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Chapter 4

Synthesis of 7-methyljuglone and diospyrin

4.1 Introduction 57

4.2 Materials and methods 58

4.2.1 Materials 58

4.2.2 Methods 59

4.2.2.1 Synthesis of 7-methyljuglone 59

4.2.2.1.1 Effect of different quantities of reagents on 7-methyljuglone formation 59

4.2.2.1.2 Effect of different quantities of catalyst on 7-methyljuglone formation 60

4.2.2.1.3 Influence of temperature on 7-methyljuglone formation 60

4.2.2.1.4 Effect of altering reaction times 61

4.2.2.2 Epoxidation of 7-methyljuglone 61

4.2.2.2.1 Effect of reaction time on epoxide formation 61

4.2.2.2.2 Influence of time before acidification 61

4.2.2.2.3 Effect of amount of acid on epoxide formation 62

4.2.2.3 Synthesis of diospyrin 62

4.2.2.3.1 Oxidative dimerisation of 7-methyljuglone **62**

4.2.2.3.2 Buffered reaction between hydroquinone and 7-methyljuglone **62**

4.2.2.3.3 Epoxide condensation **63**

4.2.2.3.3.1. Addition of an Bronsted-Lowry acid to the epoxide 63

4.2.2.3.3.2 Addition of an Lewis acid and steam distillation 63

4.3 Results 64

4.3.1 Synthesis of 7-methyljuglone 64

4.3.1.1 Effect of different quantities of reagents on 7-methyljuglone formation 64

4.3.1.2 Effect of different catalyst ratios 64

4.3.1.3 Influence of temperature on 7-methyljuglone formation 65

4.3.1.4 Effect of altering stirring times 65

4.3.2 Epoxidation of 7-methyljuglone 66

4.3.2.1 Influence of reaction time on epoxide formation 66

4.3.2.2 Effect of time before acidification 66

4.3.2.3 Effect of the amount of acid 67

4.3.3 Synthesis of diospyrin 68

4.3.3.1 Oxidative dimerisation of 7-methyljuglone 68

4.3.3.2 Buffered reaction between hydroquinone and 7-methyljuglone 68

4.3.2.3 Epoxide condensation 68

4.3.2.3.1 Addition of Bronsted-Lowry acid 68

4.3.2.3.2 Addition of Lewis acid and steam distillation 69

4.4 Discussion and conclusions 70

4.5 References 74

Chapter 4
Synthesis of 7-methyljuglone and diospyrin

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 previous studies a fairly simple organic compound, and its dimers, was isolated from *Euclea natalensis* A.DC. This compound, 7-methyljuglone**,** was first synthesized by Cooke & Dowd, (1952) and was re-investigated and improved by Musgrave & Skoyles (2001), in a two-step procedure. They used the Friedel-Crafts acylation reaction between maleic anhydride and 4-chloro-3-methylphenol to form 8-chloro-7-methyljuglone. This chlorinated product was then reduced during the second step with tin chloride to form 7-methyljuglone**.** Both steps in this synthetic pathway was low yielding and difficult to perform. The overall yield of the two-step procedure (3 % calculated from the publication) is not satisfactory. The compound was synthesized in a one-step procedure (also employing a Friedels-Craft acylation reaction) during a previous study (Van der Kooy, 2003). Although the one-step synthesis saved valuable time and costs, it only gave yields of less than 1 %. It was attempted to improve the yield in this part of the study (Van der Kooy & Meyer, 2006).

Only one publication could be found regarding the synthesis of diospyrin (Yoshida & Mori, 2000). This synthetic route entails a 14 step sequence with yields of less than 4 %. 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 and a plumbagin dimer, respectively. Due to the structural similarities between neodiospyrin and the plumbagin dimer with diospyrin**,** this synthetic pathway was further investigated. In addition a new 3-step synthetic route with 7-methyljuglone epoxide as intermediate was investigated.

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-methyljglone and some of its dimers this is almost certainly the case.
- Plant material does not have to be used, which will save plant resources from exploitation.
- Another advantage is the increase in yields. Theoretically 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 complex compounds are worked with.

In this chapter the one-step synthetic pathway is revisited. Although the Friedel-Crafts reaction in this case is a low yielding procedure, the aim of any synthetic route should be to obtain reproducible and high yields. In addition to 7-methyljuglone, the chemical synthesis of diospyrin was also investigated with three different approaches.

4.2 Materials and methods

4.2.1 Materials

All chemicals used were of AR purity. The products of the different reactions were tested on silica gel TLC (hexane:ethyl acetate 5:2) and HPLC using phenomenex RP18 column (150 X 4.6 mm, 4 μ) with 62.5 % acetonitrile and 5 % acetic acid as mobile phase. A flow rate of 1 ml/min was employed with the detection system a UV6000LP PDA detector. The NMR analysis was done on a Varian Mercury-Plus 200 MHz with $CDCl_3$ as internal standard and referenced to 7.24 ppm.

4.2.2 Methods

4.2.2.1 Synthesis of 7-methyljuglone

To obtain the highest yielding method, the ratios between reagents and parameters such as stirring time and temperature of the reaction was tested. Different ratios of anhydrous aluminium chloride and sodium chloride were melted and different ratios of *m*-cresol and maleic anhydride was added into it. After addition, the mixture was vigorously stirred and added to a mixture of 12 N hydrochloric acid and crushed ice. The resulting precipitate was filtered off after 20 min, washed with water and dried. The crude brown material was extracted repeatedly with hexane. The different yields of 7-methyljuglone were calculated (from HPLC) and from this data the optimal conditions for this synthetic pathway were obtained.

4.2.2.1.1 Effect of different quantities of reagents on 7-methyljuglone formation

To establish the optimum ratio of the two starting reagents in the reaction, it was decided to use different quantities in molar equivalents. The reagents were added to $AlCl_3$ and NaCl at 180ºC. After 2 min of stirring it was added into 30 ml of HCl and crushed ice. After 20 min the mixture was filtered and washed with water. The reaction was repeated with different ratios and the appearance of the product noted. The products were extracted and tested on TLC for 7-methyluglone formation. Table 4.1 gives the different ratios that were used.

Table 4.1: Ratios of maleic anhydride and *m*-cresol tested to obtain the highest yield. Masses are in grams with molar equivalents in brackets (1 eq).

Maleic anhydride	*m*-Cresol	$AlCl_3$	NaCl
1.00g (1.00)	0.37g (0.30)	12.00g (8.80)	2.40g (4.10)
1.00g (1.00)	0.74g (0.60)	12.00g (8.80)	2.40g (4.10)
1.00g (1.00)	1.11g (1.00)	12.00g (8.80)	2.40g (4.10)
1.00g (1.00)	2.22g (2.00)	12.00g (8.80)	2.40g (4.10)

4.2.2.1.2 Effect of different quantities of catalyst on 7-methyljuglone formation

The optimum ratio of reagents was used (1.00 g of maleic anhydride and 1.11 g of m-cresol) and the catalyst ratios were changed. The same procedure was followed and the reaction repeated with the different ratios. The appearance of the melt was noted and the amount of 7-methyljuglone was established with the use of HPLC.

Table 4.2. Ratio of catalyst that was used to obtain the highest yields

$AlCl_3$ (grams)	NaCl (grams)	Appearance	Temperature (ºC)
12.0	0.0	Does not melt	160
12.0	1.2	Dark brown	180
12.0	2.4	Light brown	180
12.0	3.6	Turns sticky after 1.5 min of stirring	180

4.2.2.1.3 Influence of temperature on 7-methyljuglone formation

The effect that the reaction temperature had on 7-methyljuglone formation was tested by using the optimum amount of reagents and catalyst and by changing the temperature during three separate reactions. The temperatures used and the appearance of the crude product is given in Table 4.3.

Table 4.3: Effect of different temperatures on 7-methyljuglone formation. The optimum ratio of maleic anhydride and m-cresol were used

Maleic anhydride (grams)	m-cresol (grams)	$AlCl_3$ (grams)	NaCl (grams)	Temp ºC	Appearance
1.00	1.11	12.00	2.40	200	Dark brown
1.00	1.11	12.00	2.40	175	Light brown
1.00	1.11	12.00	2.40	150	Yellow

4.2.2.1.4 Effect of altering reaction times

The optimum conditions were used and the reaction times changed. The reaction was repeated and the mixture stirred for 30 s, 1 min, 2 min and 3 min after which it was added into HCl (30 ml) and crushed ice. The products were filtered and extracted with hexane after which it was tested on HPLC.

