Characterisation, synthesis and antimycobacterial activity of naphthoquinones isolated from *Euclera natalensis*

by

Frank van der Kooy

Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE (Plant Physiology)

In the Department of Botany, Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

February 2004

Supervisor: Prof. J.J.M. Meyer

© University of Pretoria
Contents

1 Introduction

1.1 Background

  1.1.1 Natural product chemistry  2
  1.1.2 Treatment of *Mycobacterium tuberculosis*  5

1.2 *Euclea natalensis*  6

1.3 Objectives of this study  8

1.4 Scope of this thesis  8

1.5 References  9

2 Isolation of naphthoquinones from *Euclea natalensis*

2.1 Introduction  12

2.2 Materials and methods  13

  2.2.1 Material  13
  2.2.2 Methods  13
  2.2.2.1 Column chromatography  13
  2.2.2.2 Thin layer chromatography  14
  2.2.2.3 High performance liquid chromatography  14
2.2.2.4 Isolation of naphthoquinones

2.2.2.5 Concentration of naphthoquinones in the plant material

2.2.2.5.1 Optimum extraction solvent

2.2.2.5.2 Determination of yields from root bark and inner part of root from two geographical regions

2.3 Results

2.3.1 Isolation of naphthoquinones

2.3.1.1 7-methyljuglone isolation

2.3.1.2 Diospyrin and mamegakinone isolation

2.3.1.3 Shinanolone isolation

2.3.1.4 Neodiospyrin isolation

2.3.1.5 Isodiospyrin isolation

2.3.1.6 Isolation of 5-hydroxy-4-methoxy-2-napthaldehyde

2.3.1.7 Isolation of lupeol and betulin

2.3.1.8 Concentration of naphthoquinones in the plant material

2.3.1.8.1 Optimum extraction solvent

2.3.1.8.2 Determination of yields from root bark and inner part of root from two geographical regions

2.4 Optimising the isolation of diospyrin and 7-methyljuglone

2.5 Discussion and conclusions

2.6 References
3 Identification of naphthoquinones isolated from *Euclea natalensis*

3.1 Introduction

3.2 Materials and methods

3.2.1 Materials

3.2.2 Methods

3.2.2.1 Ultraviolet-visible spectrophotometry (UV-vis)

3.2.2.2 Mass spectrometry (MS)

3.2.2.3 Nuclear magnetic resonance spectroscopy (NMR)

3.3 Results

3.3.1 Identification of diospyrin

3.3.2 Identification of 7-methyljuglone

3.3.3 Identification of shinanolone

3.3.4 Identification of neodiospyrin

3.3.5 Identification of isodiospyrin

3.3.6 Identification of mamegakinone

3.3.7 Identification of 5-hydroxy-4-methoxy-2-napthaldehyde

3.4 Discussion and conclusions

3.5 References
4 Synthesis of 7-methyljuglone

4.1 Introduction 61

4.2 Materials and methods 62
   4.2.1 Material 62
   4.2.2 Methods 62
   4.2.2.1 Synthesis of 8-chloro-7-methyljuglone 62
   4.2.2.2 Synthesis of 7-methyljuglone 62
   4.2.2.3 Alternative synthetic pathway for the preparation of 7-methyljuglone 63

4.3 Results and discussion 63
   4.3.1 Synthesis of 8-chloro-7-methyljuglone 63
   4.3.2 Synthesis of 7-methyljuglone 64
   4.3.3 Alternative synthetic pathway for the preparation of 7-methyljuglone 65

4.4 Conclusions 67

4.5 References 74

5 Synthesis of neodiospyrin and diospyrin

5.1 Introduction 76

5.2 Materials and methods 76
5.2.1 Material .......................... 76
5.2.2 Methods .......................... 77
  5.2.1.1 Reduction of 7-methyljuglone .... 77
  5.2.2.2 The methyljuglone-hydroquinone reaction .... 77
  5.2.2.3 Diospyrin synthesis ............ 77
5.3 Results and discussion .......... 78
  5.3.1 Reduction of 7-methyljuglone .... 78
  5.3.2 The methyljuglone-hydroquinone reaction .... 78
  5.3.3 Diospyrin synthesis ............ 81
5.4 Conclusions ....................... 82
5.5 References ......................... 85

6 Enzymatic synthesis of diospyrin

6.1 Introduction ....................... 87
6.2 Materials and methods .......... 87
  6.2.1 Material ......................... 88
  6.2.2 Methods ......................... 88
  6.2.2.1 Germination of E. natalensis seedlings .... 88
  6.2.2.2 Cell-free extract of E. natalensis seedlings .... 88
  6.2.2.3 Cell-free extract with the addition of 8-chloro-7-methyljuglone 89
6.3 Results ......................... 89
6.3.1 Germination of *E. natalensis* seedlings 89
6.3.2 Cell-free extract of *E. natalensis* seedlings 89

6.4 Discussion and conclusions 94
6.5 References 99

7 Antimycobacterial activity of the isolated compounds

7.1 Introduction 101
7.2 Materials and methods 101
   7.2.1 Materials 101
   7.2.2 Methods 102
7.3 Results and discussion 103
7.4 Conclusions 108
7.5 References 109

8 General discussion and conclusions

8.1 Isolation and identification of naphthoquinones 111
8.2 Synthesis of naphthoquinones 112
Chapter 1

Introduction

1.1 Background

1.1.1 Natural product chemistry

1.1.2 Treatment of *Mycobacterium tuberculosis*

1.2 *Euclea natalensis*

1.3 Objectives of this study

1.4 Scope of this thesis

1.5 References
Chapter 1

Introduction

1.1 Background

1.1.1 Natural product chemistry

Natural product chemistry or the research into secondary metabolites from higher plants and other organisms has been conducted for centuries. According to Taiz & Zeiger (1998) plants produce a large and diverse array of organic compounds that appear to have no direct function in growth and development. These substances are known as secondary metabolites or natural products. Unlike primary metabolites, such as chlorophyll, amino acids, nucleotides or carbohydrates, secondary metabolites have no generally recognised role in the processes of photosynthesis, respiration, solute transport and other metabolic pathways. Secondary metabolites also differ from primary metabolites in having a restricted distribution in the plant kingdom. A particular secondary metabolite may only be found in a certain plant species or a taxonomically related group of species whereas primary metabolites are found throughout the plant kingdom.

Plants use these secondary metabolites in order to defend themselves against herbivores and pathogenic microbes. In addition to defence, these secondary metabolites may also play an important role in other functions, such as structural support (e.g. lignins) or pigmentation (e.g. anthocyanins).

There are 3 classes of important secondary compounds:

a) Terpenes – consisting of isopentane units (5-carbon elements).
b) Phenolics containing a hydroxyl functional group on an aromatic ring.

\[
\text{OH} \\
\begin{array}{c}
\text{C} \\
\text{H}
\end{array}
\]

\[\text{Caffeine}\]

c) Nitrogen-containing compounds – e.g. alkaloids like caffeine, found in coffee.

From these three classes of secondary metabolites, thousands of different compounds have been isolated and characterised. Each one has got a different function (many functions are however unknown), which relates to the chemical structure of the compound, in the plant.

The compounds that were investigated during this study fall into the phenolic/terpene groups. The exact biosynthetic pathway of the naphthoquinones has not yet been confirmed and four different biosynthetic pathways for the formation of these compounds have been described (Mallavadhani et al., 1998). According to Chapman & Hall (2003), twelve secondary metabolites have been isolated from *Euclea natalensis*. Nine of these compounds are naphthoquinones. The other three compounds are two dihydroxyursanoic acids (lactone derivatives) and the other one tetrahydroxyflavanone arabinopyranoside. The chemical structures of the nine naphthoquinones are given in Fig. 1.1.

During this study three compounds have been isolated and characterised from *Euclea natalensis* for the first time. These compounds, neodiospyrin, mamegakinone and 5-hydroxy-4-methoxy-2-napthaldehyde have been isolated previously from other biological sources.
Fig. 1.1: Naphthoquinones previously isolated from *Euclea natalensis* (Chapman & Hall, 2003).
1.1.2 Treatment of *Mycobacterium tuberculosis*

Mycobacteria are believed to be amongst the oldest bacteria on earth. They are free-living organisms to be found in soil, animal dung, water, mud flats and attached to grasses and algae (Evans, 1998). It has been speculated that cattle were the source of human tuberculosis infection and that *Mycobacterium tuberculosis* is a mutant form of *M. bovis*.

The current treatment of tuberculosis (TB) patients relies on a combination of drugs (chemical structures provided in Fig.1.2) that must be administered over a period of 6 months. In 1944 streptomycin was discovered and found to be active (bacteriostatic) against *Mycobacterium tuberculosis* (Schatz & Waksman, 1944). Due to antibiotic resistance after 2-3 months, the drug had to be taken according to a special rhythm or regime. Soon after, para-aminosalicylic acid was found to have bacteriostatic activity against TB (Lehman, 1946), and it was found that the combination of the two drugs could be administered without the development of resistance. In 1952 a new drug was discovered, isoniazid, and it was found that in combination with streptomycin it was the most effective remedy available at the time. With modern drug therapy (including pyrazinamide (found in 1954), ethambutol (1962) and rifampicin (1969)), it was found that all that was necessary to treat TB, was to take the correct drugs in the correct dosage for the correct duration, which may be as “short” as 6 months. The problems that developed with above mentioned treatment regimes, is that the cost and duration of treatment meant that many people were not cured completely. This caused the disease to remain infectious and to become multi-drug resistant (MDR). Immunosuppresion induced by AIDS also caused a big upsurge in the number of cases.

Therefore, there is a need to find new drugs against TB and especially MDR TB., which will be relatively cheap and that will shorten the duration of treatment. The two naphthoquinones, diospyrin and 7-methyljuglone, previously isolated (Lall & Meyer, 2000) in our laboratory did show bacteriostatic activity against MDR strains of tuberculosis. These two compounds also showed that the duration of treatment could probably be considerably shortened than treatment with current drugs.
1.2 *Euclea natalensis*

It has been reported that the roots of *Euclea natalensis* A.DC. are used by indigenous people of southern Africa for various bacterial infections. Powdered root bark of this species is used as an ingredient in medicines to treat urinary tract infections, venereal diseases and dysmenorrhea. The custom of cleaning teeth and gums with chewed roots of *E. natalensis* is also practiced widely. The Zulu tribe of South Africa use the root bark to treat TB related symptoms such as chest diseases, bronchitis, pleurisy and asthma.

*E. natalensis* is a shrub or small to medium size tree from the Ebenaceae family. It occurs in a variety of habitats, including coastal and inland forests as well as bushveld (Fig. 1.3.a, Van Wyk & Van Wyk, 1997). The leaves are elliptic to obovate-oblong, glossy dark green above and densely covered with woolly hairs below. The margins of the leaves appear wavy as shown in Fig. 1.3.b (Van Wyk & Van Wyk, 1997).
Fig. 1.3: Distribution map (a) and leaves (b) of *E. natalensis* (Van Wyk & Van Wyk, 1997)
1.3 Objectives of this study

- The isolation of naphthoquinone compounds from *E. natalensis*.
- Characterisation of the new compounds
- Development of methods to increase the extraction yields of the most bioactive compounds.
- Synthesis of the most bioactive compounds.
- Investigation of the biochemical pathway for naphthoquinone formation in the plant.

1.4 Scope of this study

The isolation of naphthoquinones and related compounds are described in chapter 2. This chapter also deals with the optimisation of the yield of the most active compounds. Chapter 3 describes the characterisation of the compounds isolated in chapter 2 and gives a general overview on the methods used to identify organic compounds. The synthesis of the most active compound, 7-methyljuglone, is described in chapter 4 (an improved synthetic pathway is also included in this chapter). Chapter 5 deals with the biomimetic synthesis of neodiospyrin, a compound newly isolated from *E. natalensis*, and an attempt to synthesise diospyrin. Chapter 6 includes the enzymatic synthesis of diospyrin and the initial investigation of the biochemical pathway for naphthoquinone biosynthesis in the plant. The activity of the isolated compounds against *M. tuberculosis* can be found in chapter 7. Chapter 8 consists of the general discussion and conclusions and chapter 9 of the summary.
1.5 References

Software copyright © Hampden Data Services Ltd.


Lall, N. & Meyer, J.J.M. 2000. Antibacterial activity of water and acetone extracts of


chemotaxonomy of *Diospyros*. *Phytochemistry*. 49: 901-951

Schatz, A. & Waksman, S.A. 1944. Effect of streptomycin and other antibiotic
substances upon mycobacterium tuberculosis and related organisms. *Proceedings of
the Society for Experimental Biology and Medicine*. 57: 244-245.

Sunderland, Massachusetts.

Van Wyk, B. & Van Wyk, P. 1997. Field guide to trees of Southern Africa, pp184-
185. Struik, McKenzie street, Cape Town.

Chapter 2

Isolation of naphthoquinones from *Euclea natalensis*

2.1 Introduction

2.2 Materials and methods

2.2.1 Material

2.2.2 Methods

2.2.2.1 Column chromatography

2.2.2.2 Thin layer chromatography

2.2.2.3 High performance liquid chromatography

2.2.2.4 Isolation of naphthoquinones

2.2.2.5 Concentration of naphthoquinones in
the plantmaterial

2.2.2.5.1 Optimum extraction solvent

2.2.2.5.2 Determination of yields from root bark and
inner part of root from two geographical regions

2.3 Results

2.3.1 Isolation of naphthoquinones

2.3.1.1 7-methyljuglone isolation

2.3.1.2 Diospyrin and mamegakione isolation

2.3.1.3 Shinanolone isolation

2.3.1.4 Neodospyrin isolation
2.3.1.5 Isodiospyrin isolation 23

2.3.1.6 Isolation of 5-hydroxy-4-methoxy-2-napthaldehyde 23

2.3.1.7 Isolation of lupeol and betulin 24

2.3.1.8 Concentration of naphthoquinones in the plantmaterial 24

2.3.1.8.1 Optimum extraction solvent 24

2.3.1.8.2 Determination of yields from root bark and inner part of root from two geographical regions 29

2.4 Optimising the isolation of diospyrin and 7-methyljuglone 32

2.5 Discussion and conclusions 32

2.6 References 35
Chapter 2

Isolation of naphthoquinones from *Euclea natalensis*

2.1 Introduction

The isolation of the two known naphthoquinones, diospyrin and 7-methyljuglone, from *Euclea natalensis* A.DC. (Lall & Meyer, 2000) and the discovery of their antituberculosis activity, led to the further investigation of the chemical composition of this species. These naphthoquinones also showed significant activity against drug-resistant TB.

Various authors used chloroform to extract naphthoquinones from various plant species and from different plant parts (Ferreira *et al.*, 1973, Van der Vuyver *et al.*, 1974). It was therefore decided to investigate the chloroform extract of the roots for other naphthoquinones, which may show activity against TB.

