line. **Fig 9.15 (b)** shows the influence that cisplatin

(50 μM) has on the H157 cell line after 24 h by killing up to 25% of the cell culture. Thus, our positive control shows a significant increase in F-dUTP staining and is denoted by an increase in the spreading of the cells toward the Y-axis in the dot plot diagram. Compound **133** induced DNA fragmentation in 52% of the H157 cells **[Fig. 9.15 (c)]** compared to its tri-aryl analogue **135** which only killed 19% of the cell population **[Fig. 9.15 (d)]**. Compound **146** was far more active and the majority of the cell population (87%) was in a late stage of apoptosis as illustrated in **fig. 9.15 (e)** compared to its regioisomer, which exhibited a reduction in activity as only 31% of the cell population underwent DNA fragmentation **[Fig. 9.15 (e)]**. This is visible from the amount of dots in the R2 region in the dot plot diagrams of the four test compounds. These results correspond to what we observed in the APOPercentage ™ assay after 24 h of incubation with the four test compounds and clearly demonstrate the different apoptotic potential of these compounds against the H157 cell line.

9.4.2.2 KMST-6

The results for the DNA fragmentation analyses performed on the KMST-6 cell line are depicted in **fig. 9.16**. The untreated control showed that 6% of the cell population were in the late stages of apoptosis after 24 h **[Fig. 9.16 (a)]**, whereas the positive control induced with 50 μM of cisplatin **[Fig. 9.16 (b)]**, showed only a slight increase compared to the untreated control, with 10% of the cells being apoptotic. **Fig. 9.16(c)** shows that 16%, of the non-carcinogenic cell line under investigation, was in a late apoptotic phase when induced with compound **133**, and compound **135 [Fig. 9.16(d)]**, the tri-aryl analogue of compound **133**, induced DNA fragmentation in 35% of the KMST-6 cell population after 24 h of incubation. **Fig. 9.16(e)** illustrates the DNA strand breaks in the KMST-6 cell line when treated with compound **146**, which in turn induced DNA fragmentation in 36% of the cell population, whereas the dot plot diagram **[Fig. 9.16(f)]** of compound **147**, the regioisomer of compound **146**, exhibited 42% of DNA fragmented cells. These results confirm the reduced activity of the respective compounds against the non-cancerous cell line KMST-6 exhibited by these compounds in the APOP*ercentage* assay.