4.2.2.2. Epoxidation of 7-methyljuglone

Twenty five mg of 7-methyljuglone was dissolved in 10 ml of ethanol at 0 ºC. To this mixture a mixture of sodium perborate (62.5 mg) in water (7 ml) was added. After 2 min the pH was adjusted to 5 with 2 M HCl. A saturated mixture of NaCl in water (5 ml at 0 ºC) was added and the mixture extracted with hexane after 4 min. The crude product was tested on HPLC and NMR.

4.2.2.2.1 Effect of reaction time on epoxide formation

It was noted that two dimers also appeared during the epoxidation reaction. One of these had a similar retention time on the HPLC than diospyrin**.** This compound might have formed due to the reaction time or the over acidification of the extract. The analysis was therefore repeated and the solution added to hexane at different time intervals. Ten mg of 7-methyljuglone were dissolved in 4 ml EtOH at 0 ºC. To this mixture sodium perborate (25 mg) in water (2.8 ml) was added. The pH was adjusted with 2 M HCL to pH 5 after 2 min, after which 2 ml of NaCl was added. The reaction was continued in separate experiments for 1, 4, 10, 20 and 60 min. Extracting the products after the different time intervals with hexane stopped the reactions, after which it was tested on HPLC.

4.2.2.2.2 Influence of time before acidification

The reaction was repeated as described above with the exception that the pH was adjusted after 30 s, 60 s, 120 s, 240 s and 300 s (5 drops HCl) in separate experiments. After the addition of acid the NaCl solution was added and the products extracted after 4 min. The products were tested on HPLC for the formation of the dimeric forms and the conversion into the epoxide.

4.2.2.2.3 Effect of amount of acid on epoxide formation

The same reaction was repeated with the exception that the amount of acid used varied. The reaction mixture was added to different volumes of acid after 4 min. The following quantities were used: no acid, 5 drops, 10 drops, 20 drops. After the addition of the acid, 4 ml NaCl was added.

4.2.2.3 Synthesis of Diospyrin

The synthesis of diospyrin has been achieved recently by Yoshida & Mori (2000). The synthesis entails 14 steps with very low overall yields which makes this method not commercially viable. There are however a couple of ways to dimerise a compound of this nature. Van der Vjiver & Gerritsma (1974) reported that neodiospyrin and mamegakinone forms through oxidative dimerisation when 7-methyljuglone is exposed to air in a silica gel matrix. This method was investigated. The synthesis of neodiospyrin in a phosphate buffer, which was achieved in previous studies, was also reinvestigated by changing the pH of the buffer. The aim was to stop the formation of neodiospyrin and to synthesise diospyrin. A third reaction was investigated through which 7-methyljuglone was epoxidised and allowed to react with itself with the loss of water.

4.2.2.3.1 Oxidative dimerisation of 7-methyljuglone

Synthetic 7-methyljuglone (5 mg) was added to 1 g of silica gel. The mixture was stirred and air was passed over it for 6 weeks. Samples were collected at weekly intervals by dissolving 100 mg of the mixture in chloroform and filtering the silica off. The filtrate was injected into the HPLC with an authentic diospyrin standard.

4.2.2.3.2 Buffered reaction between 7-methyljuglone and its hydroquinone

7-Methyljuglone (25 mg) was dissolved in 2.5 ml of tetrahydrofuran (THF). This was added to a solution of tin chloride (125 mg), 2.5 ml of THF and 8.75 ml of 4 M hydrochloric acid (HCl) at 60 ºC. The solution was stirred for 3 hours. After stirring, the THF was evaporated by opening the vessels for 15 min at 60 ºC, after which the THF 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.

A phosphate buffer was prepared by dissolving 6.8 g of KH_2PO_4 in 1 L of water. The pH was adjusted separately to 4.6, 5.6, 6.6, 7.6 and 8.6 with 3 % KOH. Unreduced 7-methyluglone (25 mg) was dissolved in the minimum amount of MeOH and 25 ml of phosphate buffer. The solution was stirred while adding the reduced 7-methyljuglone (hydroquinone), dissolved in the minimum amount of MeOH, into the reaction vessel. The solution was stirred for 30 min. 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 diospyrin and neodiospyrin standard.

4.2.2.3.3 Epoxide condensation

4.2.2.3.3.1. Addition of an Bronsted-Lowry acid to the epoxide

The 7-methyljuglone epoxide was dissolved in an acetone / water mixture and the acetone evaporated. A couple of drops of 4 M HCl were added to the water mixture. The reaction was heated to 60 ºC for 1 hour after which a brown solid precipitate appeared. The solid precipitate was filtered off and tested on NMR and HPLC.

4.2.2.3.3.2. Addition of an Lewis acid and steam distillation

The 7-methyljuglone epoxide (10 mg) was dissolved in the 20 ml of THF. This was added to a round bottom flask containing 56 mg of SnCl2 in 20 ml of water. The mixture was heated to 60 °C without vacuum on a rotary evaporator. The mixture was tested on HPLC after 1 min. 30 min and 2 hours. After 2 hours the vacuum was switched on and the distillate (yellow colour) and reaction mixture were tested on HPLC. The reaction was continued for 2 days without vacuum and THF was added when solid material appeared. After 2 days, 10 mg of 7-methyljglone in THF was added to the reaction mixture. The mixture was again distilled and tested on HPLC.

4.3 Results

4.3.1 Synthesis of 7-methyljuglone

4.3.1.1 Effect of different quantities of reagents on 7-methyljuglone formation

The products of the various reactions were tested on TLC plates and on HPLC with an authentic 7-methyljuglone standard. The different ratios of reactants gave the best result when a 1:1 molar equivalent was used. This translates into using 1.0 g of maleic anhydride and 1.11 g of *m*-cresol. With the other reactions none or very small amounts of 7-methyljuglone was obtained. The appearance of more by-products was also seen when the other ratios were used.

4.3.1.2 Effect of different catalyst ratios

The catalyst quantities were tested at four different ratios. During the first analysis no salt was used. The $AlCl_3$ did however not melt and the temperature stayed at 160 ºC. Therefore the salt was needed to assist in the melting of $AlCl_3$. It was previously thought that the salt might play a role in the reaction itself. Table 4.4 gives the ratios used with the resulting 7-methyljuglone formation.

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Table 4.4: Ratio of catalyst used

$AlCl_3$ (grams)	NaCl (grams)	Appearance	Reaction temperature (ºC)	Presence of 7-methyljuglone
12.0	0.0	Does not melt	160	No
12.0	1.2	Sticky darker brown	180	Yes
12.0	2.4	Brown	180	Yes
12.0	3.6	Turns sticky-solid after 1.5 min	180	Yes

The ratio of 12 g $AlCl_3$ and 2.4 g of NaCl appeared to give 7-methyljuglone in the largest amount. The appearance of the amount of by-products was also noted in each reaction.

4.2.1.3 Influence of temperature on 7-methyljuglone formation

The reaction was repeated with the optimum ratios of reactants and catalyst at 3 different temperatures. The presence of 7-methyljuglone and any by-products were tested on TLC. The appearance of the crude product was also indicative of the amount of 7-methyljuglone present. The reaction at 200 ºC gave the best results while at 150 ºC no 7-methyljuglone formed (Table 4.5).

Table 4.5: Effect of different temperatures on 7-methyljuglone formation. The optimum ratio of maleic anhydride and m-cresol were used

Temperature (ºC)	Appearance	Presence of 7-methyljuglone
200	Dark brown	Yes –very little by-products
175	Light brown	Yes – more by-products
150	Yellow	No

4.1.2.4 Effect of altering stirring times

The time of the reaction was also tested. After addition of the reactants to the molten catalyst the mixture was stirred for 30 s, 60 s, 120 s and 240 s after which it was added into HCl and ice. The appearance of the product and the amount of catalyst was noted. Stirring time of 60-120 s gave the best results. Longer stirring time resulted in a very sticky mixture with lower amounts of 7-methyljuglone.