This chapter will deal primarily with the isolation of the naphthoquinones (their structure elucidation will be described in chapter 3) and the optimization of the extraction and isolation procedures. It was important to increase the yields of the naphthoquinones for the *in vivo* mouse trials of these anti-TB compounds. This chapter will also deal with experiments towards finding the plant parts with the highest concentration of the active compounds, as well as comparing the concentration of naphthoquinones from two different locations.
2.2 Materials and methods

2.2.1 Material

The *E. natalensis* root material was collected in Gaza province in southern Mozambique and at Trafalgar, southern Kwazulu-Natal, at various times throughout the year. The samples were compared to a voucher specimen (N.L. 22), which was deposited at the H.G.W.J. Schweickerdt herbarium at the University of Pretoria. Material collected during the summer months were used to compare the concentrations of compounds at the two different locations.

2.2.2 Methods

Various techniques are available for the purification and identification of natural products. During this study the compounds were purified by means of column chromatography and compared to standards with the use of thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

2.2.2.1 Column chromatography

A dry column technique was employed to fractionate the crude extract. This technique was used throughout this study unless otherwise stated. Dry silica gel (silica gel 60, 0.08mm, Merck, Darmstadt) was added to a glass column of choice. The sample size will dictate the column size. A good estimate is to use 500 g of silica gel for every 5 g of sample (Houghton & Raman, 1998). The dry extract was re-dissolved in a minimum amount of chloroform and added to dry silica powder in a suitable glass container. This mixture was stirred until all the chloroform evaporated, after which it was added on top of the silica gel inside the glass column. Clean dry silica powder was added on top of this to act as a physical buffer, when adding the mobile phase. The mobile phase was added to the column without disturbing the extract containing silica band, and the eluting fractions were collected.
2.2.2.2 Thin layer chromatography (TLC)

The simplest and cheapest of these techniques is the TLC method. If the isolated sample gives the same retardation factor (Rf) value as a known standard after using about 5 different mobile phases, it can be concluded that the sample is most probably the same as the standard. The Rf value is measured as the distance moved by the compound divided by the distance moved by the solvent font.

2.2.2.3 High performance liquid chromatography (HPLC)

A second more expensive technique is that of HPLC. A sample is applied into an injection port, which is connected to a specific column (usually silica) through which the mobile phase continuously flows. Separation of different compounds occurs due to their different affinities for the stationary phase (the silica inside the column) and the mobile phase. The compound with the greatest affinity for the mobile phase will elute first from the column, whereas the compound with the greatest affinity for the stationary phase will elute last from the column. The eluting compounds are detected with a suitable detector and plotted on a graph of detector signal versus time. The time between introduction of the sample and the tip of the peak on the graph is called the retention time (RT). The RT of any compound will remain the same given that exactly the same parameters such as mobile phase, stationary phase, temperature of column/mobile phase and pH of mobile phase are used. If the RT of an unknown compound is the same as a standard compound, under a number of different conditions, then the two compounds are probably the same. (It is important to confirm this with the use of a different technique such as NMR spectroscopy) (McMaster, 1994).

2.2.2.4 Isolation of naphthoquinones.

The root material was air-dried at room temperature and ground into a fine powder. The powdered root material (1.5kg) was extracted with 10 L of chloroform for 48 hours at
room temperature. The solid root material was filtered of and re-extracted in 10L of chloroform. This procedure was repeated three times. Using a rotary-evaporator the extract was dried to yield 25 g of crude extract (yield 1.7%). The crude extract was spotted on a TLC plate (Merck, Kieselgel F256, 20x20cm) with authentic standards diospyrin, 7-methyljuglone and shinanolone and developed in hexane:ethyl acetate (5:2). The TLC plate was examined under UV light at 254 and 366 nm, to confirm the presence of naphthoquinones. After the examination under UV light the TLC plate was dipped in vanillin (7.5g vanillin, 250ml acetone, 5ml H$_2$SO$_4$) and heated at 105°C for 2 minutes. The Rf values, colouration and the spot morphology were compared to the reference standards. A dry silica gel column was prepared and the crude extract was fractionated with a 9:1 hexane: ethyl acetate (1.5L) mobile phase. This was followed with an 8:2 ratio (1.5L) until a 1:1 ratio was reached.

2.2.2.5 Concentration of naphthoquinones in the plant material

2.2.2.5.1 Optimum extraction solvent

Three solvents (acetone, chloroform and petroleum ether (60-80°C boiling range)) were tested for their ability to extract naphthoquinones from the root material of *E. natalensis* (all three solvents were previously used by various authors). Fifteen grams of the grounded root material was added to 300 ml of each of the three solvents. It was left to stand for one day (24 hours) while shaking occasionally. The root material was filtered off and the filtrate was dried using a rotary evaporator. This yielded the crude extracts of the three samples (Table 2.1). Five mg of each of the samples was dissolved in 2ml of acetonitrile with the use of an ultrasonic bath. Ten μl was injected into the HPLC using the following conditions: 62.5% acetonitrile: 5% acetic acid as mobile phase. A photo diode array detector set on 254, 325 and 430 nm was used for detection of the naphthoquinones. A Luna RP 18, 150mm, 3 μm column was used as the stationary phase. To determine the concentrations of the two naphthoquinones standard curves for diospyrin and methyljuglone were prepared using four concentrations each.
2.2.2.5.2 Determination of yields from root bark and inner part of root from two geographical regions.

One gram of root material, which was mechanically separated into their root bark and the remaining root (inner part of root) from the different regions, were grinded and extracted in 40 ml of chloroform. The extracts were left to stand for 60 hours after which the solids were filtered off and the extracts dried. The concentration of 7-methyljuglone and diospyrin in these four samples were calculated in the same way as described in section 2.2.2.5.1, and expressed in mg/kg of root material. Five μl of a 5mg/ml sample was injected into the HPLC using the standard conditions.

2.3 Results

2.3.1 Isolation of naphthoquinones.

The yield of the crude extract ranged from 1.2% - 3.5% in subsequent extractions. The crude extract was spotted on a TLC plate (Merck, Kieselgel F256, 20x20cm) with authentic standards diospyrin (Rf = 0.78), 7-methyljuglone (Rf = 0.88) and shinanolone (Rf = 0.31) and developed in hexane:ethyl acetate (5:2). Using a UV light at 254 and 366 nm, it was concluded that the crude extract contained all three the standard compounds (Fig. 2.1). This was confirmed by dipping the TLC plate in vanillin (7.5g vanillin, 250ml acetone, 5ml H₂SO₄) and after heating comparing the Rf values, colouration and the spot morphology with the reference standards. The collected fractions (121) were tested on TLC with the naphthoquinone standards. According to the TLC profile fractions 5-39 contained 7-methyljuglone (11.0g), 41-87 diospyrin (1.5g) and 89-121 shinanolone (1.7g). The similar fractions were combined and the solvents evaporated. Fig. 2.2 schematically represents the isolation steps followed.
Fig. 2.1: TLC profile of the crude extract under visible light (left) and under UV light (right). Lane 1: crude extract, lane 2: 7-methyljuglone, lane 3: shinanolone, lane 4: diospyrin and lane 5: neodiospyrin.

2.3.1.1 7-Methyljuglone isolation

A dry silica column was prepared for the fractionation of the crude 7-methyljuglone fraction (1 g). As eluent, 2 L of hexane was used, followed by 2 L of hexane: ethyl acetate (9:1 ratio). Thirty-four fractions were collected of which fractions 3-24 contained 7-methyljuglone. These fractions were combined, dried (4 g) and subjected to another dry silica column using hexane: ethyl acetate in the following ratios 1:0; 9.75:0.25 - 9:1 (1 L aliquots). This solvent system was employed to increase the time needed for the completion of the column in an attempt to improve the efficiency of the separation. Fractions 4-24 of 56 fractions were combined and dried to yield 1.77 g of partially pure 7-methyljuglone.
Fig. 2.2: Schematic representation of the purification steps for the isolation of the different compounds.
It was decided to use a size exclusion chromatographic technique to purify the partially pure 7-methyljuglone and for this a Sephadex LH-20 column was prepared. The Sephadex material was mixed with methanol (MeOH) and allowed to swell for 1.5 hours, after which it was added into a glass column and allowed to settle for a further 30 min. The crude 7-methyljuglone sample was dissolved in the minimum amount of MeOH and added to the top of the column. As eluent MeOH was used. Thirty fractions were collected and tested for purity employing the standard TLC methods. The separation was inefficient and yielded only a very small quantity of pure 7-methyljuglone. All the fractions were combined and dried.

Another dry silica column was prepared with hexane: chloroform (6:4) (1L) followed by a 1:1 ratio (1L) as eluents. Fractions 1-11 contained a known compound identified by previous researchers as lupeol. Fractions 12 – 25 (1.2g) contained 7-methyljuglone and were combined and dried. An isocratic mobile phase of hexane: chloroform (9:1) was employed in another dry silica column. Fractions 15-45 were combined as semi-pure 7-methyljuglone. The 7-methyljuglone containing fractions (15-45) were dried and re-dissolved in MeOH. This mixture was heated while water was added drop-wise. The addition of water was stopped as soon as the mixture’s saturation point was reached.

This is usually the point where the mixture becomes turbid. The mixture was cooled down on ice and left to stand overnight to give ample time for crystal formation. The crystals were filtered off and washed with cold 90% MeOH, to yield 265 mg (0.017%) of 7-methyljuglone. The sample was authenticated by means of TLC with mobile phase hexane: ethyl acetate (5:1) and $^1$H NMR (described in chapter 3).

2.3.1.2 Diospyrin and mamegakinone isolation

A dry silica column was prepared and 1.5 g of the crude diospyrin-containing fraction was subjected to it. An isocratic mobile phase of hexane: ethyl acetate (8:2) was employed. Fractions 17-34 were combined, dried and re-dissolved in MeOH. Diospyrin
is insoluble in MeOH and could therefore be filtered off. This procedure, also called a washing procedure, was repeated twice. It yielded 460mg (0.03%) of pure diospyrin. The sample was authenticated by means of NMR and TLC using hexane: ethyl acetate (5:2) as mobile phase. Mamegakinone crystallized from chloroform out of this crude diospyrin-containing fraction. The crystals were filtered of and tested on TLC for purity. The sample was authenticated by means of NMR analysis.

2.3.1.3 Shinanolone isolation

The impure shinanolone fraction (89-121) from the first column (2.3.1) was mixed with silica and subjected to a silica column using hexane: ethyl acetate in an 8:2 ratio (3L) as the mobile phase. The mobile phase ratio was changed to 7:3 (1L) and thereafter to 6:4 (1L) ratio. The collected fractions were spotted on a TLC plate and developed using hexane: ethyl acetate (5:3) as the mobile phase. The fractions containing shinanolone were combined according to the TLC profile. Depending on the purity of this semi-pure fraction it may be crystallized from aqueous methanol. In this case the semi-pure fraction was not pure enough and therefore a Sephadex LH-20 column was prepared in methanol. The semi-pure shinanolone fraction was dissolved in methanol and added to the column. Fractions 3-11 contained predominantly shinanolone. This fraction was chromatographed on a silica gel column using chloroform: methanol (9.7:0.3) as eluent. Sixty-one fractions were collected. Fractions 41-61 yielded pure shinanolone. The fractions 2-39 (1.2g) contained significant amounts of shinanolone and an attempt was made to crystallize the shinanolone in this fraction by adding hot methanol to it until everything dissolved. Water was added drop wise until the solution became saturated. The solution was left to cool down and 490 mg (0.032%) of shinanolone crystallized from the solution. The shinanolone was tested for purity on a TLC plate using hexane: ethyl acetate (5:3) as eluent and with $^1$H NMR.
2.3.1.4 Neodiospyrin isolation

Neodiospyrin was isolated from the same fraction containing diospyrin. The pure diospyrin obtained in 2.3.1.2 did contain significant quantities of neodiospyrin but these two compounds cannot be separated by means of a TLC analysis. The Rf value, colour and spot morphology of both compounds appears exactly the same. Four different solvent systems were tested on TLC in an attempt to separate the two compounds. A reverse phase TLC plate (Merck, DC-Alufolien, 20x20cm, RP-18, F254s) was developed with ethanol: acetic acid: water (6.5:0.5:3) as the mobile phase. No separation occurred, except when the same plate was redeveloped twice in the same solvent system. The two compounds could be separated on HPLC using the same solvent system as described above or 62.5% acetonitrile: 5% acetic acid. The retention time of neodiospyrin was 10.3 minutes while that of diospyrin was 12.7 minutes (Fig. 2.3).

According to the literature (Tezuka et al., 1972, Van der Vuyver & Gerritsma 1976) this mixture of naphthoquinones can be separated by means of their dimethyl ether derivatives. This method does change the chemical structure and would therefore influence the activity of the compound. It was therefore decided that a different separation technique should be developed. An attempt was made to separate the compounds by preparing a preparative reverse phase silica column with acetonitrile 62.5% acetic acid 5% as mobile phase. There was however no adequate separation. A washing procedure was employed by washing the mixture of diospyrin and neodiospyrin with methanol. Neodiospyrin dissolves slightly better in methanol than diospyrin (neodiospyrin is therefore slightly more polar than diospyrin).
Fig. 2.3: Chromatogram showing the separation that can be achieved between neodiospyrin (Rt = 10.3) and diospyrin (Rt = 12.8) with the HPLC.

This difference was employed to separate the two compounds further. The methanol filtrate was dried and washed with hexane at 40 °C for 30 min (neodiospyrin is slightly more insoluble in hexane than diospyrin because of its polarity). The insoluble material was filtered off and tested on HPLC for purity. It contained 80% pure neodiospyrin with 20% diospyrin. The process was repeated and yielded 90% pure neodiospyrin. To test for purity HPLC analysis was employed using 62.5% acetonitrile: 5% acetic acid as mobile phase. A photo diode array detector set on 254, 325 and 430 nm was used for detection of the naphthoquinones. A Luna RP 18, 150mm, 3 µm column was used as the stationary phase.
2.3.1.5 Isodiospyrin isolation

Isodiospyrin was isolated from the crude diospyrin-containing fraction (fractions 41-87). It has a slightly lower Rf value (0.72) than diospyrin and therefore appears in the fractions after the diospyrin containing fractions. The fractionation of diospyrin (described in 2.3.1.2) yielded isodiospyrin in fractions 34-38. The concentration of this compound appeared to be lower than that of diospyrin in this fraction. The fractions were combined and dried using a rotary evaporator. The dry material was re-dissolved in the minimum amount of chloroform and left to stand. Isodiospyrin crystallized out of this solution and its structure was confirmed by $^1$H NMR.