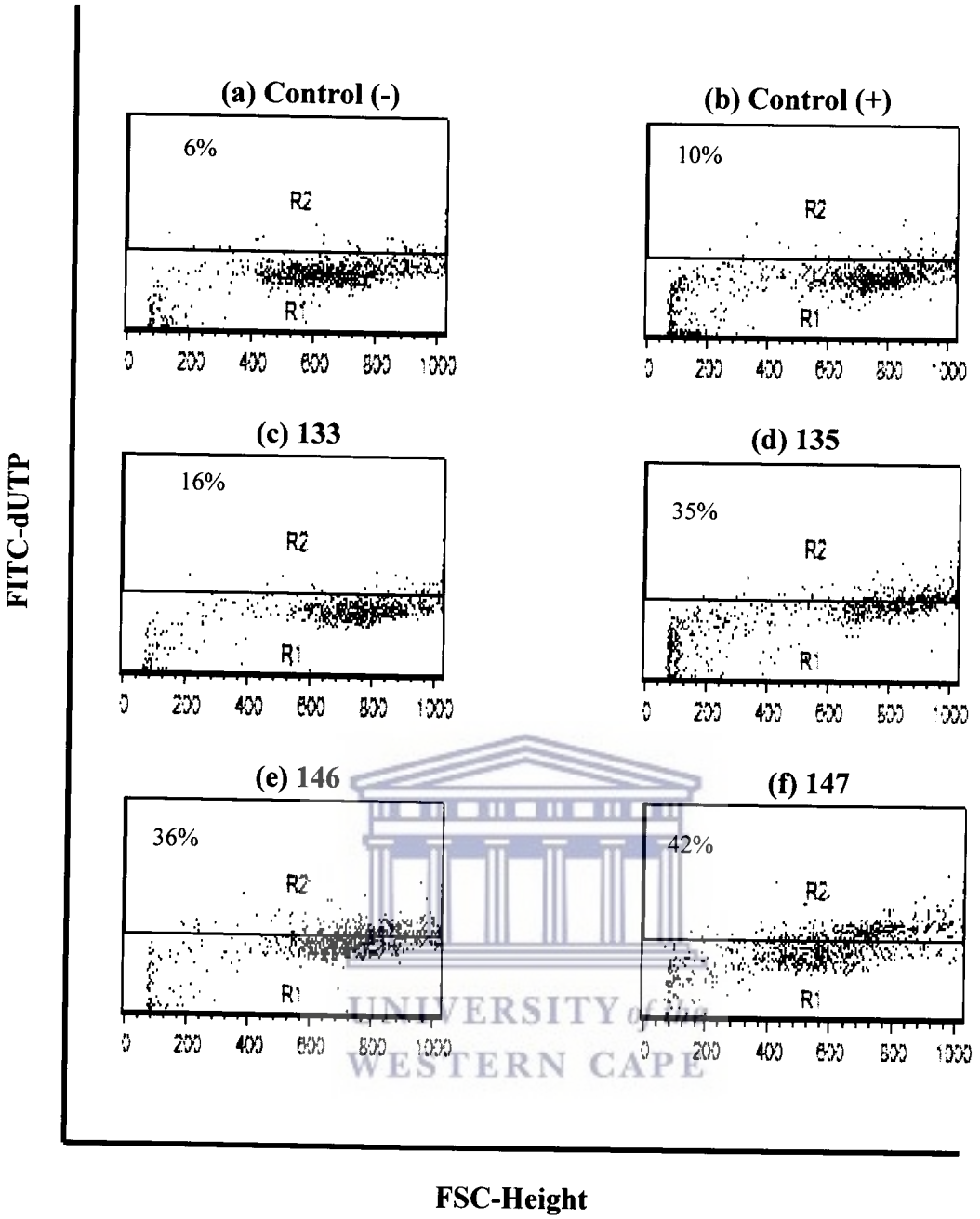


Fig. 9.16 DNA fragmentation in KMST-6 cells after 24 h

9.5 Conclusion

Several of the synthesized compounds were screened for their ability to induce apoptosis in five human cancer cell lines viz., H157, Hek239T, HeLa, Jurkat, MCF-7 and two non cancerous cell lines viz., CHO (animal) and KMST-6 (human). The APOP*ercentage* ™ assay revealed that in addition to the redox ability of the quinone carbonyl groups, the *peri* methoxy or hydroxyl group also plays a role in the apoptotic activity. The majority of the compounds proved to be active against the cancerous cell

lines whereas a reduction in their activity is observed against the non-carcinogenic human cell line KMST-6. An independent evaluation, the DNA fragmentation test, confirmed both a degree of selectivity viz., being not too active against non-cancerous cell lines on the one hand whereas on the other having a high apoptotic potential against all five of the cancer cell lines evaluated.

In the present investigation, the effect of the test compounds on expression of apoptosis regulating genes (p53, Bax, bcl-2, etc.) is unknown and thus a more detailed study could shed some light on the underlying mechanism of action of these compounds.

CHAPTER 10

CONCLUSION

Several biaryl systems, based on the framework of juglone, were successfully synthesized utilizing the Suzuki methodology. Subsequent biological evaluation against *Mycobacterium tuberculosis* and various cancer cell lines has shown in addition to the carbonyl group, that the methoxy and hydroxy group, *peri* to the carbonyl moiety, does indeed play a role in the activity of these compounds.

In conclusion, quinones play a pivotal role in energy metabolism and their widespread use as antibiotics, anti-parasitic agents, anti-tumour agents, and a variety of other agents makes it imperative to understand their effects on cellular function and the molecular mechanisms involved. Until this is not clarified, it is not possible to use a rational approach to search or design more effective quinone agents. And the current approach of random screening and analogue development will continue to dominate future research.

CHAPTER 11

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