The reaction was repeated with all the optimum conditions (1 g maleic anhydride, 1.11 g m-cresol, 12 g $AlCl_3$, 2.4 g NaCl, at 200 ºC with stirring time of 120 s). The resulting precipitate was filtered and washed with water. After drying the solid material it was extracted with hexane. A silica column was prepared with hexane ethyl acetate 9:1 as eluent. The 7-methyljuglone fractions were collected and a ^{1}H-NMR was performed. Fig 4.1 gives the proton NMR spectrum.

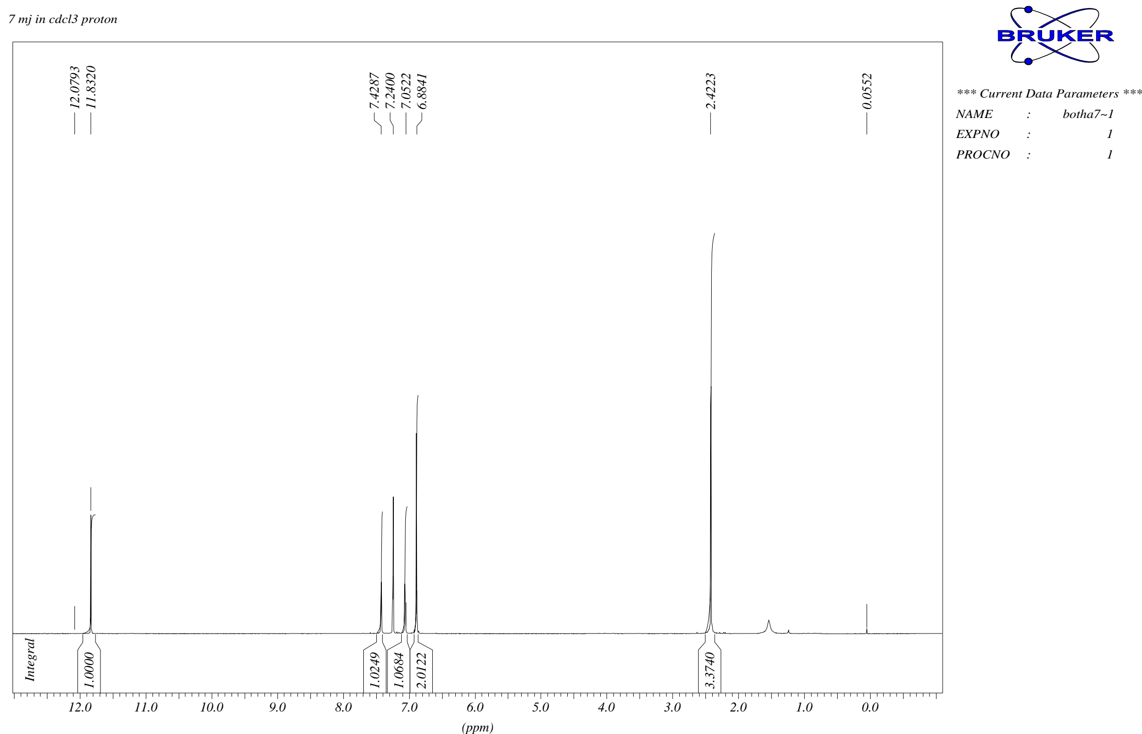

Fig. 4.1: ^{1}H-NMR spectrum of the synthetic 7-methyljuglone**.**

4.3.2 Epoxidation of 7-methyljuglone

The epoxidation of 7-methyljuglone yielded approximately a 60:40 ratio of the epoxide to the unreacted 7-methyljuglone as tested on HPLC. It was therefore necessary to improve this method.

4.3.2.1 Influence of reaction time on epoxide formation

The analysis was repeated and the solution stopped by adding it to hexane at five different time intervals. The resulting products were tested on HPLC. No difference in the epoxide formation was noted. The two dimers that formed were also not influenced by the reaction time. The addition of the acid therefore effectively stopped the reaction.

4.3.2.2 Effect of time before acidification

The addition of acid to the reaction mixture was tested at different time intervals. At 240 and 300 s the 7-methyljuglone was completely converted into its epoxide. It did not however influence the formation of the dimeric forms.

4.3.2.3 Effect of the amount of acid

The reaction was repeated and it was decided to change the amount of acid that was added. Five different volumes of acid were added and the resulting products were tested on HPLC. The epoxide itself did form in all the cases, but no difference was noted in the dimer formation. The crude products were tested on NMR to identify the dimers. The hydroxy proton shifts of mamegakinone and diospyrin could be seen (Fig. 4.2). It is therefore a by-product of the epoxidation step. It was however noted that the presence of 7-methyljuglone was needed before the dimers formed. It is therefore plausible that the epoxide reacts with 7-methyljuglone to give diospyrin and mamegakinone.

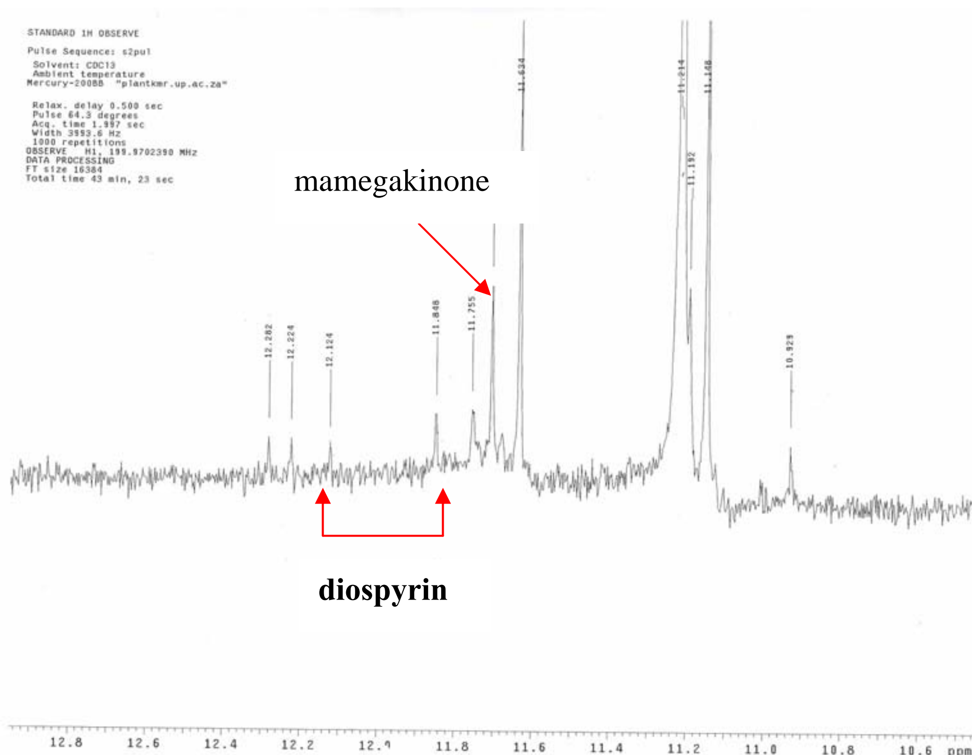

Fig 4.2 Hydroxy proton shifts, on the ^{1}H-NMR spectrum, indicating the presence of diospyrin and mamegakinone

4.3.3 Synthesis of diospyrin

4.3.2.1 Oxidative dimerisation

After aerating the sample in silica gel for 6 weeks it was tested on HPLC. The sample did appear to undergo some major changes. A very small amount of 7-methyljuglone was still present but a lot of other compounds did form. None of these compounds was diospyrin.

4.3.2.2 Buffered reaction between hydroquinone and 7-methyljuglone

The colour of the reactions was important (Table 4.5). A purple colour indicated the appearance of the 7-methyljuglone potassium salt. This appears to be a crucial step in the formation of the dimers. Neodiospyrin did form, but there was no trace of diospyrin. The atomic radius of the potassium atom (227 pm) is large compared to the rest of the molecule. It might therefore be possible that the carbon 6 atom is sterically hindered to take part in the reaction. Future experiments should therefore make use of smaller atoms (Na = 186 pm, Li = 152 pm).