2.3.1.6 Isolation of 5-hydroxy-4-methoxy-2-napthaldehyde

The Rf value of 5-hydroxy-4-methoxy-2-napthaldehyde (0.78) on TLC is exactly the same as that of diospyrin, using hexane: ethyl acetate (5:2) as eluent. Under UV light, 5-hydroxy-4-methoxy-2-napthaldehyde, appears light blue while diospyrin appears dark brown. It is also possible to separate them due to the solubility of 5-hydroxy-4-methoxy-2-napthaldehyde in methanol and the insolubility of diospyrin in methanol. During the washing procedure in the purification process of diospyrin, 5-hydroxy-4-methoxy-2-napthaldehyde remained in the methanol filtrate while diospyrin and neodiospyrin were mainly un-dissolved.

The methanol filtrate was dried and re-dissolved in the minimum amount of MeOH. Water was added drop-wise and the solution was left to stand for crystallization to take place. The crystals were filtered off and tested on TLC for purity. The crystals proved to be impure. The filtrate and the impure crystals were then mixed in aqueous methanol.
The methanol was evaporated and the water solution containing 5-hydroxy-4-methoxy-2-napthaldehyde subjected to solid phase extraction using a RP 18 column.

The mixture was added to the column and the water with the water soluble impurities were removed by suction. The column was air dried for 5 minutes and 5-hydroxy-4-methoxy-2-napthaldehyde was washed out of the column with acetone. The compound was sufficiently pure for structural elucidation.

2.3.1.7 Isolation of lupeol and betulin

These two compounds were isolated from the crude 7-methyljuglone fraction (fractions 5-39 of the first column). During the fractionation of 7-methyljuglone, lupeol crystallized spontaneously out of the ethyl acetate: hexane mixture, which was used as eluent. The white crystals were filtered off and identified by means of TLC co-spotted with an authentic standard. The Rf value of lupeol is exactly the same as for 7-methyljuglone when using hexane: ethyl acetate (5:2) as eluent. Lupeol however appears dark blue when heated with vanillin, while 7-methyljuglone has a brownish appearance. Betulin does however have a smaller Rf value compared to 7-methyljuglone and crystallized spontaneously from the solvent mixture used for the isolation of 7-methyljuglone. The identification was done by comparing the filtered crystals with an authentic specimen on TLC.

2.3.1.8 Concentration of naphthoquinones in the plant material

2.3.1.8.1 Optimum extraction solvent

Three solvents (acetone, chloroform and petroleum ether (60-80°C boiling range)) were tested for their ability to extract naphthoquinones from the root material of E. natalensis. Table 2.1 gives the concentrations of the naphthoquinones extracted with the different solvents. The percentage area of the peaks for each of the naphthoquinones was compared to those of standards to determine which solvent would give the optimum yield
(Fig. 2.4-2.6). To determine the concentrations of the two naphthoquinones standard curves for diospyrin and 7-methyljuglone were prepared using four concentrations each.

Table 2.1. Yield of crude extracts, 7-methyljuglone and diospyrin by using different organic solvents. The results are expressed in mg/ kg of root material.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Root material extracted (g)</th>
<th>Crude yield in g/kg (%)</th>
<th>7-Methyl juglone (mg/kg)</th>
<th>Diospyrin (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>15.23</td>
<td>27.60 (2.76%)</td>
<td>54.88</td>
<td>209.60</td>
</tr>
<tr>
<td>Chloroform</td>
<td>15.01</td>
<td>49.97 (5.00%)</td>
<td>18.00</td>
<td>503.70</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>15.24</td>
<td>23.62 (2.36%)</td>
<td>9.19</td>
<td>196.06</td>
</tr>
</tbody>
</table>
Fig 2.4: Acetone extract chromatogram showing 7-methyljuglone at RT 3.657 and diospyrin at RT 8.478
Fig 2.5: Chloroform extract chromatogram showing 7-methyljuglone at RT 3.632 and diospyrin at RT 8.353
Fig 2.6: Petroleum ether extract chromatogram showing 7-methyljuglone at RT 3.723 and diospyrin at RT 8.724.
Material collected during the summer from southern Mozambique (Gaza province) and southern KwaZulu-Natal (Trafalgar) were compared by means of HPLC. The root bark and the remaining inner part of the root of each of these samples were separated and compared to determine which contained the highest concentration of diospyrin and 7-methyljuglone. Table 2.2 gives the concentrations of the naphthoquinones from the different regions. The chromatograms of the samples are given in Fig. 2.7-2.10.

**Table 2.2.** Concentration (mg/kg) of diospyrin and 7-methyljuglone in the root bark and inner root of two different specimens collected at Mozambique (Mozam) and southern KwaZulu-Natal (Trafal).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rootbark</th>
<th>Inner root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mozam</td>
<td>Trafal</td>
</tr>
<tr>
<td>diospyrin</td>
<td>3680 ± 87</td>
<td>1280 ± 29</td>
</tr>
<tr>
<td>7-methyljuglone</td>
<td>414 ± 9</td>
<td>1800 ± 35</td>
</tr>
</tbody>
</table>
Fig 2.7: Chromatogram showing the constituents of the root bark extract from material collected in Mozambique.

Fig 2.8: Chromatogram showing the constituents of the inner root extract from material collected in Mozambique.
**Fig 2.9:** Chromatogram showing the constituents of the root bark extract from material collected at Trafalgar.

**Fig 2.10:** Chromatogram showing the constituents of the inner root extract from material collected Trafalgar.
2.4 Optimising the isolation of diospyrin and 7-methyljuglone

The isolation procedure for the purification of 7-methyljuglone and diospyrin has been improved by employing washing procedures and liquid: liquid-partitioning procedures. The purification of diospyrin as described in paragraph 2.3.1.2 was improved by washing the crude diospyrin fraction with MeOH at 60°C. This step was repeated until diospyrin were pure as confirmed with HPLC analysis. In this procedure only one silica column for the purification of diospyrin is used. Diospyrin can also be purified further, if needed, by crystallization from chloroform/petroleum ether.

7-Methyljuglone can be purified by means of two silica columns and liquid:liquid partitioning. A dry silica column was employed as described in section 2.3.1.1. The crude 7-methyljuglone-containing fraction was then subjected to another dry silica column. As eluent, hexane: ethyl acetate (9:1) was used isocratically. The fractions containing 7-methyljuglone were combined and dried. This fraction was re-dissolved in hexane (200ml) and placed in a glass-separating funnel. 90% MeOH (200ml) was added to the hexane solution and the mixture was shaken for two minutes. The solution was left to stand for approximately 5 minutes to give time for the two immiscible solvents to separate. The aqueous MeOH layer was tapped into a separate container while the organic (hexane) layer was left in the separating funnel. The organic layer was extracted three times with aqueous MeOH. The aqueous MeOH mixture contains predominantly 7-methyljuglone and the hexane layer lupeol and other hydrocarbon impurities. The 7-methyljuglone was dried and crystallized, if needed, from aqueous MeOH or chloroform/petroleum ether.

2.5 Discussion and conclusions

The previously isolated naphthoquinones, 7-methyljuglone and diospyrin were present in all the samples collected at different times of the year. The concentration of these naphthoquinones differed in the root bark and inner root and also from the different
locations. These experiments did not give conclusive evidence for the best plant part or extraction solvent and should be repeated in future to ensure more accurate results. In future a larger sample size will be used to examine the optimum extraction solvent. The influence of different extraction times and different extractant volumes will also be investigated. This will yield information that will improve the optimum extraction of naphthoquinones from *E. natalensis*.

The variation of diospyrin and 7-methyljuglone in *E. natalensis* at the two different locations appears to be conflicting. In the Mozambique sample, diospyrin is in a higher concentration in the root bark while 7-methyljuglone has a higher concentration in the remaining inner root. The Trafalgar sample indicated that 7-methyljuglone and diospyrin was concentrated predominantly in the inner root. The explanation for these differences might be that the collected sample from Mozambique might have been under attack from an external pathogen and that diospyrin was therefore concentrated in the root bark. The high concentration of the naphthoquinones in the Trafalgar sample might indicate a systemic infection. Other factors like the age of the roots, soil conditions and climate might also influence the results. In future three to five trees at a specific location will be collected in order to minimise the risk of infections and differences in root age etc. This will give more accurate results.

In future, experiments will be conducted using an ultrasonic bath at 50°C, to try and improve the yields from the roots. The solubility of the naphthoquinones improves with higher temperatures and the agitation caused by the ultrasonic bath should increase the crude yield. The extraction solvent to be used depends on the compound that needs to be isolated. Chloroform appears to be the best solvent for diospyrin extraction and acetone for 7-methyljuglone extraction. Unconfirmed analysis has shown that the acetone crude extract proved to have a better activity against TB. This can be due to the higher concentration of 7-methyljuglone in the extract.

In addition to the known naphthoquinones several other very similar compounds have been isolated. These compounds, especially neodiospyrin can give false positives.
Diospyrin and neodiospyrin could not be separated on a TLC plate using two different stationary phases (silica-gel and reversed phase silica-gel) and at least five different mobile phases. (hexane: ethyl acetate 5:1 ; 5:2 ; 5:3 ; hexane: chloroform 5:1 and hexane: ethyl acetate: MeOH 5:1:0.5. on the silica-gel stationary phase and MeOH: acetic acid: water 8:0.5:1.5 and 7:0.5:2.5 ; acetonitrile: acetic acid: water 7:0.5:2.5 on the reversed phase silica-gel.) The two different compounds do however appear to have exactly the same Rf values with the same colouration patterns when sprayed with vanillin. The spot appearance also looks the same. With the use of washing procedures (MeOH and hexane) it was possible to separate the two compounds.

The use of the HPLC proved to be invaluable in this work. Different solvent systems were used to improve the separation of the compounds as well as to keep the backpressure of the system at reasonable levels. The first solvent system employed was: ethanol: water: acetic acid (6.5: 3: 0.5). This system gave a backpressure of 150 bar at a flow rate of 0.6 ml/min. It was decided to employ an acetonitrile: water: acetic acid (62.5: 32.5: 5) system in order to decrease the backpressure. This system with a flow rate of 1 ml/min gave a backpressure of 75 bar. This did however have an influence on the retention times of the different compounds. 7-Methyljuglone had a RT of 3.8 min. while diospyrin a RT of 10 min. and neodiospyrin appeared at a RT of 8 min. Due to temperature variations of the column and the solvent, the retention times of the compounds differed with as much as 2 minutes when data from the winter months were compared with those in the summer months. The data from the early mornings also differed from those in the early afternoons due to temperature fluctuations. It was therefore decided that the diospyrin standards as well as 7-methyljuglone should be injected before and after each day's analysis. The UV spectra of the compounds were always compared with the standards if there was any doubt.

According to Chapman and Hall (2003) two of the isolated naphthoquinones, diospyrin and isodiospyrin shows antiparasitic and cytotoxic activity respectively. The other isolated compounds do not have any known activity.
2.6 References


Chapter 3
Identification of naphthoquinones isolated
from *Euclea natalensis*

3.1 Introduction 37

3.2 Materials and methods 38

3.2.1 Materials 38

3.2.2 Methods 39

3.2.2.1 Ultraviolet-visible spectrophotometry (UV-vis) 39

3.2.2.2 Mass spectrometry (MS) 40

3.2.2.3 Nuclear magnetic resonance spectroscopy (NMR) 40

3.3 Results 42

3.3.1 Identification of diospyrin 42

3.3.2 Identification of 7-methyljuglone 46

3.3.3 Identification of shinanolone 46

3.3.4 Identification of neodiospyrin 48

3.3.5 Identification of isodiospyrin 50

3.3.6 Identification of mamegakinone 52

3.3.7 Identification of 5-hydroxy-4-methoxy-2-napthaldehyde 53

3.4 Discussion and conclusions 55

3.5 References 58
Chapter 3
Identification of naphthoquinones isolated from *Euclea natalensis*

3.1 Introduction

Various techniques have been employed to identify organic compounds. The simplest and cheapest form of identification is to make use of chromatographic techniques. These methods employ comparison of the unknown compounds with known compounds (standards). During this study the TLC and HPLC chromatography techniques have been used to identify unknown compounds with reference standards. There are also various spectroscopic techniques available. The mass spectroscopy instrument yields information on the size and chemical formula of the unknown compound. Infrared spectroscopy (IR) yields information on what kind of functional groups are present in the compound. Another spectroscopic technique is that of ultraviolet spectroscopy. This gives information on whether there is a conjugated π electron system present in the compound (double and single bond alternation).

Lastly, nuclear magnetic resonance spectrometry gives the most valuable information. It yields information on the hydrogen-carbon framework of the unknown compound. It is however very important to make use of more than one of these techniques to identify unknown compounds. By so doing, mistakes can be minimized and the structure of the unknown compound can be confirmed. All of the above techniques, excluding IR, have been used throughout this study, and will be explained more thoroughly in the next paragraphs.
3.2 Materials and methods

3.2.1 Materials

All the isolated compounds were purified to above 80% purity (tested on HPLC and TLC). Most of the NMR analysis was performed with deuterated chloroform (D-chloroform) unless otherwise stated. The samples were dried in a rotary evaporator and re-dissolved in D-chloroform. The NMR tubes were washed with chloroform and dried for at least 1 hour at 105°C. The solution was filtered and injected into the NMR tube after which it was sealed with parafilm. All the analyses were done on a Brucker 300 MHz NMR instrument. Fig 3.1 shows the chemical structure of diospyrin and indicates the numbering system that was used throughout this dissertation.

![Diospyrin structure](image)

**Fig. 3.1:** Molecular structure of diospyrin indicating the numbering system.
3.2.2 Methods

3.2.2.1 Ultraviolet-visible spectrometry

There are several different spectroscopic techniques available. The UV-visible spectrometer is the cheapest and could be used to correctly identify some known organic compounds. The UV spectroscopy is however only applicable to conjugated systems (McMurry, 1996). A conjugated system is found were multiple bonds (double bonds) alternate with single bonds. For other molecules like alkanes, which only contain single bonds, the energy needed for excitation is too high and therefore a very short wavelength is needed. This is not yet possible with today’s instruments.

The molecules in a given sample absorb electromagnetic radiation in a certain region of the light spectrum. This causes transitions between electronic energy levels. As a molecule absorbs energy, an electron is promoted from an occupied orbital to an unoccupied orbital of greater potential energy. The most probable transition is from the highest occupied molecular orbital to the lowest unoccupied molecular orbital. For an single atom that absorbs UV the absorption spectrum will consist of very sharp line. This is caused by the energy transition between two very discreet energy levels. For molecules that contain lots of atoms the UV absorption will occur over a wide range of wavelengths (McMurry, 1996).