Table 4.5: The formation of the dimer, neodiospyrin at different pH's. The colour of the reaction was also noted.

pH	4.6	5.6	6.6	7.6	8.6
Reaction colour	Orange	Green-brown	Dark purple – brown	Purple-brown green	Purple -green
Dimer formation	no	no	Yes - neodiospyrin	Yes - neodiospyrin	no

4.3.2.3 Epoxide condensation

4.3.2.3.1 Addition of Bronsted-Lowry acid

The epoxide reacted rapidly with HCl to form the expected 2-chloro-3-hydroxy-7-methyljuglone. NMR analysis done on the crude product indicated that this was the major product. No dimers occurred during any stage of this reaction. The use of HCl will unfortunately always give the chlorinated product. It was therefore decided that a Lewis acid should be used.

4.3.2.3.2 Addition of Lewis acid and steam distillation

After 2 days the formation of a small amount of diospyrin in the reaction vessel was noted. The distillate contained 7-methyljuglone, 7-methyluglone epoxide and the reduced epoxide. The reaction vessel contained small amounts of diospyrin. The major constituents of the vessel were still the epoxide and 7-methyljuglone. The sample was tested on HPLC and it appeared to be diospyrin. An authentic standard of diospyrin was injected with the sample and only one well-resolved peak at the correct retention time was noted. The sample was subjected to NMR and small hydroxy proton peaks at the correct chemical shifts (Lillie & Musgrave, 1977) were noted. The signal to noise ratio was very low and therefore the NMR analysis can not be seen as conclusive proof of diospyrin formation. Fig 4.3 gives the HPLC chromatogram indicating the presence of diospyrin at rt 9.496 min.

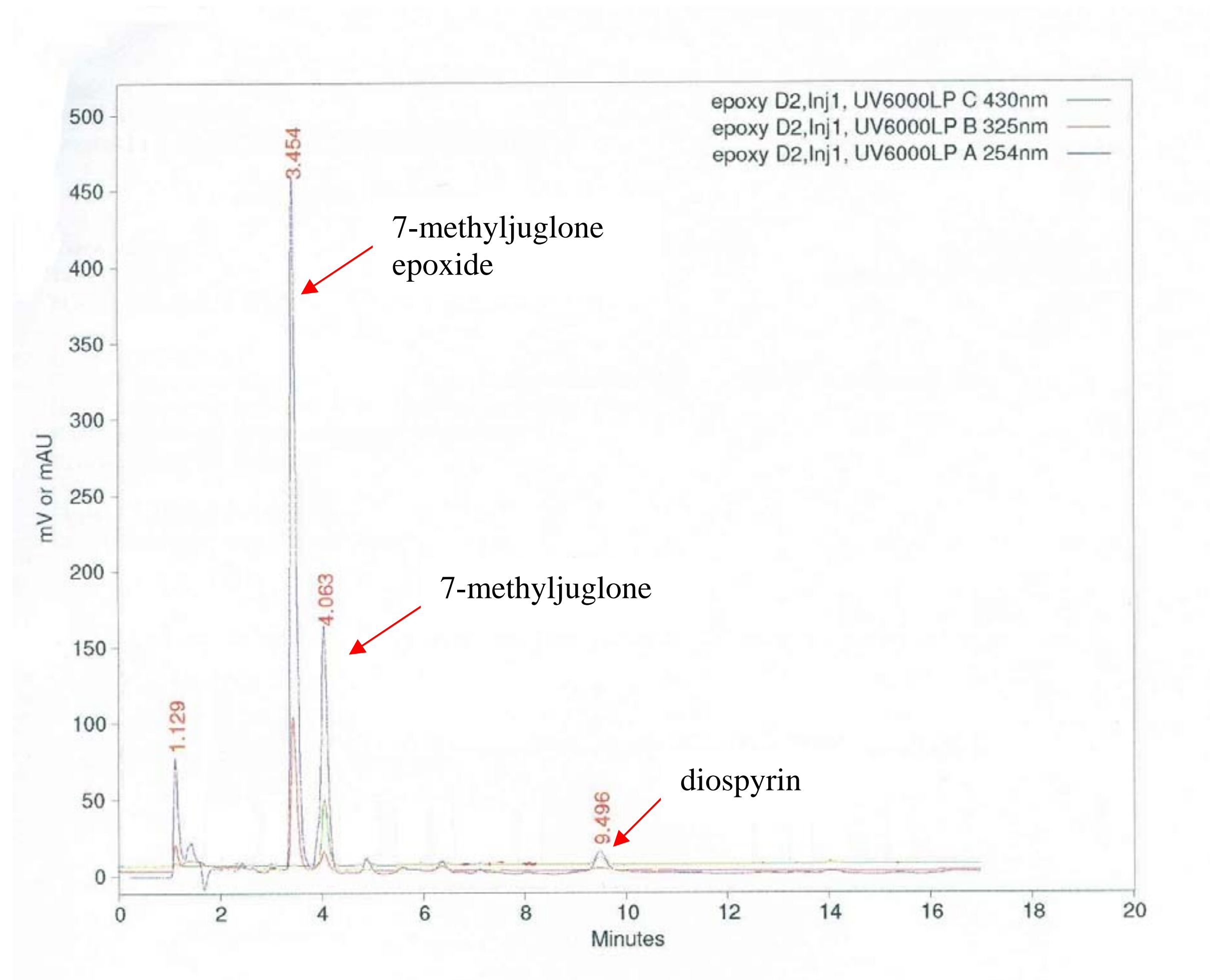

Fig. 4.3: The presence of a small amount of diospyrin, on the HPLC chromatogram, can be seen at a retention time of 9.496 min.

4.4 Discussion and conclusions

The synthesis of 7-methyljuglone remains a low yielding method (16 %). It was however improved in such a way that the one-step synthesis can be used instead of the published two-step synthesis. With the variation in the amount of starting reagents used, it was quite clear that a 1:1 molar equivalent gave the best yields. The catalyst with the salt also gave a clear indication that the salt is needed in order to melt the mixture. It therefore plays an important part in the viscosity of the mixture. The previously used temperature of 180 ºC appears to be correct. It might be significant that temperatures above this could dramatically increase the yields. We did however not attempt any temperatures in excess of 200 ºC due to safety concerns (The flash points of the different chemicals were taken into account in this decision). In future a reactor can be used which can be operated under reduced pressure and thereby higher temperatures. The effect of the stirring time on the reaction indicated that the optimum time was between 1 and 2 minutes. The purification step proves to be considerably more difficult as the *m*-cresol (unreacted) and 7-methyljuglone overlaps in the current column system that was used. This can however be improved on with the use of liquid - liquid separation or a different mobile phase in the column system. This was however not the purpose of the experiment.

The reaction mechanism is not yet fully understood. Fig. 4.4 illustrates a probable mechanism. The mechanism for the reaction involves either the formation of an acylium ion as the reactive electrophilic species, but it might also involve an electrophilic complex between the acid anhydride and the aluminium chloride (Hanneford *et al*., 1989). We propose that an electrophilic complex forms between the catalyst and the ketone group. This creates ring opening with nucleophilic attack in the *ortho* position from the aromatic ring to the partially positively charged carbon. Lower temperatures favours attack from the *para* position (Benson *et al*. 2001). A second molecule of $AlCl_3$ forms a complex with the second ketone group as the process is repeated. This reaction sequence probably occurs in a single step with two $AlCl_3$ molecules reacting at once with the anhydride. The end result is 7-methyljuglone with the loss of one molecule of water.

Diospyrin synthesis via oxidative dimerisation did not yield the correct results. No diospyrin formed after 6 weeks even when the experiment was repeated with added $FeCl_3$ as catalyst. There are still some variations that could be tried but it was abandoned in favour of the

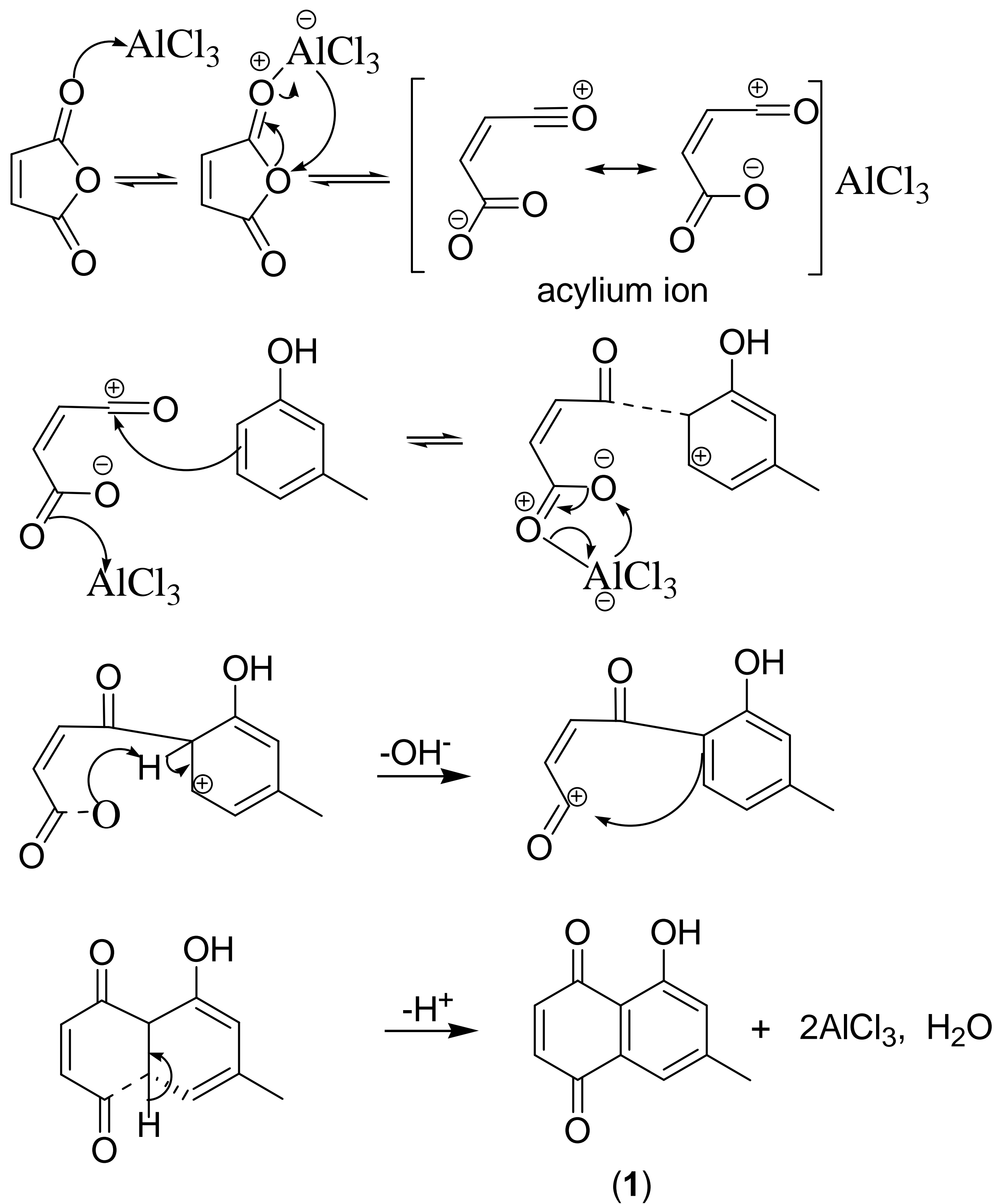

Fig. 4.4: Proposed reaction mechanism for 7-methyljuglone synthesis

epoxide and reductive coupling of 7-methyljuglone in buffer reactions. The reaction time becomes important when the synthesis is commercialised.

The buffer reaction still shows promise and it should be repeated with the use of different bases. The pH can also be adjusted in smaller steps. In future a lithium or sodium base should be used in the buffer and the pH should be changed in smaller steps from pH 5 to pH 7. The occurrence of neodiospyrin in high yields should make it possible to alter conditions so that diospyrin forms instead.

The epoxidation of 7-methyljuglone and the improvement of the method led to the occurrence of two dimers. The one dimer could positively be identified as mamegakinone (due to its hydroxy proton shift). Unfortunately the hydroxy proton shift of one hydroxyl ion of diospyrin overlaps with approximately 4 other similar compounds. The other resonance peak was clearly present. Fig. 4.5 illustrates the mechanism of diospyrin formation. In theory it is possible to reduce an epoxide group to a double bond with the use of steam distillation. This process eliminates one molecule of water.

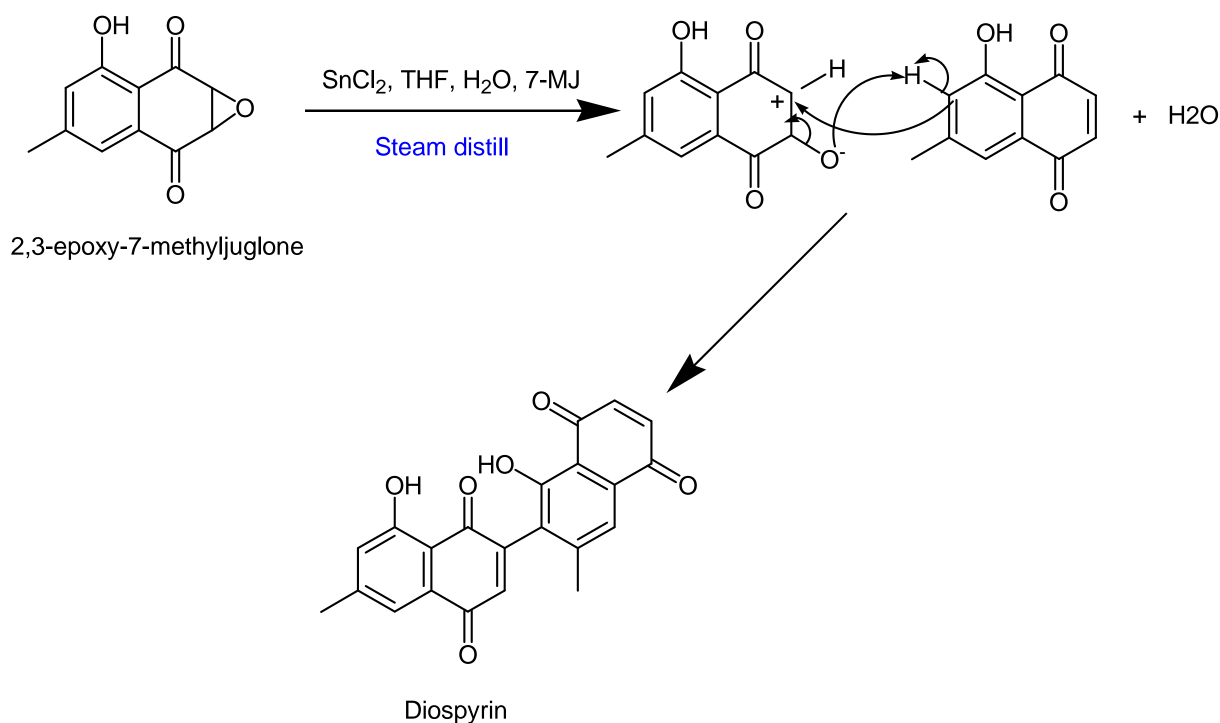

Fig. 4.5: Possible mechanism for diospyrin formation

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University of Pretoria etd – Van der Kooy, F (2007)

Chapter 5

Stability of naphthoquinones

5.1 Introduction 76

5.2 Materials and methods 77

5.2.1 Materials 77

5.2.2 Methods 77

5.2.2.1 Stability in dimethylsulfoxide 77

5.2.2.2 Stability in BACTEC buffer solution 78

5.2.2.3 Stability in vero cell toxicity bioassay buffer 78

5.2.2.4 Stability in 20 % DMSO/Agar mixture 78

5.2.2.5 Stability in broth used for mode of action studies 78

5.3 Results 79

5.3.1 Stability in dimethylsulfoxide 79

5.3.2 Stability in BACTEC buffer solution 82

5.3.3 Stability in vero cell toxicity bioassay buffer 83

5.3.4 Stability in 20 % DMSO/Agar mixture 84

5.3.5 Stability in broth used for mode of action studies 84

5.4 Discussion and conclusions 84

5.5 References 87

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 5

Stability of naphthoquinones

5.1. Introduction

The often overlooked chemical analysis that bioactive compounds should be subjected to, is their stability in solvents. Most published bioassays are accepted by scientists and the methodology can therefore be used to screen any new bioactive compound that has been found. Unfortunately these new bioactive compounds do not always remain stable during these bioassays. If it is found that the compounds are not stable, it is necessary to adapt bioassays, or change the molecules structure to be able to get an accurate result.