Because there are so many possible energy transitions, the UV spectrometer cannot distinguish between all the closely spaced lines. The spectrometer will therefore give a broad band of absorption. This method was employed to determine the Lambda maximum ($\lambda_{\text{max}}$) for the naphthoquinones (the peak of the broad band), and to confirm the authenticity of the standards.
3.2.2.2 Mass spectrometry (MS)

The mass spectrometer gives information on the molecular mass of the compound. In addition to the mass, the fragmentation pattern can also give valuable information on the structure of the compound. According to Morrison & Boyd (1992), the mass spectrometer bombards the molecule with high-energy electrons and thereby converting some of the molecules into ions. These ions contain a specific charge and are accelerated in an electric field, which separates them according to their mass: charge ratio. Ions with a specific mass: charge ratio will hit a detector, which can determine the amount of ions striking it. The detectors output is amplified and plotted as a bar graph. The most abundant ion will give rise to the tallest peak, which is called the base peak. All the other peaks are shown relative to the base peak.

The ion that misses only one electron will give rise to the molecular ion (M+) peak. This peak will give the actual mass of the molecule. Naturally occurring isotopes will give rise to M+1 and M+2 peaks. These peaks will give masses that are heavier than the actual molecule due to the fact that molecules in nature contain heavier isotopes in their molecular structures. The abundance of these peaks will however be much less than the M+ peak.

3.2.2.3 Nuclear magnetic resonance spectroscopy (NMR)

The most powerful structural elucidation technique today is nuclear magnetic resonance (NMR) spectrometry (McMurry, 1996; Pavia et al., 2001). This instrument gives information on the hydrogen and carbon framework of the molecule. It can also be employed to detect some inorganic nuclei like $^{19}$F and $^{31}$P. Most commonly, only the $^{13}$C and $^1$H spectra are used. The coupling between the nuclei can also be investigated with the use of 2D analysis. During this study mostly $^1$H NMR was used for comparative analysis.
Many atomic nuclei have a property called spin. Any nucleus with an odd mass or an odd atomic number will possess a property called spin angular momentum and a magnetic moment. Each nucleus will have a nuclear spin quantum number (I), which is a physical constant. For the hydrogen atom (proton) this number is ½ (Pavia et al., 2001). The nucleus is also a charged particle and by moving, it will generate a magnetic field. The nucleus has therefore a magnetic moment (μ). For the hydrogen atom the nucleus might have a clockwise (+1/2) or a counter clockwise (-1/2) spin, with the nuclear magnetic moments pointed in opposite directions. In an applied magnetic field, all the nuclei will be either aligned with the external field or will be aligned against the external magnetic field. The alignment with the magnetic field will be at a lower energy state, and energy will be needed to flip the proton over into the less stable alignment, against the magnetic field.

This energy, depending on the strength of the external magnetic field, is applied by electromagnetic radiation in MHz. This energy flips the proton into the higher energy state and the proton is said to be in resonance with the applied radiation. Protons attached to different atoms will however "feel" different field strengths (effective field strength). This effective field strength depends on the electronic environment in which the specific proton occurs. Protons on different atoms will therefore resonate at different applied field strengths. This gives rise to resonance peaks at different positions on the NMR spectrum and is called the chemical shift of the specific proton in the molecule. The number of peaks will give information on the amount of protons in the molecule, while the position of the peaks (chemical shift) will give information of the atomic environment of each specific proton (Morrison & Boyd, 1992).

The height of the peaks can be integrated (area under peak) to give information on the amount of chemically equivalent protons (two protons that are in exactly the same environments on the molecule will give rise to one peak). Neighbouring protons give rise to spin-spin splitting patterns, which is very useful in structure elucidation. This multiplicity of peaks gives an indication of how many protons are on the neighbouring carbon atoms. The multiple peaks will have a distance between them called the j-value,
which will be exactly the same as the distance between a second multiple peak. By measuring this distance it is possible to determine which peaks are caused by neighbouring protons. All of this information can be used to build a carbon-hydrogen skeleton of the unknown molecule.

For the carbon NMR the isotope of carbon 13 is employed (odd mass). It works in exactly the same way as proton NMR but is less sensitive than proton NMR, due to the low abundance of the naturally occurring isotope (Pavia et al., 2001).

In this chapter the structures of the compounds were identified with the use of TLC, HPLC, UV-vis, MS and NMR spectroscopy.

3.3 Results

3.3.1 Identification of Diospyrin

Originally diospyrin was only compared and identified by means of TLC. This was stopped after it became clear that neodiospyrin causes false positives. These false positives occurred in all three samples that were collected at different locations throughout the year. The concentration of neodiospyrin does however show seasonal and geographical variation. A $^1$H NMR was performed as well as an HPLC analysis. The first $^1$H NMR result yielded the spectra shown in Fig. 3.2. The fact that the integration of the peaks especially the hydroxyl peaks (~12ppm) showed approximately a 1:1 ratio, gave origin to the hypothesis that a 7-methyljuglone tetramer existed (four 7-methyljuglone molecules bonded together).
Fig. 3.2: $^1$H NMR spectra indicating the existence of one tetrameric molecule, or two similar 7-methyljuglone dimers.

According to literature such tetramers do in fact exist (Tezuka et al., 1972). The ratio between the four peaks differed in subsequent NMR analysis, and it was concluded that two similar molecules existed. The diospyrin/neodiospyrin mixture was washed with MeOH until HPLC analysis confirmed the purity of diospyrin. The $^1$H NMR data for diospyrin and related quinones are summarized in Table 3.1. (Lillie & Musgrave, 1977). Fig. 3.3 shows the $^1$H NMR spectra of diospyrin.
Table 3.1: NMR data for selected naphthoquinones. Observed values are indicated in red whereas the published data (Lillie & Musgrave, 1977) are given in black.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Unit</th>
<th>H-2</th>
<th>H-3</th>
<th>H-6</th>
<th>7-Me</th>
<th>H-8</th>
<th>5-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diospyrin</td>
<td>A</td>
<td>6.88</td>
<td>6.89</td>
<td>7.11</td>
<td>7.49</td>
<td>11.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.93</td>
<td>6.94</td>
<td>7.11</td>
<td>7.49</td>
<td>11.85</td>
<td></td>
</tr>
<tr>
<td>7-Methyljuglone</td>
<td>A</td>
<td>6.89</td>
<td>6.88</td>
<td>7.06</td>
<td>7.43</td>
<td>11.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.89</td>
<td>6.88</td>
<td>7.06</td>
<td>7.43</td>
<td>11.83</td>
<td></td>
</tr>
<tr>
<td>Neodiospyrin</td>
<td>A</td>
<td>6.76</td>
<td>6.77</td>
<td>6.92</td>
<td>7.25</td>
<td>12.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.60</td>
<td>6.61</td>
<td>7.09</td>
<td>7.52</td>
<td>11.73</td>
<td></td>
</tr>
<tr>
<td>Isodiospyrin</td>
<td>A</td>
<td>6.70</td>
<td>6.72</td>
<td>6.90</td>
<td>7.28</td>
<td>12.41</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.93</td>
<td>6.94</td>
<td>6.92</td>
<td>2.01</td>
<td>12.03</td>
<td></td>
</tr>
<tr>
<td>Mamegakinone</td>
<td>A</td>
<td>6.96</td>
<td>6.97</td>
<td>7.11</td>
<td>7.48</td>
<td>11.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.96</td>
<td>6.97</td>
<td>7.11</td>
<td>7.48</td>
<td>11.70</td>
<td></td>
</tr>
</tbody>
</table>
Fig 3.3: $^1$H NMR spectra of diospyrin (a) and expanded aromatic region of diospyrin (b)
3.3.2 Identification of 7-methyljuglone

7-Methyljuglone was authenticated by means of HPLC, TLC, Mp and $^1$H NMR analysis. The $^1$H-NMR data for 7-methyljuglone are: δ = 2.37 (s, 3H, 7-Me); 6.88 (s, 2H, 2,3 H); 7.06 (d, J=0.54, 1H, 8 H); 7.42 (d J=0.54, 1H, 6 H); 11.83 (s, 1H, 5-OH) (Fig 3.4). Melting point (Mp) = 125-127°C (lit., 126.5-127°C) (Cooke & Dowd, 1952).

Fig 3.4: $^1$H NMR spectrum for 7-methyljuglone

3.3.3 Identification of shinanolone

Shinanolone was identified by comparing the HPLC retention times and the $^1$H NMR spectra with an authentic sample. The splitting patterns shown in Fig 3.5b indicate the lack of aromaticity between carbons 1-4. The resulting splitting patterns show the expected doublet of doublet (dd) at δ 4.8 ppm belonging to the hydrogen on carbon 1.
Two complicated splitting patterns (dddd) belonging to the axial (δ2.25) and equatorial (δ2.1 ppm) hydrogen atoms on carbon 2 can be seen in Fig 3.5b. Carbon 3’s hydrogen atoms gives rise to the two ddd peaks at δ2.9 and δ2.55 ppm.

**Fig 3.5a:** $^1$H NMR data of the aromatic and hydroxy region of shinanolone
Fig 3.5b. $^1$H NMR downstream region indicating the splitting patterns for shinanolone

3.3.4 Identification of neodiospyrin.

The $^1$H-NMR spectra for neodiospyrin are shown in Fig 3.6 a and b. Table 3.1 lists the chemical shifts for neodiospyrin. M.p for neodiospyrin 216-219°C (lit., 220°C) (Kumari et al., 1982).
Fig 3.6: $^1$H NMR spectra for neodiospyrin (a) and expanded aromatic region for neodiospyrin (b)
3.3.5 Identification of isodospyrin

The $^1$H NMR data for isodospyrin are: $\delta = 1.99$ (s, 3H, 7Me), 2.01(s, 3H, 7'Me), 6.69 (d, J=10.35 1H, 2 H), 6.89 (d, J=10.35, 1H, 3 H), 6.92 (s, 2H, 2',3'H), 7.28 (s, 1H, 8 H) 7.59 (s, 1H, 6'H), 12.02 (s, 1H, 5'OH) and 12.41 (s, 1H, 5OH).

Molecular mass (MM) = 374. The $^1$H NMR spectra are shown in Fig. 3.7a-c and Fig 3.7d shows the MS spectra.

Fig 3.7a: $^1$H NMR for isodospyrin
Fig 3.7b: Expanded aromatic region of isodospyrin

Fig 3.7c: Further expansion of the aromatic region of isodospyrin to indicate splitting patterns
Fig 3.7d: Molecular mass spectrum of isodiospyrin indicating a molecular mass of 374 (M⁺ peak). This peak is also the expected base peak.

3.3.6 Identification of mamegakinone

The similarities between the ¹H NMR spectra of 7-methyljuglone and mamegakinone are very easy to observe. The chemical shifts show some difference but the key distinguishing factor is the integration of the hydrogen peak at 6.96 ppm. This peak shows that there is only one aromatic proton whereas the correlating 7-methyljuglone peak at 6.89 ppm indicates the presence of two hydrogen atoms. This gives rise to the symmetric mamegakinone molecule (Lillie & Musgrave, 1977).
3.3.7 Identification of 5-hydroxy-4-methoxy-2-napthaldehyde

5-hydroxy-4-methoxy-2-napthaldehyde was identified by means of $^1$H and $^{13}$C NMR data. The $^{13}$C data were compared to published data (Likhitwitajawud et al., 1999).

The $^1$H NMR data for 5-hydroxy-4-methoxy-2-napthaldehyde (acetone): $\delta = 4.09$ (s, 3H, O-Me); 6.87 (dd, J=1.3, J=7.5, 1H, H8); 7.1 (d, J=1, 1H, H1); 7.35 (dd, J=7.5, J=7.5, 1H, H7), 7.40 (dd, J=1.3, J=7.5, 1H, H6), 7.95 (d, J=1, 1H, H3), 9.25 (s, 1H, 5-OH) and 9.95 (s, 1H, 2-CHO).

$^{13}$C NMR data (CDCl$_3$): $\delta = 56.4$ (4-OMe), 98.5 (C-3), 114.3 (C-6), 117.3 (C4a), 120.6 (C-8), 128.8 (C-7), 130.2 (C-1), 134.1 (C-2), 135.5 (C-8a), 154.5 (C-5), 156.9 (C-4), 191.4 (2-CHO).
Fig 3.9a: $^1$H NMR data for 5-hydroxy-4-methoxy-2-naphthaldehyde

Fig 3.9b: Aromatic region for 5-hydroxy-4-methoxy-2-naphthaldehyde
Fig 3.9c: $^{13}$C NMR spectra for 5-hydroxy-4-methoxy-2-napthaldehyde

3.4 Discussion and conclusions

All of the compounds have previously been isolated from different biological sources. Neodiospyrin, isodiospyrin and 5-hydroxy-4-methoxy-2-napthaldehyde have not yet been isolated from *Euclea natalensis*. The NMR data of these compounds can be found in various publications and the comparisons to these published data seems to fit well. The two compounds that still need some attention are neodiospyrin and 5-hydroxy-4-methoxy-2-napthaldehyde. The published data for these compounds seems to be insufficient. Neodiospyrin was isolated in 1972 for the first time by Tezuka and co-workers (1972) from *Diospyros kaki* roots. They methylated the mixture of diospyrin and neodiospyrin in order to separate the two compounds after numerous preparative TLC (PTLC) experiments failed to do so. These experiments were repeated by Van der Vijver and Gerritsma (1976), when they investigated the Ebenaceae family of southern Africa.
They isolated a mixture of two naphthoquinones from the roots of *D. rotundifolia* and attempted to isolate the separate compounds by means of PTLC in various solvent systems. After methylating the mixture, it separated easily with the same PTLC system. The first naphthoquinone proved to be diospyrin (NMR analysis) and the second compound neodiospyrin. The NMR data of neodiospyrin proved to be difficult to interpret, due to the fact that the 6-H peak appeared at δ 7.25 and the residual chloroform peak at δ 7.26.

Lillie & Musgrave (1977) had the same problem in establishing the correct structure for neodiospyrin. In an attempt to get rid of the residual chloroform peak, an NMR spectra was performed in acetone. The solubility of neodiospyrin in acetone is unsatisfactory but it still yielded an interpretable spectrum. From the spectrum it can be concluded that the residual chloroform peak does in fact obscure the H peak at δ 7.25. But the linkage between the two moieties could not be established from this spectrum. Various authors suggest that the bond between the two 7-methyljuglone moieties are between C3-C8’. They derivatised neodiospyrin and concluded from these experiments that the bond must be C3-C8’. The same authors do agree that the exact linkage still remains undetermined and might be either 2-8’ or 3-8’. (Kumari *et al.,* 1982; Mallavadhani *et al.,* 1998). Further investigation is needed to establish the exact chemical structure for this compound.