The various tests that have to be performed to investigate the naphthoquinones (NQ's) as potential TB drugs, makes use of several solvents. During this chapter the stability of predominantly two NQ's were tested. The NQ's, diospyrin and 7-methyljuglone, showed the best activity against TB in the BACTEC radiometric assay (Jenkins, 1998). In this assay, liquid medium is used and the growth of *Mycobacterium tuberculosis* is monitored radiometrically with the results available within 5-6 days. Only 7-methyljuglone were tested for stability in the BACTEC vials due to the radioactive nature of the ^{14}C labelled palmitic acid. The two NQ's, diospyrin and 7-methyljuglone, were also the only ones tested in mice due to the high costs of the experiment involved, while three other NQ's, juglone, menadione and plumbagin, were also included in some of the experiments listed below. The NQ's were tested in five different "solvents", namely.

a) Dimethylsulfoxide (DMSO)

b) BACTEC buffer (9H11 Middlebrook agar with OADC growth supplement)

c) Minimum essential medium (vero cell toxicity bioassay)

d) 20 % DMSO/ 7H9 broth/glycerol (*in vitro* mice work)

e) 7H9 Middlebrook broth that was used for mode of action studies.

The organic solvent DMSO was tested because it is the most commonly used solvent to dissolve hydrophobic compounds (Lipinski, 2004). It was used to dissolve the compounds and to prepare a

University of Pretoria etd – Van der Kooy, F (2007)

stock solution which was also sometimes stored for future use. The final concentration of DMSO is usually between 0.5 and 2 % in the various bioassays before it became toxic to the test organisms (Soerbye *et al*., 1993; Crawford & Braunwald, 1991). These concentrations of DMSO were used in the BACTEC and vero cell toxicity bioassays. The solutions were prepared and tested on HPLC. The stability of 7-methyljuglone in DMSO was also tested in a kinetic NMR experiment. For the *in vitro* mice work the compounds were dissolved in a mixture of DMSO and added to 7H9 Middlebrook broth in 5 % glycerol. The final concentration of the DMSO was 20 %. This mixture was also tested for stability on HPLC. All the tests were carried out in exactly the same way that they were performed during the actual bioassays.

5.2. Materials and methods

5.2.1 Materials

The BACTEC vials were obtained from Becton, Dickenson and Company, USA, while the 7H9 Middlebrook broth, OADC growth supplement, DMSO and glycerol from Sigma-Aldrich. The samples were injected into the HPLC with mobile phase 62 % acetonitrile and 5 % aqueous acetic acid. The column was a RP18 Phenomenex 150X4.6 mm, 5 μ. The PDA detector was set to 254, 325 and 430 nm.

5.2.2 Methods

5.2.2.1 Stability in dimethylsulfoxide

The five napthoquinones: diospyrin, juglone, menadione, 7-methyljuglone and plumbagin were dissolved in AR grade DMSO to a 1 mg/ml concentration. These solutions were injected into the HPLC every hour for the first 4 hours after dissolving and then daily for one week and weekly for 1 month. This experiment was repeated to confirm the results. An additional experiment was performed in duplicate, but at a concentration of 10 mg/ml for diospyrin and 7-methyljuglone, as this is the stock concentration for most bioassays used. The samples were sonicated for 30 sec before each injection. In addition, 5 mg of pure 7-methyljuglone was dissolved in deteurated DMSO and analysed in a kinetic study on a 200 MHz Varian NMR spectrometer. The spectra were acquired 1 hour apart for 6 hours.

University of Pretoria etd – Van der Kooy, F (2007)

5.2.2.2 Stability in BACTEC buffer solution

7-methyljuglone was dissolved in DMSO and injected into the BACTEC vial so that the final concentration was 100 µg/ml and 1 % DMSO. Due to cost constraints and the radioactive nature (contains ^{14}C-labelled palmitic acid) of the test, only 7-methyljuglone was tested. The vials were placed in an incubator at 37 ˚C in the dark and 20 µl was injected into the HPLC after addition of the compound. The test was performed in duplicate. The test was repeated every 30 min for the first 3 hours. As control only the BACTEC solvent with 1 % DMSO was injected without any 7-methyljuglone.

5.2.2.3 Stability in vero cell toxicity bioassay buffer

The five napthoquinones: diospyrin, juglone, menadione, 7-methyljuglone and plumbagin were dissolved in DMSO at 20 mg/ml and immediately diluted 200 times in complete Minimum Essential Medium (MEM). As control the solvent mixture was prepared without any addition of the compounds. The samples (at a final concentration of 100 µg/ml) was injected into the HPLC every 30 min for the first hour and then daily for two days. During the toxicity bioassay the stock solution was further diluted to the required concentrations. Due to the low concentration of 7-methyljuglone a stock solution of 100 µg/ml was prepared in order to achieve a good signal to noise ratio on the HPLC.

5.2.2.4 Stability in 20% DMSO/ 7H9 broth/glycerol

For the *in vivo* mice work, the compounds were dissolved in DMSO and added to 7H9 broth in 5 % glycerol to reach a final concentration of 20 % DMSO. The final concentration of diospyrin and 7-methyljuglone was 2.5 mg/ml. The standards were prepared and injected into the HPLC over a period of four days. The solutions were stored in the dark in the cold room at 4 ˚C. The test was repeated to confirm the results.

5.2.2.5 Stability in broth used for mode of action studies

7H9 broth was prepared by dissolving 2.45 g in 450 ml of distilled water. To this mixture 2 ml of glycerol was added. The solution was autoclaved and allowed to cool down to room temperature

University of Pretoria etd – Van der Kooy, F (2007)

before 50 ml of OADC growth supplement was added. The five naphthoquinones: diospyrin, juglone, menadione, 7-methyljuglone and plumbagin was dissolved in DMSO and added to the broth solution to a final DMSO concentration of 0.5 %. The stability was determined on HPLC over a period of three days. The blank sample contained only the broth with 0.5 % DMSO.

5.3 Results

5.3.1 Stability in dimethylsulfoxide

The stability of the NQ's were tested over a period of 1 month. Only menadione and plumbagin remained stable over that period. The most unstable compound appeared to be 7-methyljuglone**.** It started to break down after 30 minutes and after 1 month no traces of 7-methyljuglone could be found. Table 5.1 gives the rate of break down for the different NQ's. Fig. 5.1-5.4 contain HPLC chromatograms and the kinetic NMR spectrum of the breakdown of 7-methyljuglone in d-DMSO. The break down rate were higher at higher concentrations. Interestingly, the breakdown product were different at the different concentrations. The major product at 1 mg/ml appeared to be neodiospyrin, a dimeric form of 7-methyljuglone**.** At 10 mg/ml the major product was mamegakinone. Dimerisation therefore occurs in DMSO.

Table 5.1: The breakdown of the NQ's in DMSO over time displayed as average percentage remaining compound, as determined by HPLC.