The chemical structure for 5-hydroxy-4-methoxy-2-naphthaldehyde needs to be confirmed with the use of MS. The published $^{13}$C NMR data for this compound do in fact match the isolated compound’s $^{13}$C NMR data. The $^1$H data obtained for the compound matches the proposed structure. The peak at δ 4.09 corresponds well with methoxy groups. The integration also confirms the presence of three hydrogen atoms. The doublet of doublets at δ 6.87 with coupling constants of 1.3 and 7.5 indicates the presence of vicinal coupling between protons on neighboring carbon atoms (H7 J=6-15Hz), and long range coupling between the meta protons (HH6 J=1-3 Hz). This peak will be assigned H-8. The δ 7.1 and δ 7.95 peaks will be assigned H1 and H3 respectively. These two peaks only show meta coupling with J = 1. H7 gives rise to a dd peak at δ 7.35 with J = 7.5 and J=7.5. This
indicates the presence of two vicinal protons (H8 and H6) on neighboring carbons. H6 gives rise to the peak at $\delta$ 7.40 with coupling of $J=1.3$ and $J=7.5$. This again indicates the presence of the vicinal coupling with the hydrogen on carbon 7 ($J=7.5$) and the meta coupling with the hydrogen on carbon 8 ($J=1.3$). The singlets at $\delta$ 9.25 and 9.95 are caused by the 5-OH proton and the 2-aldehyde proton respectively.
3.5 References


Chapter 4

Synthesis of 7-methyljuglone

4.1 Introduction 61

4.2 Materials and methods 62

4.2.1 Material 62

4.2.2 Methods 62

4.2.2.1 Synthesis of 8-chloro-7-methyljuglone 62

4.2.2.2 Synthesis of 7-methyljuglone 62

4.2.2.3 Alternative synthetic pathway for the preparation of 7-methyljuglone 63

4.3 Results and discussion 63

4.3.1 Synthesis of 8-chloro-7-methyljuglone 63

4.3.2 Synthesis of 7-methyljuglone 64

4.3.3 Alternative synthetic pathway for the preparation of 7-methyljuglone 65

4.4 Conclusions 67

4.5 References 74
Chapter 4

Synthesis of 7-methyljuglone

4.1 Introduction

According to McMurry (1996) and Morrison & Boyd (1992), organic chemistry is the chemistry of carbon compounds. Although carbon is the principle atom in organic compounds, most compounds also contain hydrogen, nitrogen, oxygen or other elements. Carbon has the unique ability to bond together to form long chains and rings. Therefore carbon is able to form an immense diversity of compounds, from the simple (methane- one carbon atom) to the complex (DNA- billions of carbon atoms).

All living organisms produce organic compounds in order to be able to perform various metabolic processes. Plants do this primarily through their ability to photosynthesise. Animals use these products from plants to generate energy, grow and repair wounds. The physical and chemical properties of these compounds depend on the molecular structure of the compound. During this study a fairly simple organic compound was isolated from *Euclia natalensis* A.DC. and an attempt was made to synthesise this compound.

There are a couple of advantages in being able to synthesise a natural product:
- Under some circumstances synthesising the compound might be cheaper than isolating the compound. In the case of 7-methyljuglone this is almost certainly the case.
- Another advantage is the increase in yields. Any amount of compound can be synthesised according to the needs of future experiments.
- The synthetic compound also proves that the proposed structure of the isolated compound is correct. This advantage is of more use when more complicated compounds are worked with.
In this chapter a published synthetic pathway was used and in addition a simpler, cheaper pathway was developed. This was done through personal communication with one of the authors (Musgrave, O.C.) of the published pathway (Musgrave & Skoyles, 2001) from the Chemistry Department, Old Aberdeen University, UK.

4.2 Materials and methods

4.2.1 Material

All chemicals used were of AR purity. The products of the different reactions were tested on TLC (hexane:ethyl acetate 5:2) and HPLC using the standard conditions. The NMR analysis was done on a Brucker 300 MHz. The petroleum ether fraction refers to the 60-80°C boiling range.

4.2.2 Methods

4.2.2.1 Synthesis of 8-chloro-7-methyljuglone

Anhydrous aluminium chloride (80g) and sodium chloride (16g) was heated to 180°C. This molten mixture was stirred while adding a mixture of 4-chloro-3-methylphenol (4g) and maleic anhydride (8g) into it. After the addition the mixture was vigorously stirred for 2 minutes and added to a mixture of 12N hydrochloric acid (250ml) and crushed ice. The resulting precipitate was filtered off after one hour, washed with water and dried. The crude brown material was extracted repeatedly with petroleum ether and yielded mainly 8-chloro-7-methyljuglone (NQ). The product was purified by column chromatography (silica-gel) using hexane: ethyl acetate (9:1) isocratically. This yielded 1.2 g (10%) of 95% pure NQ.

4.2.2.2 Synthesis of 7-methyljuglone

A solution of NQ (100mg) in tetrahydrofuran (THF) (10ml) was added drop-wise to a solution of stannous chloride (0.5g) in 4M hydrochloric acid (35ml) and THF (10ml) at 60°C. The reaction mixture was kept at 60°C for three hours, cooled, and quickly
filtered into a solution of ferric chloride (2.5g) and water (20ml). The resulting yellow precipitate was filtered off and dried. TLC and HPLC analysis of this crude yellow precipitate indicated the formation of 7-methyljuglone (51mg). The product was purified with silica gel column chromatography and the structure was confirmed as 7-methyljuglone with \(^1\)H NMR.

4.2.2.3 Alternative synthetic pathway for the preparation of 7-methyljuglone

An alternative pathway for the preparation of 7-methyljuglone was also investigated. In this pathway m-cresol was used instead of 4-chloro-3-methylphenol as a starting material. AlCl\(_3\) (40 g) and 8 g of NaCl were melted at 140°C on a hotplate. A mixture of m-cresol (2 g) and maleic anhydride (4g) was added to it. The reaction mixture was stirred for 3 minutes and poured into 12 M hydrochloric acid (125ml) and crushed ice. The resulting brown precipitate was filtered off, washed with water and dried. This yielded 2 g of crude material. The material was crushed and then extracted with petroleum ether for 1 hour. The extract was tested on TLC with an authentic standard and it appeared to contain 7-methyljuglone as the main product. The extract was applied to a silica gel column with hexane: ethyl acetate 9:1 as mobile phase. Fractions 5-18 contained pure 7-methyljuglone.

4.3 Results and discussion

4.3.1 Synthesis of 8-chloro-7-methyljuglone

The \(^1\)H NMR data for NQ: \(\delta=2.45\) (3H, s, CH\(_3\), 7-Me), 6.87 (2H, s, H-2 and H-3), 7.16 (1H, s, H-6) 12.50 (1H, s, OH). (Fig. 4.1) The published yields of the intermediate product (NQ) varied between 15-22%. The yield of the product depends on the correct temperature, efficiency of stirring and the correct pH. Depending on the purity of the crude product the NQ can be crystallised from the crude product using chloroform/petroleum ether or aqueous methanol.
Fig. 4.1: $^1$H NMR spectra of 8-chloro-7-methyljuglone

4.3.2 Synthesis of 7-methyljuglone

The NMR data for 7-methyljuglone are given in chapter 3, section 3.3.2. The yield of the product (51%) indicates that the reduction step can be improved. According to the author (Musgrave) of the published pathway the yields varied between 35-85%. By accurately controlling the temperature and the stirring of the reaction mixture it should be possible to obtain reproducible yields of 70%. Fig. 4.2 indicates the overall reaction mechanism and Fig. 4.3 the Friedel-Crafts acylation reaction.
4-chloro-3-methylphenol + maleic anhydride

8-chloro-7-methyljuglone → 7-methyljuglone

1. SnCl₃, HCl, THF 60°C
2. FeCl₃, H₂O

Fig. 4.2: Schematic representation of 7-methyljuglone synthesis.

4.3.3 Alternative synthetic pathway for the preparation of 7-methyljuglone

An alternative pathway for the preparation of 7-methyljuglone was also investigated. In this pathway m-cresol was used instead of 4-chloro-3-methylphenol as the starting material. This decreases the amount of steps needed for the synthesis of 7-methyljuglone to one step. This also means that the costs and time involved decreases accordingly. Before the experiment was conducted the reaction mechanism was investigated and it was concluded that the temperature would play a very important role during this reaction (Morrison & Boyd, 1996). The two possible scenarios was that the reaction will yield 7-methyljuglone as the chief product or that it will yield 6-hydroxy-8-methyljuglone. This depends on the acylium ion attacking the ortho position or the para position during the reaction.
The extract was tested on TLC with an authentic standard and it appeared to contain 7-methyljuglone as the main product. One of the by-products appeared to be m-cresol, which indicates that the reaction was not completed. (This by-product was in a much higher concentration when the reaction was done at 180°C. The synthesis of MJ therefore looks temperature dependent). A $^1$H NMR analysis was performed on the purified compound (Fig. 4.4).
Fig. 4.4: $^1$H NMR spectra of the synthetic 7-methyljuglone.

### 4.4 Conclusions

Two proposed reaction mechanisms for the formation of NQ are shown in Fig. 4.5a and 4.5b. The use of sodium chloride is not yet completely understood. It might therefore play a role in the formation of the second acid chloride as shown in step 4. Alternatively, the AlCl$_3$ might form a complex with the resulting carboxylic acid and thereby activate the carbonyl carbon to be attacked by the nucleophilic ring system (Fig. 4.5c) with the loss of one molecule of water. The AlCl$_3$ complex is broken up by the addition of concentrated hydrochloric acid. This mechanism will also yield the NQ as product.
Fig. 4.5a. Reaction mechanism for the preparation of 8-chloro-7-methyljuglone.
Fig. 4.5b. Alternative reaction mechanism for the preparation of 8-chloro-7-methyljuglone (continued on page 68).
**Fig. 4.5b.** Alternative reaction mechanism for the preparation of 8-chloro-7-methyljuglone (continued from page 67).

**Fig. 4.5c** Alternative mechanism for step 5
During the various attempts to synthesise 7-methyljuglone the influence of correct temperature and the efficiency of stirring became apparent. By using a hotplate and a glass thermometer the temperature could not be controlled that accurately. This can be overcome with the use of an oil bath instead of a hotplate in future.

The molten aluminium chloride/sodium chloride mixture was too viscous for a magnetic stirrer. The mixture was therefore stirred mechanically which made the process inefficient. To overcome this a new stirring technique needs to be developed.

The published synthesis (Musgrave & Skoyles, 2001) did not give a reaction mechanism for the Friedel-Crafts reaction between maleic anhydride and 4-chloro-3-methylphenol. It referred to this reaction as a classical reaction described by Cooke & Dowd, 1953. This publication did however not contain any reaction mechanisms for this first step of the reaction. According to McMurry (1996) and Morrison & Boyd (1992), anhydrides react in the same way as acid chlorides except that the reactions are a little bit slower. None of these sources gives a reaction mechanism for the anhydride reaction. The mechanism for the acid chloride reaction is explained by McMurry (1996). The reaction between the acyl chloride and aluminium chloride yields a resonance-stabilised electrophile, the acylum ion. This ion is then attacked by the aromatic ring to yield a substituted ketone in the ortho position. Usually only half of the anhydride will appear in the product, the other half forms an carboxylic acid. In the case of cyclic anhydrides (used during this synthesis) the carboxylic acid will appear in the product.

The proposed mechanisms attempt to explain how this carboxylic acid is converted into an acid chloride for the Friedel-Crafts reaction to occur for the second time. The first mechanism (Fig. 4.5a) explains the formation of the acylum ion catalysed by aluminium chloride (step 1). This acyl ion is then attacked by the aromatic ring and the proton is substituted with the acyl ion forming a ketone in the ortho position (steps 2,3). In addition a carboxylic acid is formed in the form of its carboxylate ion (COO-). This carboxylate ion must then be converted into an acid chloride in order to acylate for a second time in the meta position. A nucleophilic acyl substitution reaction of carboxylic acid to the corresponding acid chloride occurs. This nucleophilic chloride atom is generated during the first Friedel-Crafts acylation step or its origin might be
from sodium chloride (step 4). The formation of a second acylium ion with the subsequent aromatic attack and substitution occurs at the meta position as already described (steps 5,6).

A second mechanism is proposed through an esterified intermediate (Fig. 4.4b). It was noticed that when the maleic anhydride (white powder) and the 4-chloro-3-methylphenol (white powder) were combined it rapidly changed to a yellow moist mixture. This did not happen during the dry winter months or when the reactions were done with fresh stock. Therefore a certain percentage of maleic anhydride (indicated as 20%) might be in the corresponding acid form (step 1). The maleic acid forms an ester with the phenol, explaining the yellow colour, and in the process water is given off, explaining the wet appearance (Morrison & Boyd, 1992), (step 2). (Usually this reaction occurs in the presence of a small amount of a mineral acid.)

After the addition of this esterified starting material to AlCl₃ at 180°C a Fries rearrangement occurs as shown in steps 3 and 4. There are a few hypothesis of how this reaction occurs but at this point it is speculative. This rearrangement occurs at the ortho position rather than the para position. (The para position is blocked by the chlorine atom when the published synthesis is used. During the alternative synthesis this position is open for para substitution).

According to Benson et al. (2001) and Harjani et al. (2001) the amount of catalyst and the temperature of the reaction will dictate the preferred substitution. They found that an excess of catalyst and a temperature of 140°C favour the ortho substitution. The resulting carboxylic acid is converted to its acid chloride by the action of sodium chloride to yield the acid chloride and sodium hydroxide (steps 5, 6). The acid chloride is converted to the acylium ion as described earlier and the meta substitution occurs to form a ketone and AlCl₃ and HCl (steps 7, 8).

The yields of the 8-chloro-7-methyljuglone varied between 5-20% during separate preparations. If the alternative pathway is correct then the presence of maleic acid as the reactive species might explain the low yield. The low yields might also be explained through ineffective stirring and inaccurate temperatures. To test this maleic acid can be used in future experiments instead of maleic anhydride.
The reduction of 8-chloro-7-methyljuglone is schematically presented in Fig. 4.6 (Musgrave & Skoyles, 2001). The 8-chloro-7-methyljuglone is first reduced to its napthoquinol in step 1. In the presence of an acid a proton is supplied to the carbon atom carrying the chlorine atom. Loss of a proton and expulsion of the chlorine atom followed by a further proton loss would give 7-methyljuglone.

```
\[ \text{SnCl}_2 \text{HCl, THF} \]
```

**Fig. 4.6. Reduction of 8-chloro-7-methyljuglone**

The alternative preparation for 7-methyljuglone was done in exactly the same way as the published synthesis except that the starting material and the temperature used differed. During this synthesis the focus was qualitative and not quantitative. No yield is therefore reported. Future experiments will focus on increasing the yields and by better understanding of the reaction mechanisms involved.
4.5 References


Chapter 5

Synthesis of neodiospyrin and diospyrin

5.1 Introduction 76

5.2 Materials and methods 76
   5.2.1 Material 76
   5.2.2 Methods 77
       5.2.1.1 Reduction of 7-methyljuglone 77
       5.2.2.2 The methyljuglone-hydroquinone reaction 77
       5.2.2.3 Diospyrin synthesis 77

5.3 Results and discussion 78
   5.3.1 Reduction of 7-methyljuglone 78
   5.3.2 The methyljuglone-hydroquinone reaction 78
       5.3.3 Diospyrin synthesis 81

5.4 Conclusions 82

5.5 References 85
Chapter 5

Synthesis of neodiospyrin and diospyrin

5.1 Introduction

The dimeric form of 7-methyljuglone occurs naturally in *E. natalensis*. Four of these dimers have been isolated thus far from *E. natalensis* (diospyrin, isodiospyrin, neodiospyrin and mamegakinone). The activity of diospyrin against multidrug resistant strains of TB necessitated the need to be able to synthesise these dimers. A multi-step synthetic pathway for the synthesis of diospyrin has been published by Yoshida & Mori in 2000. This pathway consists of eleven steps and therefore ten intermediate compounds, which must be extracted and purified, in order to complete the synthesis. Keeping in mind the costs, time and the experience needed for such a synthesis it was decided that an alternative pathway was needed. Kumari *et al.* (1982) and Sankaram *et al.* (1975), published a two-step pathway for the synthesis of neodiospyrin. Due to the structural similarities between neodiospyrin and diospyrin this synthetic pathway was further investigated.