Compound	3min	30min	1 day	1week
diospyrin	100.0	100.0 ± 0.4	68.9 ± 1.8	<5.0
juglone	100.0	100.0 ± 0.5	12.1 ± 2.8	<5.0
menadione	100.0	100.0 ± 0.3	100.0 ± 0.1	100.0 ± 0.3
7-methyljuglone	100.0	75.0 ± 2.4	7.5 ± 1.2	<5.0
plumbagin	100.0	100.0 ± 0.8	100.0 ± 0.4	100.0 ± 0.5

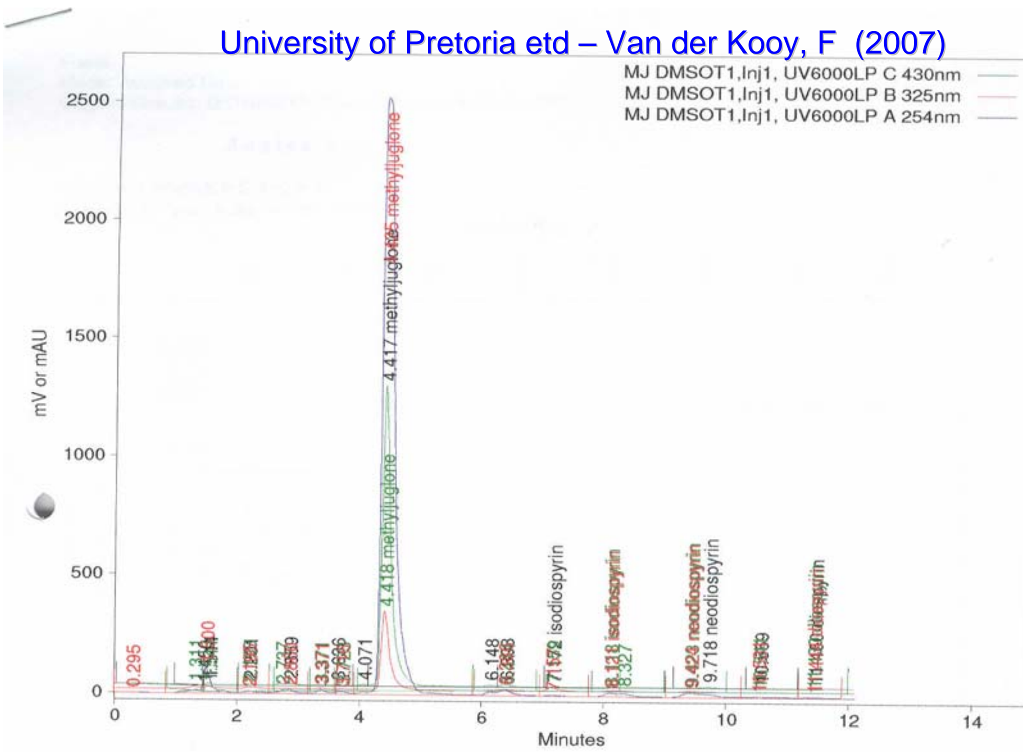

Fig. 5.1: HPLC chromatogram of 7-methyljuglone at a concentration of 10mg/ml in DMSO after 3 minutes.

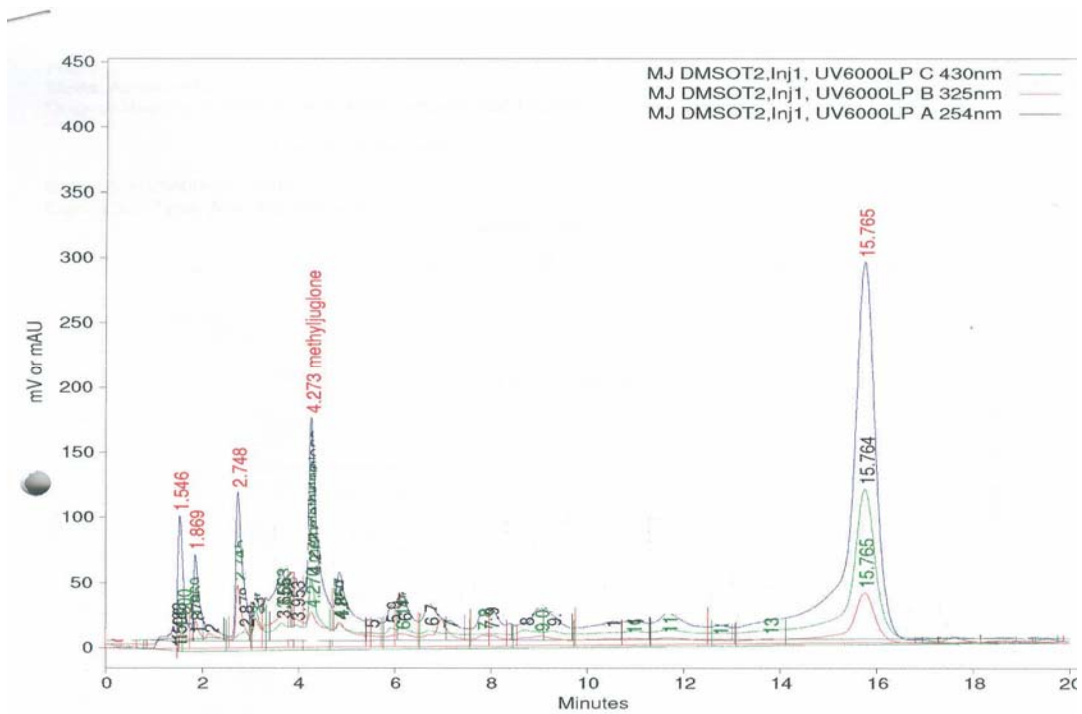

Fig. 5.2: HPLC chromatogram 7-methyljuglone after 24 hours at a concentration of 10mg/ml in DMSO.

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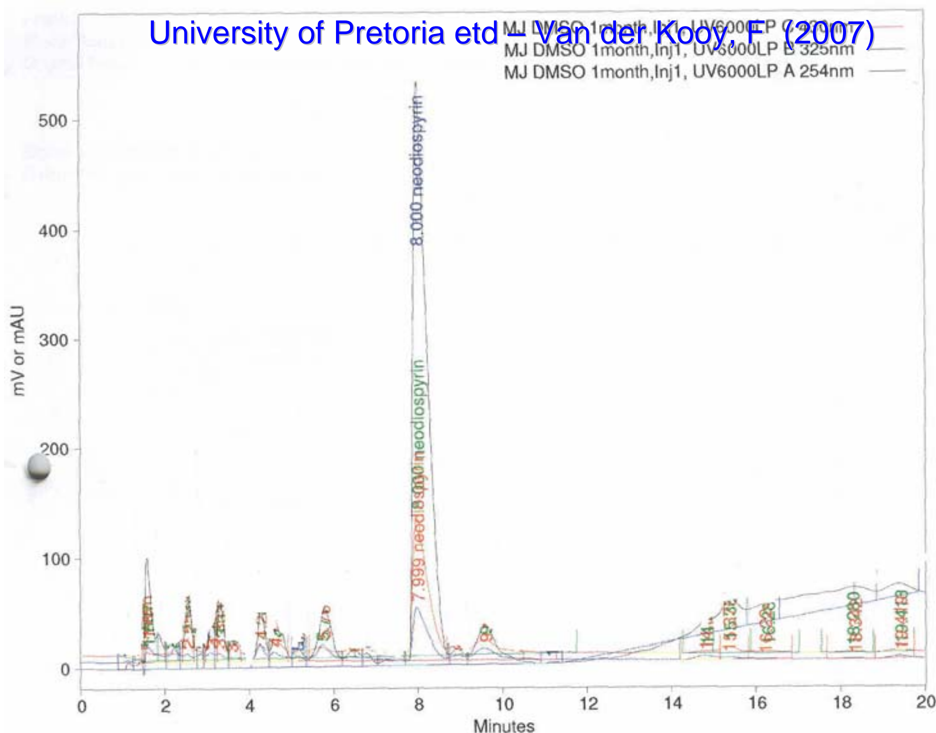

Fig. 5.3: HPLC chromatogram of 7-methyljuglone at a concentration of 1mg/ml in DMSO after 1 month. The breakdown product is different from the 10mg/ml concentration. It appears to be the dimeric compound, neodiospyrin.