5.2 Materials and methods

5.2.1 Material

The products of the various reactions were tested on HPLC with authentic standards. TLC was also employed as a rapid analysis to verify the existence of the intermediates. The final products were analysed by NMR and compared with authentic samples. All chemicals used were of AR purity.
5.2.2 Methods

5.2.2.1 Reduction of 7-methyljuglone

7-Methyljuglone (25mg) was dissolved in 2.5ml of tetrahydrofuran (THF). This was added to a solution of tin chloride (125mg), 2.5ml of THF and 8.75ml of 4M hydrochloric acid (HCl) at 60°C. The solution was stirred for 3 hours. After stirring, the THF was evaporated by opening the vessels for 15 min at 60°C, after which it was completely evaporated with the use of a rotary evaporator. The reduced 7-methyljuglone was recovered by washing out the excess HCl and tin chloride in a reverse phased silica gel column (solid phase extraction). The column was prepared by conditioning it with methanol (MeOH) and then with water. The sample was introduced and the HCl and tin chloride removed by washing the column with water. The reduced 7-methyljuglone remained in the silica column and was collected by washing the column with MeOH.

5.2.2.2 The methyljuglone-hydroquinone reaction

A phosphate buffer was prepared by dissolving 6.8 g of KH₂PO₄ in 1 L of water. The pH was adjusted to 6.6 with 3% KOH. Unreduced 7-methyljuglone (25mg) were dissolved in the minimum amount of MeOH and 25ml of phosphate buffer. The solution was stirred while adding the reduced 7-methyljuglone (hydroquinone), dissolved in the minimum of MeOH, into the reaction vessel. The solution turned dark purple after 5 minutes and remained purple/brown for the remaining 25 minutes of the reaction. The resulting precipitate that formed was filtered off, washed with water and dried. This crude product was tested on HPLC for purity with an authentic neodiospyrin standard.

5.2.2.3 Diospyrin synthesis

Exactly the same procedure was used during this experiment as described in sections 5.2.2.1 and 5.2.2.2. The only difference was that 8-chloro-7-methyljuglone was used instead of 7-methyljuglone. The 8-chloro7-methyljuglone was prepared as described
by Musgrave & Skoyles (2001). The resulting product was tested on HPLC for purity. The possibility that this product was the chlorinated diospyrin was investigated and an attempt was made to reduce the compound as described by Musgrave & Skoyles (2001).

It was decided to modify the reaction conditions in order to synthesise diospyrin. The first parameter that needed to be adjusted was the pH of the phosphate buffer. In an attempt to measure the pH of the roots of *E. natalensis*, 1 g of seedling roots were homogenised and dissolved in 10 ml of distilled water. The pH of the supernatant was measured at different time intervals.

5.3 Results and discussion

5.3.1 Reduction of 7-methyljuglone

Kumari *et al.* (1982) used 100mg of 7-methyljuglone in 20ml of methanol and reduced it with hydrogen gas over an Adams catalyst in order to obtain the hydroquinone. Due to the availability of these chemicals it was decided to use a different reduction method as described by Musgrave & Skoyles (2001). The reduction of 7-methyljuglone did not pose any problems and it was isolated with the use of a reversed phased silica column.

5.3.2 The methyljuglone-hydroquinone reaction

Fig 5.1 illustrates the chromatogram of the product. The compound with an RT of 10.489 is neodiospyrin. Trace amounts of 7-methyljuglone can be seen at an RT of 5.667. The proposed reaction mechanism is schematically presented in Fig. 5.2. Step 1 illustrates the reduction of 7-methyljuglone into its hydroquinone.
Fig. 5.1: Chromatogram indicating neodiospyrin as the sole product.

The hydroxy group on carbon 5 is an ortho and para directing activator and therefore two intermediates are possible. The negative charge (lone pair of electrons) is shared by either the ortho or para carbon, and thereby forming the hydroquinone intermediates.

In step two the unreduced 7-methyljuglone is added to the reduced hydroquinone. The 7-methyljuglone can form an enolate ion intermediate and thereby activate the carbon 3 atom. The oxygen on carbon 1 carries three lone pair electrons and has a negative charge. The carbon 3 atom carries a net positive charge and is open for nucleophilic attack by the hydroquinone. The bond forms between C-3 and C-8’ after which the hydroquinone becomes oxidised as indicated by the red arrows (arrows indicate electron flow). Hydrogen atoms are expelled during this process.
Fig. 5.2: Reaction mechanism for the synthesis of neodiospryn.
5.3.3 Diospyrin synthesis

Exactly the same procedure was used during this experiment as described in section 5.2.2. The only difference was that 8-chloro-7-methyljuglone was used instead of 7-methyljuglone. The reason for this is that an attempt was made to block the C-8' position with the chlorine atom. By doing this it will not be possible for the bond between C-3 and C-8' to form, and therefore theoretically no neodiospyrin will form. The resulting product was tested on HPLC for purity and it was found that a single product was formed (Fig 5.3). The possibility that this product was the chlorinated diospyrin was investigated and an attempt was made to reduce the compound as described by Musgrave & Skoyles (2001). The resulting product was a mixture of compounds without any traces of diospyrin or neodiospyrin.

It was decided to modify the reaction conditions in order to synthesise diospyrin. The first parameter that needed to be adjusted was the pH of the phosphate buffer. In an attempt to measure the pH of the roots of E. natalensis 1 g of seedling roots were homogenised and dissolved in 10 ml of distilled water. The pH of the supernatant was measured to be 5.1. After 2 hours the pH gradually increased to 5.6. In future a phosphate buffer at different pH values (starting at 5.5) will be tested to see if this will have an influence on diospyrin formation.
Fig 5.3: Chromatogram indicating the presence of an unknown compound formed during diospyrin synthesis.

5.4 Conclusions

The synthesis of neodiospyrin was repeated twice and both times the major product was neodiospyrin. It was confirmed with TLC, HPLC and $^1$H NMR. The proposed reaction mechanism tries to explain why the carbon-carbon bond is formed between C-3 and C-8'. In diospyrin this bond is between C-2 and C-6'.

In the first step of the synthesis 7-methyljuglone is reduced to its hydroquinone by the action of tin chloride (Musgrave & Skoyles, 2001). It is proposed that the hydroxy group on carbon 5 activates the ortho and para positions of the ring by donating an electron pair to the aromatic system. This will put a negative charge on carbons 6 or 8.
The reason that only carbon 8 takes part in the reaction might be due to temperature and pH factors as published by Harjani et al. (2001). A higher temperature seems to favour the *ortho* substitution whereas a lower temperature favours the *para* substitution. (These reactions were all done at room temperature.)

The unreduced 7-methyljuglone forms an enolate ion intermediate, which puts a positive charge (electrophilic site) on carbon 3' (step 2). The ketone group on carbon 4' forms a hydrogen bond with the hydroxy group on carbon 5' and thereby cannot form the enolate ion. (This activates only carbon 3'). A nucleophilic addition with the reduced 7-methyljuglone, as the nucleophile, takes place and neodiospyrin is formed by a charge transfer (electron flow) as shown in steps 4-5.

The attempt of blocking carbon 8 with the chlorine atom did not work. Cl is an *ortho* and *para* deactivating group. It will therefore be unlikely for substitution to take place at the *meta* position (C-6). A single product did form, which was neither the reduced 8-chloro-7-methyljuglone nor its oxidised form. It also did not appear to be diospyrin or neodiospyrin after HPLC testing. The possibility that it might be a chlorinated dimer of 7-methyljuglone must therefore be investigated in future experiments.

The acidity of phenols might also be an important aspect in this synthetic pathway. Phenols are weak acids that dissociates in aqua’s solutions to yield a phenoxide ion and H$_2$O$^+$. This phenoxide ion is resonance stabilised. The negative charge is delocalised over the *ortho* and *para* positions of the aromatic ring. The colour of the reaction mixture changes to purple when the phenoxide ion is formed. This effect was noticed during the synthesis. The dilute base in this case was potassium hydroxide used to change the pH of the phosphate buffer to 7.6. The purple coloration was only noticed when the reduced 7-methyljuglone was added to the unreduced 7-methyljuglone in the phosphate buffer (which showed no purple colour). This might give further evidence that in the unreduced 7-methyljuglone the hydrogen in the hydroxy group on carbon 5 has bonded with the oxygen on carbon 4 via hydrogen bonding. The electrophilic site will therefore be on carbon 3 and not carbon 2.

The phenoxide ion (purple colour) only forms when the reduced 7-methyljuglone is added to the buffer solution. In the reduced 7-methyljuglone there are three hydroxy
groups, which can take part in the formation of the phenoxide ion. There will therefore be no hydrogen bonding between the hydroxy on carbon 5 and the hydroxy on carbon 4. At the temperatures and pH used during this synthesis the para position (C6) was favoured for substitution. The influence of the electron donating methyl group on carbon 7 might also affect the preferred substitution at the para position.

In future experiments the pH and the temperature of the reaction mixture will be altered in an effort to produce diospyrin instead of neodiospyrin. The possibility that the solvent MeOH plays a role in dimer formation, by forming formaldehyde, will also be investigated.
5.4 References


Chapter 6

Enzymatic synthesis of diospyrin

6.1 Introduction 87

6.2 Materials and methods 87
   6.2.1 Material 88
   6.2.2 Methods 88
   6.2.2.1 Germination of E. natalensis seedlings 88
   6.2.2.2 Cell-free extract of E. natalensis seedlings 88
   6.2.2.3 Cell-free extract with the addition of 8-chloro-7-methyljuglone 89

6.3 Results 89
   6.3.1 Germination of E. natalensis seedlings 89
   6.3.2 Cell-free extract of E. natalensis seedlings 89

6.4 Discussion and conclusions 94

6.5 References 99
Chapter 6

Enzymatic synthesis of diospyrin

6.1 Introduction

Plants produce a large amount of primary and secondary metabolites during the processes of growth, wound repair and for defensive purposes. These metabolites are synthesised in the plant via biochemical pathways. The products of photosynthesis are channelled to specific cells where enzymatic reactions convert them into specific compounds. In this case the biochemical pathway for the formation of naphthoquinones are not yet completely understood. The hypothesis is that a specific enzyme(s) is responsible for the formation of 7-methyljuglone dimers (diospyrin, neodiospyrin, namegakinone etc.). An attempt was made to isolate these enzymes, (in the form of a cell-free extract) responsible for naphthoquinone formation, and add 7-methyljuglone into this enzyme mixture. The possible formation of diospyrin can then be monitored using the HPLC. Once the enzyme(s) can be isolated, it will be possible to semi-synthesise diospyrin by using 7-methyljuglone as the starting material. During this chapter numerous attempts were made to isolate the responsible enzyme(s) and thereby synthesise diospyrin.

The 2-6' bond between the two 7-methyljuglone units seemed to be very difficult to form, therefore diospyrin will be very difficult to chemically synthesise in the lab. During the previous chapter attempts were made to synthesise diospyrin but failed. Seeds of *Euclea natalensis* A.DC. were collected in Tembe elephant park and were germinated to produce seedlings. A cell-free extract was produced from these seedling roots and 7-methyljuglone added to the extract. The production of small amounts of neodiospyrin, diospyrin and isodospyrin were noticed.
6.2 Materials and methods

6.2.1 Material

_E. natalensis_ seeds were collected at Tembe elephant park, northern KwaZulu-Natal. A specimen was compared to a voucher specimen (N.L.22) deposited at the H.G.W.J. Schweikerdt herbarium at the University of Pretoria.

6.2.2 Methods

6.2.2.1 Germination of _E. natalensis_ seedlings

Four batches of seeds (20 each) were washed separately in 0.5% (2 min and 5 min) and 1% (2 min and 5 min) solution of sodium hypochlorite (NaOCl). The seeds were thoroughly washed with distilled water to get rid of the excess NaOCl, after which the seeds were placed on moist paper and incubated at 37°C for six weeks. The light regime was 16 hours dark and 8 hours light. After 6 weeks the containers were collected and the germination percentage calculated for the different treatments.

6.2.2.2 Cell-free extract of _E. natalensis_ seedlings

The germinated seedling's roots were cut off and placed in a mortar on ice. The total weight of the roots was 4.26 g. A phosphate buffer was prepared with 0.68g of KH₂PO₄, 0.19g EGTA and 19.7μg of BSA in 100ml of distilled water (Meyer & O'Hagan, 1992). The pH was adjusted to 7.6 with KOH (3%). Phosphate buffer (5ml) was added to the mortar and the root material was grinded until a brown slurry remained. The resulting slurry was filtered using a Whatman no. 42 filter paper. The filter paper was washed with another 5 ml of buffer.

The cell-free extract was placed in a suitable glass container (covered with tinfoil) and 1 ml of a 0.2mg/ml 7-methyljuglone was added to it. Before the addition, 5 μl of the cell-free extract was injected into the HPLC. This sample acted as the control. The cell-free extract with the added 7-methyljuglone was placed in an incubator at 30°C and tested for activity after each hour.
6.2.2.3 Cell-free extract with the addition of 8-chloro-7-methyljuglone

This experiment was performed in order to block the 8-carbon in participating in the dimerisation process. It should therefore be possible to force diospyrin formation instead of neodiospyrin formation. A stock solution of 8-chloro-7-methyljuglone (prepared according to Musgrave & Skoyles, 2001) (0.2mg/ml stock) was added to the enzyme extract. The solubility of the 8-chloro-7-methyljuglone in the buffer was not satisfactory. It eventually disappeared from the chromatogram probably due to precipitation. There was no effect on the dimers present in the cell-free extract.

6.3 Results

6.3.1 Germination of *E. natalensis* seedlings

Table 6.1 gives the germination percentage of the seeds. The treatment of 0.5% NaOCl for a period of 5 minutes yielded the highest germination percentage.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% NaOCl 2min.</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>0.5% NaOCl 5 min.</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>1% NaOCl 2 min.</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>1% NaOCl 5min.</td>
<td>50 ± 15</td>
</tr>
</tbody>
</table>

6.3.2 Cell-free extract of *E. natalensis* seedlings

The cell-free control sample contained only 7-methyljuglone and trace amounts of diospyrin, neodiospyrin and mamegakinone and isodiospyrin. After 24 hours the 7-methyljuglone concentration was reduced to almost 25% of its original value while the concentration of the dimers increased (Fig 6.1-6.6). There was also an increase in an unknown compound at RT 2.16. (This might be the reduced form of 7-methyljuglone or a juglone-enzyme complex.)
The total areas of the four dimers compared with the area of 7-methyljuglone were used to calculate if the enzyme reaction occurred. The separate increases were too small to give accurate data on dimerisation. In Table 6.2 the areas obtained from the chromatograms are given.

**Table 6.2:** Areas obtained from the chromatograms indicating the decrease in 7-methyljuglone (MJ) and the increase in the dimers over time (hours).

<table>
<thead>
<tr>
<th>Sample name</th>
<th>MJ area at 254 nm</th>
<th>Dimers area at 254 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract control</td>
<td>395 2385</td>
<td>1053 5946</td>
</tr>
<tr>
<td>Extract plus MJ time 0</td>
<td>977 0565</td>
<td>1320 1775</td>
</tr>
<tr>
<td>Extract plus MJ time 1</td>
<td>730 3341</td>
<td>1191 4622</td>
</tr>
<tr>
<td>Extract plus MJ time 2</td>
<td>615 8461</td>
<td>1221 7073</td>
</tr>
<tr>
<td>Extract plus MJ time 3</td>
<td>839 4170</td>
<td>1309 4880</td>
</tr>
<tr>
<td>Extract plus MJ time 18</td>
<td>235 2560</td>
<td>1311 6844</td>
</tr>
</tbody>
</table>

The 7-methyljuglone peak area decreased from 977 0565 (after external 7-methyljuglone was added) to 235 2560 (76% decrease). The concentration of the dimers increased from 1053 5946 to 1311 6844 (19.7% increase). Fig 6.1-6.6 illustrates the chromatograms for the above-mentioned reaction. During all of the repeat experiments this trend was observed (7-methyljuglone decreasing and the dimers increasing).
Fig 6.1: The cell-free extract before 7-methyljuglone was added (control).

Fig 6.2: The cell-free extract after adding external 7-methyljuglone (time 0).
Fig 6.3: The cell-free extract after 1 hour.

Fig 6.4: The cell-free extract after 2 hours.
Fig 6.5: The cell-free extract after 3 hours.

Fig 6.6: The cell-free extract after 18 hours.
6.4 Discussion and conclusions

The germination of the seedlings did not pose any problems. It appears that a treatment of 5% NaOCl for 5 minutes yielded the best results. On average the germination percentage was between 75-85% with this treatment.

The phosphate buffer unfortunately extracted some of the naturally occurring naphthoquinones. This means that the reaction might take place without any addition of external 7-methyljuglone. Therefore a filter method or centrifugation is needed to get rid of the naphthoquinones in the extract. Once the extract is free of naphthoquinones the addition of 7-methyljuglone will conclusively prove if any dimers will form or not. The decrease in 7-methyljuglone in the extract proved that either the 7-methyljuglone precipitated over time, was converted into dimers, or was complexing with something else (enzymes).

When the cell-free extract was left to stand for 10 days (at 30°C) all the naphthoquinones disappeared from the chromatogram. This might be due to precipitation or the action of micro-organisms in the extract. In future the extract needs to be stirred gently to keep everything in suspension. (This will avoid early precipitation). The action of microbes can be avoided by filtering the extract with a 0.22-micron filter paper.

Another potential problem is that of dilution and air bubbles. Once 7-methyljuglone is added to the cell-free extract the sample is diluted. This will mean that a difference in the dimer concentration should occur before and after the addition of 7-methyljuglone. This did in fact happen. To counter the problem of dilution, a 1 ml standard of the cell free extract was kept as a standard and 1 ml of buffer was added to it. The reaction extract (1ml) was also diluted with 1 ml of 7-methyljuglone containing buffer. This kept the standard and the reaction mixture at the same concentration. Air bubbles in the HPLC syringe and in the system as a whole could influence the peak area of the chromatogram. The only solution to this problem is to be very careful before injecting any samples. The air bubbles will fortunately have the same influence on all the compounds of interest.
The cell free experiment was repeated at least 5 times. All of the results suggested that dimerisation occurred. The evidence is however not yet conclusive. The decrease in 7-methyllumuglone does not match the increase in the concentration of the dimers. For every dimer to form, two 7-methyllumuglone units are needed. Therefore the 7-methyllumuglone peak will disappear twice as fast as the appearance of dimer peaks. The increase in the dimers (20%) can therefore be doubled to compare it to the decrease of the 7-methyllumuglone peak. According to this calculation 40 % of the 7-methyllumuglone was used for dimer formation. The remaining 36 % cannot be accounted for.

The assumption was made that all four dimers will absorb UV light in exactly the same way as diospyrin. This could mean that isodiospyrin, neodiospyrin and mamegakine appear to be less than what they actually are. To solve this problem a standard curve for all four dimers needs to be done.

The cell-free experiments were conducted in order to synthesise diospyrin. From the results it can be concluded that dimerisation does occur albeit in a small scale. To be able to synthesise diospyrin, a different buffer (different pH) can be used in future.

The addition of 8-chloro-7-methyllumuglone instead of 7-methyllumuglone did not work to stop neodiospyrin from forming and to increase the possible formation of diospyrin. This was unfortunately due to the insolubility in the buffer solution.

Another hypothesis is that the reaction is not due to enzymes but due to a simple chemical process (redox reaction). In the previous chapter the synthesis of neodiospyrin in a phosphate buffer was achieved. This was done by reducing 7-methyllumuglone and adding it to an unreduced 7-methyllumuglone form, in a phosphate buffer. The possibility that only a co-factor is needed, was tested by adding a common co-factor, nicotinamide adenine dinucleotide (NAD) to 7-methyllumuglone. The assumption was that this co-factor might reduce 7-methyllumuglone and thereby react with itself to form diospyrin. After the addition of NAD, 7-methyllumuglone was quickly converted into a “reduced” form. (Thereby forming the hydroquinone of methyljuglone and NADH). This peak appeared at the same retention time as
shinanolone (semi-reduced 7-methyljuglone) (Fig 6.7 a & b). The UV spectra of this compound differed from that of shinanolone. This is however where the reaction stopped. Addition of 7-methyljuglone did not have any effect on the expected dimerisation.

NADH was also added to 7-methyljuglone in phosphate buffer with the hypothesis that no reduction of 7-methyljuglone will occur. The same “reduction” product formed after 24 hours (Fig 6.8 a & b). This means that NAD and NADH appear to have the same effect on 7-methyljuglone.

Fig 6.7a: 7-Methyljuglone and NAD in phosphate buffer after 5 minutes.
Fig 6.7b: 7-methyljuglone reduced by NAD to an unknown product with the same RT as shinanolone after 30 min.

Fig 6.8a: 7-methyljuglone and NADH in phosphate buffer after 5 min.
Fig 6.8b: 7-methyljuglone reduced to unknown compound with the same RT as shinanolone after 30 min.

According to Morrison & Boyd (1992) quinones seem to take part in redox cycles essential to living organisms and that certain quinones undergo redox cycling and cause depletion of the co-factors such as NADH.

The experiments conducted with the co-factor NAD proves that it probably takes part in the biochemical pathway for producing naphthoquinones. The investigation of the enzymatic synthesis of diospyrin from 7-methyljuglone and the biochemical pathway for quinone synthesis will be continued during PhD studies.
6.5 References


Chapter 7

Antimycobacterial activity of the isolated compounds

7.1 Introduction 101
7.2 Materials and methods 101
  7.2.1 Materials 101
  7.2.2 Methods 102
7.3 Results and discussion 103
7.4 Conclusions 108
7.5 References 109
Chapter 7

Antimycobacterial activity of the isolated compounds

7.1 Introduction

According to the World Health Organisation, over 8 million cases of TB occurred in 1992. In sub-Saharan Africa, 1.2 million of these cases were documented. The rest of the infections (95%) occurred predominantly in the developing world. During the pre-chemotherapy era, TB had a mortality rate of 50-60%. Today the death rates are not as high because a significant proportion of cases are detected and treated (Snider et al., 1994). The search for new and improved drugs to treat TB is therefore an ongoing process and with the advances in detection systems, has yielded quite a few potential anti-TB drugs in the past decade.

The introduction of radiometric techniques in the field of mycobacteriology is a recent development. This detection system has opened new possibilities for determining the susceptibility of mycobacterium, on the basis of minimal inhibitory concentration (MIC), of plant extracts or drugs. If the BACTEC radiometric assay liquid medium is used, the growth of Mycobacterium tuberculosis is monitored radiometrically and the results are available within 5-6 days (Jenkins, 1998). The conventional method monitored the growth of M. tuberculosis on solid agar medium, the mycobacterial colonies were counted and only after 3-4 weeks the results were available (Jenkins, 1998). The accuracy and reproducibility of the BACTEC method compares well with the conventional method (Siddiqi et al., 1981). During this study the isolated compounds and synthetic 7-methyljuglone have been tested against various strains of mycobacterium using the BACTEC radiometric method.

7.2 Materials and methods

7.2.1 Materials

The TB strains used in this chapter were obtained from the Medical Research Council of South Africa.
The strains used were:

-H37Rv 27294: drug sensitive strain
-WC 64/02: sensitive to isoniazid, rifampicin, streptomycin and ethambutol
-DP 481/01: resistant to isoniazid and rifampicin
-DP 492/01: resistant to isoniazid, rifampicin, streptomycin and ethambutol
-WH 51/02: sensitive to isoniazid, rifampicin, streptomycin and ethambutol

The chemical structures of the abovementioned commercial drugs are shown in Fig. 1.2.

The following compounds, isolated as described in chapter 2, were tested against these strains: diospyrin, 7-methyljuglone, shinanolone, isodiospyrin and neodiospyrin. Synthetic 7-methyljuglone and the intermediate compound, 8-chloro-7-methyljuglone, (synthesis described in chapter 4) were also tested against the H37Rv strain of TB. In addition to these compounds the crude chloroform extract of E. natalensis root material and mixtures of 7-methyljuglone, diospyrin and shinanolone were tested for activity. The mixtures of compounds were tested to establish if any synergistic activity was present.

7.2.2 Methods

The medium used during these studies was the 7H12 Middlebrook TB medium (Middlebrook et al., 1977). It consists of an enriched Middlebrook 7H9 broth base supplemented with bovine serum albumin, catalase, casein hydrolysate and 14C-labelled substrate (palmitic acid) as a source of carbon. Growth of the mycobacterium leads to the consumption of the carbon source (palmitic acid), with subsequent release of labelled 14CO2. This labelled CO2 moves into the atmosphere above the medium in the sealed vial, the BACTEC TB 460 instrument detects the amount of 14CO2 and records it as a growth index (GI) on a scale of 0-999 (Heifets & Good, 1994).

In preparing strains of mycobacterium for MIC determination, a vial containing 7H12 Middlebrook TB medium was inoculated with homogenised cultures in a special diluting fluid. (Middlebrook-Dubos 7H9 broth having the no.1 McFarland standard optical density.) When growth in this vial reached a GI reading of 400-500, the 7H12
broth culture was used undiluted to inoculate a set of vials. The isolated compounds were added into the vials together with PANTA, an antimicrobial supplement. This was added to suppress rapidly growing contaminants that might otherwise overgrow the slow growing mycobacterium.

The principles of the radiometric proportion method states that the concentration of the compound that produces a daily GI increase or a final GI increase, lower than the 1:100 control sample can be considered to be the concentration at which the compound inhibits the growth of the mycobacterium with more than 99%. The MIC is defined as the lowest concentration of a drug that inhibits more than 99% of the bacterial population.

Inoculated vials were incubated at 38°C and each vial was assayed daily at the same hour until cumulative results were interpretable. The GI value of the control vial was compared with the readings from the vials containing the compounds. The control vial contains a 1:100 dilution of the inoculum and when it reached a reading of 30 the readings were stopped. The difference in GI readings for the last two days (ΔGI) was used. If ΔGI readings of any of the compounds were less than the control vial, that compound was considered to be active. All the compounds were tested in triplicate.

7.3 Results and discussion

The antimycobacterial results for diospyrin, neodiospyrin, isodiospyrin, 7-methyljuglone, 8-chloro-7-methyljuglone, shinanolone and the crude extract are given in Tables 7.1-7.7. These compounds were tested against various TB strains and are compared to different commercial TB drugs.

Table 7.1 gives the MIC values for the naphthoquinones and some commercially available drugs. From this table it can be seen that 7-methyljuglone has the lowest MIC of the tested naphthoquinones. It compares very well with the commercial drugs especially with Ethambutol (MIC of 7-methyljuglone 0.5µg/ml compared with Etambutol 1.25µg/ml). The MIC of the synthetic 8-chloro-7-methyljuglone (10-50...
μg/ml) had a worse than expected activity against TB. The addition of the chlorine atom on carbon 8 of 7-methyljuglone appears to decrease the activity.

**Table 7.1:** Antimycobacterial activity of 3 commercial drugs compared to naphthoquinones alone and in combination against the H37Rv drug sensitive strain.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory Concentration (MIC) in μg/ml</th>
<th>Lowest concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>0.062</td>
<td>0.0155</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.125</td>
<td>0.0625</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>1.25</td>
<td>0.625</td>
</tr>
<tr>
<td>7-Methyljuglone</td>
<td>0.50</td>
<td>0.250</td>
</tr>
<tr>
<td>Diospyrin</td>
<td>5.0-10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Crude chloroform extract</td>
<td>10.0-50.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Shinanolone</td>
<td>50.0-100.0</td>
<td>10.0</td>
</tr>
<tr>
<td>8-chloro-7-methyljuglone</td>
<td>10.0-50.0</td>
<td>10.0</td>
</tr>
<tr>
<td>a7-MJ+ bDio</td>
<td>10.0-50.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7-MJ+Dio+ cShi</td>
<td>10.0-50.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*a* = 7-methyljuglone  
*b* = diospyrin  
*c* = shinanolone

**Table 7.2:** Activity of naphthoquinones alone and mixed against a multidrug sensitive TB strain (WC64/02).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory Concentration (MIC) in μg/ml</th>
<th>Lowest concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>&gt;10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>7-Methyljuglone</td>
<td>1.0-5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Diospyrin</td>
<td>5.0-10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>a7-MJ+ bDio</td>
<td>&gt;10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7-MJ+Dio+ cShi</td>
<td>5.0-10.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*a* = 7-methyljuglone  
*b* = diospyrin  
*c* = shinanolone
Table 7.3: Activity of naphthoquinones against an isoniazid/rifampicin resistant strain (DP481/01) of TB.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory Concentration (MIC) in μg/ml</th>
<th>Lowest concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>&lt;1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>&gt;0.25</td>
<td>0.0625</td>
</tr>
<tr>
<td>7- Methyljuglone</td>
<td>5.0-10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Diospyrin</td>
<td>&lt;1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7-MJ+7&quot;Dio</td>
<td>&gt;10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7-MJ+Dio+5&quot;Shi</td>
<td>1.0-5.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(a=7\)-methyljuglone  
\(b=\) diospyrin  
\(c=\) shinanolone

Table 7.4: Activity of naphthoquinones against multidrug resistant TB strain (DP492/01).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory Concentration (MIC) in μg/ml</th>
<th>Lowest concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>5.0-10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>&gt;2.0</td>
<td>0.10</td>
</tr>
<tr>
<td>7-Methyljuglone</td>
<td>1.0-3.0</td>
<td>0.20</td>
</tr>
<tr>
<td>Diospyrin</td>
<td>5.0-10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>7-MJ+7&quot;Dio</td>
<td>&gt;50.0</td>
<td>10.0</td>
</tr>
<tr>
<td>7-MJ+Dio+5&quot;Shi</td>
<td>5.0-10.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(a=7\)-methyljuglone  
\(b=\) diospyrin  
\(c=\) shinanolone
Table 7.5: Activity of naphthoquinones against the WH51/02 strain, which is sensitive to all commercial drugs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory Concentration (MIC) in µg/ml</th>
<th>Lowest concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>&gt;10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>&lt;0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>7-Methyljuglone</td>
<td>5.0-10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Diospyrin</td>
<td>&gt;10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(^a)7-MJ(^b)Dio</td>
<td>5.0-10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7-MJ+Dio(^c)Shi</td>
<td>5.0-10.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) = 7-methyljuglone  
\(^b\) = diospyrin  
\(^c\) = shinanolone

Table 7.6 Summary of MIC values of naphthoquinones activity against the various TB strains.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory Concentration (MIC) in µg/ml</th>
<th>H37Rv</th>
<th>WC 64/02</th>
<th>DP 481/01</th>
<th>DP 492/01</th>
<th>WH 51/02</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.125</td>
<td>0.125</td>
<td>&gt;0.25</td>
<td>&gt;2.0</td>
<td>&lt;0.50</td>
<td></td>
</tr>
<tr>
<td>7-Methyljuglone</td>
<td>0.20-0.50</td>
<td>1.0-5.0</td>
<td>5.0-10.0</td>
<td>1.0-3.0</td>
<td>5.0-10.0</td>
<td></td>
</tr>
<tr>
<td>Diospyrin</td>
<td>5.0-10.0</td>
<td>5.0-10.0</td>
<td>5.0-10.0</td>
<td>5.0-10.0</td>
<td>&gt;10.0</td>
<td></td>
</tr>
<tr>
<td>Crude Extract</td>
<td>10.0-50.0</td>
<td>&gt;10.0</td>
<td>&lt;1.0</td>
<td>5.0-10.0</td>
<td>&gt;10.0</td>
<td></td>
</tr>
<tr>
<td>(^a)7-MJ(^b)Dio</td>
<td>10.0-50.0</td>
<td>&gt;10.0</td>
<td>&gt;10.0</td>
<td>&gt;50.0</td>
<td>5.0-10.0</td>
<td></td>
</tr>
<tr>
<td>7-MJ+Dio(^c)Shi</td>
<td>10.0-50.0</td>
<td>5.0-10.0</td>
<td>1.0-5.0</td>
<td>5.0-10.0</td>
<td>5.0-10.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) = 7-methyljuglone  
\(^b\) = diospyrin  
\(^c\) = shinanolone
Table 7.7: Activity of isodospyrin, neodospyrin and synthetic 7-methyljuglone against the H37Rv TB strain.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MIC (µg/ml)</th>
<th>ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isodospyrin</td>
<td>5.0</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>Neodospyrin</td>
<td>10.0</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>Synthetic 7-MJ</td>
<td>3.0</td>
<td>Bacteriostatic</td>
</tr>
</tbody>
</table>

The mixtures of 7-methyljuglone, diospyrin and shinanolone showed that there are probably no synergistic effect between these compounds. The MIC values for the mixtures were higher than 7-methyljuglone and diospyrin tested separately. Table 7.2 indicates the activity of the naphthoquinones against a TB strain that is sensitive to four commercial drugs. Due to the high MIC of shinanolone (50-100 MIC) this compound was not tested against this strain. From this table it can be seen that 7-methyljuglone has the lowest MIC value of the naphthoquinones tested and that the mixtures of naphthoquinones had a lower activity than the compounds tested separately.

Table 7.3 gives the MIC values of naphthoquinones tested against a TB strain that showed resistance to two commercial drugs. From this table it can be seen that diospyrin and the crude extract has got the lowest MIC values. This will indicate that there are probably unknown compounds in the crude extract that will show very good activity against this specific strain of TB. In future studies these compounds will be isolated and tested for activity against this particular strain of TB. Tables 7.4 and 7.5 indicates that 7-methyljuglone showed the lowest activity of the naphthoquinones against the specific TB strains used. Overall 7-methyljuglone appears to have the lowest MIC value of the naphthoquinones tested. It compares well with the commercially available drugs and therefore further studies into the chemistry and mode of action of naphthoquinones and especially 7-methyljuglone are needed.
Table 7.7 gives the activity of isodospyrin and neodospyrin. This test was done only once in triplicate and can therefore only be considered to be indicative of the potential activity for these compounds. The activity of these two dimers appears to be similar to diospyrin. These two compounds might also be responsible for the low MIC value of the crude extract against the DP481/01 strain. In future they will also be tested against this strain.

The activity of the synthetic 7-methyljuglone appears to be slightly lower than the naturally occurring 7-methyljuglone. This can be due to impurities or the slow breakdown caused by trace amounts of catalyst (AlCl₃) remaining in the synthetic compound. Unconfirmed tests of mamegakinone showed an MIC of >100µg/ml and this compound can therefore be considered as inactive.

7.4 Conclusions

From the results it can be concluded that 7-methyljuglone showed the most promising activity against the various TB strains tested. Only in one instance did diospyrin (and crude extract) have a better MIC value than 7-methyljuglone. This was against the DP481/01 TB strain. This indicates that there is a lot of variation between the mycobacterium strains and that all of the naphthoquinones should be tested against all of the different strains. This will also yield some information on the mode of action of naphthoquinones. In future the difference between the TB strains should be studied and attempts must be made to connect the structure of the compound with the difference in TB strain.

It must also be concluded that the roots of *E. natalensis* does have unknown compounds that will show activity against TB. Future attempts must therefore be directed towards finding these unknown compounds.
7.5 References


Chapter 8

General discussion and conclusions

8.1 Isolation and identification of naphthoquinones 111
8.2 Synthesis of naphthoquinones 112
8.3 Antimycobacterial activity of naphthoquinones 112
8.4 Future studies 113
8.5 References 115
Chapter 8

General discussion and conclusions

8.1 Isolation and identification of naphthoquinones

In the introduction to this dissertation, the naphthoquinones previously isolated from *E. natalensis* were described and illustrated in Fig.1.1 (Chapman & Hall, 2003). In this study three compounds have been isolated for the first time from this ethno-botanically selected species. Two of which are naphthoquinones (neodiospyrin and mamegakinone), and one naphthalene based aromatic compound (5-hydroxy-4-methoxy-2-napthaldehyde).

In chapter 2 the isolation procedures for all the isolated compounds were described. The dry silica column chromatography technique was used in the isolation of all the compounds (Houghton & Raman, 1998). This method might be revised in future, due to the potential loss of the compounds (absorption onto the silica) and the resulting lower yields. Currently a new column matrix is being tested which can improve the yields and decrease the isolation time.

The HPLC system that is currently in use must also be standardised so that the RT of the various compounds remain constant. This will be achieved by controlling the column and solvent temperature. The experiments described in section 2.2.2.5 need to be repeated in order to statistically validate the yields obtained from different solvents and from different root parts. These experiments do however give some indication as to which solvent will give the highest yields of a certain naphthoquinone. The differences in naphthoquinone concentration in the two different plant specimens suggest that there is a huge natural variation caused by the time of collection, location of collection and even time before extraction (This will however be very difficult to standardise). The method optimisation, as described in section 2.4, does considerably shorten the purification time of the naphthoquinones. It is still important to improve on this technique in order to increase yields even further.
The structural elucidation process of the naphthoquinones did not pose any big problems (Chapter 3). All of the isolated compounds have been isolated before from various plant species. Through comparative studies the structures could be confirmed.

8.2 Synthesis of naphthoquinones

Chapter 4 described the synthesis of the most active compound, 7-methyljuglone as described by Musgrave & Skoyles, 2001. The exact reaction mechanism for the first step (section 4.3.1.) is still under review and various alternatives were given in this case. The yields of the synthetic product are far too low to commercialise at this point in time. The yields can be increased by effective stirring and accurate measurements of the temperature and pH. The alternative synthetic pathway does show some promise in reducing time and costs. This work will be continued during future studies.

The synthesis of neodiospyrin and the enzymatic synthesis of diospyrin described in chapter 5 and 6 will be investigated further. The difficult C-C bond between carbon 2 and carbon 6', to form diospyrin, will be achieved either enzymatically or chemically. The use of the phosphate buffers at different pH and temperatures should give more information on how to synthesise diospyrin. Up to now the synthesis of neodiospyrin was surprisingly easy whereas the synthesis of diospyrin was practically impossible.

8.3 Antimycobacterial activity of naphthoquinones

Chapter 7 dealt with the activity of some of the isolated compounds. The activity of neodiospyrin and isodiospyrin appears to be comparable with diospyrin and 7-methyljuglone. The activity of the naphthoquinones against various TB strains indicated that 7-methyljuglone showed the best activity. All of the other naphthoquinones did show good activity against all the strains. The mixtures of naphthoquinones tested indicated that no synergistic effect was taking place. The activity of diospyrin and the crude extract against the resistant DP481/01 strain
indicated that there are some unknown compounds in the root material that should show activity against TB. These compounds, which might be the different dimers (neodiospyrin and isodiospyrin), should be isolated and tested for activity. The synthesis of isodiospyrin therefore also seems to be important for future studies.

8.4 Future studies

Future studies will concentrate on the possible mode of action of these naphthoquinones. One potential target is the mycolic acids, which are high molecular weight, α-alkyl, β-hydroxy fatty acids, found on the mycobacterial cell wall. A typical characteristic of the mycolic acids is that they undergo a reverse Claisen-type condensation reaction (Besra & Chatterjee, 1994), (Fig 8.1). Naphthoquinones ability to take part in redox cycling can cause these compounds to become proton cirlers as illustrated below.

![Diagram of the reverse Claisen condensation reaction](image)

**Fig 8.1:** Reverse Claisen condensation reaction caused by 7-methyljuglone yielded a meroaldehyde and a hydroxy group.

This reaction might degrade the cell wall and thereby destroy the bacterium. When mycolic acid and 7-methyljuglone are mixed in a phosphate buffer, the reaction should give 3 products (7-methyljuglone, meroaldehyde and the hydroxyl group), instead of the two starting reagents (7-methyljuglone and mycolic acid). With the use
of the HPLC this reaction can be followed. This system would enable us to search for potential anti-TB compounds without the use of the BACTEC radiometric testing system. The advantage would be that compounds could be screened for activity before they are tested against the mycobacterium. This work will be continued during future studies.
8.5 References


Chapter 9

Summary

Characterisation, synthesis and antimycobacterial activity of naphthoquinones isolated from *Euclea natalensis*

by

Frank van der Kooy

Supervisor: Prof. J.J.M. Meyer
Department of Botany
MAGISTER SCIENTIAE (Plant Physiology)

TB is still one of the world’s biggest killers. Immunosuppression induced by AIDS caused a rise in the incidence of TB during the past decade. The search for new drugs to effectively treat TB remains one of the big challenges facing the scientific community. Drugs from plants have been used for centuries to treat various human diseases with varying degrees of success. South Africa with its big resource of plants and ethnobotanical knowledge is an ideal place to screen for anti-TB compounds. The Zulu tribe of South Africa used the root bark of *Euclea natalensis* A.DC. to treat TB
related symptoms. Naphthoquinones isolated from *E. natalensis* proved to have good activity against TB.

Nine compounds were isolated from the chloroform extract of *E. natalensis* root material. Three of these compounds were newly isolated from this species (mamegakinone, neodiospyrin and 5-hydroxy-4-methoxy-2-napthaldehyde). The structures of the isolated compounds were confirmed using NMR methods and where possible the HPLC and TLC results were compared to authentic standards.

Most of the compounds were tested for anti-TB activity with only mamegakinone, lupeol and betulin not showing any activity (5-hydroxy-4-methoxy-2-napthaldehyde still needs to be tested). The activity of the naphthoquinones, especially 7-methyljuglone, diospyrin, isodiospyrin and neodiospyrin, show promise that these compounds could develop into an affordable medicine to treat TB. The activity of the crude extract against the resistant DP481/01 TB strain showed that there are probably unknown active compounds remaining in the extract.

The most active compound, 7-methyljuglone, was synthesised and an improved synthetic pathway was developed. The synthesis of naphthoquinones remains important in order to produce the compounds on a larger scale. This will make further studies into the mode of action, biosynthesis, bioactivity etc. of these compounds possible. Attempts were made to synthesise diospyrin with 7-methyljuglone as the starting material. These experiments failed up to now. By altering the reaction parameters such as pH and temperature it should be possible to synthesise diospyrin in future attempts.
Neodiospyrin were synthesised from reduced 7-methyljuglone. This synthesis will yield information on the naphthoquinone chemistry and on how to synthesise diospyrin and isodiospyrin. The enzymatic synthesis of naphthoquinones was also investigated with the use of a cell-free extract. These experiments indicated that it might be possible to enzymatically synthesise diospyrin and the other dimers.
Chapter 10

Acknowledgements

I would like to thank the following people and institutions:

Prof. J.J.M. Meyer for his guidance, comments and suggestions during the course of this research project.

Dr. Namrita Lall, Mr Fanie de Meillon, Quentin Kritzinger, Rene Swart and the other students in our group, for assistance and comments during the course of this research project.

Prof. J.H.I. Martinez with the help and guidance on the HPLC and NMR.

Prof. O.C. Musgrave for the assistance with the synthesis of 7-methyljuglone.

Mr. Eric Palmer, Department of Chemistry for the NMR analysis.

Mr. Benedict Bapela for the testing of the various compounds against TB.

The National Research Foundation and the University of Pretoria for financial support.