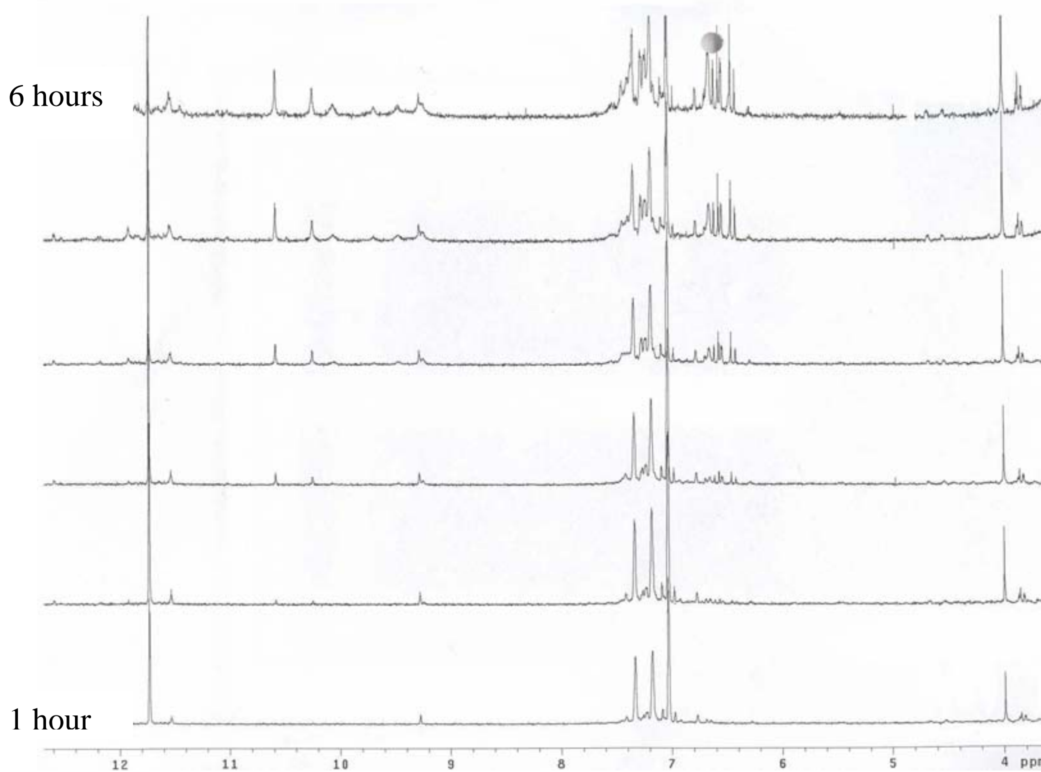

Fig. 5.4: ^{1}H-NMR kinetic study of 7-methyljuglone in DMSO. The scans were taken 1 hour apart, starting from the bottom.

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5.3.2 Stability in BACTEC buffer

The activity of 7-methyljuglone was tested in the BACTEC vial at a concentration of 0.5-5 µg/ml. Due to these low concentrations it was decided to use 100 µg/ml to be able to detect it easily on the HPLC. The compound broke down completely within 80 min. Fig. 5.5-5.7 displays the chromatograms of these tests.

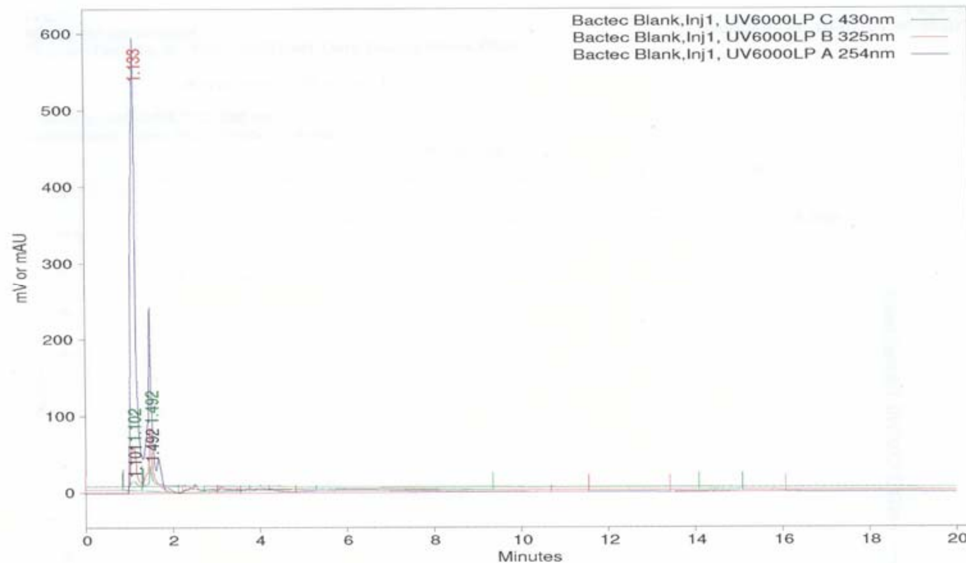

Fig. 5.5:HPLC chromatogram of the BACTEC sample without 7-methyljuglone

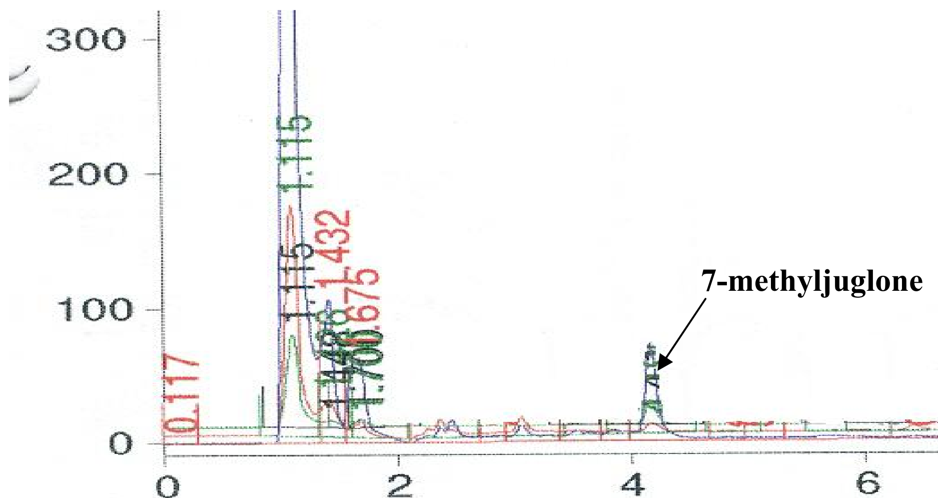

Fig. 5.6: HPLC chromatogram directly after addition of 7-methyljuglone to the BACTEC vial.

Fig 5.7: HPLC chromatogram after 80 minutes. No trace of 7-methyljuglone could be seen. The expanded region as indicated verifies that 7-methyljuglone had broken down.

The concentration of 7-methyljuglone decreased by 40 % during the first 40 min. After 80 min. no traces of the compound could be detected, even after sonication for 15 min before injection.

5.3.3 Stability in vero cell toxicity bioassay buffer

All five NQ's disappeared from the HPLC chromatogram after 2 days. Only menadione and 7-methyljuglone could be found in small amounts after 1 day. After two days all five the compounds broke down completely. Table 5.2 gives the areas of the peaks at different time intervals.

Table 5.2: The time-dependant breakdown of the NQ's in MEM expressed as average percentage area as determined on the HPLC

Compound	Area 0 min	Area 60 min	Area day 1	Area day 2
diospyrin	100	46 ± 2.3	<1	<1
juglone	100	93 ± 1.1	<1	<1
menadione	100	100 ± 2.0	26 ± 1.0	<1
7-methyljuglone	100	74 ± 1.2	23 ± 1.3	<1
plumbagin	100	<1	<1	<1

5.3.4 Stability in 20% DMSO/ broth mixture

The compounds appeared to remain stable, although it was slightly insoluble. Without sonication before each injection, 7-methyljuglone precipitated at a rate of 30 % per day. The precipitation of diospyrin was slightly larger. Before each injection the samples were sonicated and shaken. No breakdown occurred within four days for both compounds.

5.3.5 Stability in 7H9 Middlebrook broth used for mode of action studies

All five the NQ's slowly disappeared from solution (Table 5.3). The compounds appeared to remain stable for 8 hours after which it started to break down. After 24 hours no trace of the compounds could be found. After sonication there was still no trace of the NQ's.

Table 5.3: The time-dependant breakdown of the NQ's in 7H9 Middlebrook broth expressed as average percentage area as determined on the HPLC

Compound	Area 0 min	Area 60 min	Area 8 hours	Area day 1
diospyrin	100	98 ± 1.3	95 ± 0.9	<1
juglone	100	96 ± 2.1	93 ± 0.7	<1
menadione	100	99 ± 0.6	89 ± 1.2	<1
7-methyljuglone	100	100 ± 0.4	92 ± 1.9	<1
plumbagin	100	96 ± 1.2	91 ± 2.1	<1

5.4 Discussion and conclusions

The stability of the NQ's in DMSO gave very perculiar results. It appears that the rate of breakdown is concentration dependant. diospyrin, juglone and 7-methyljuglone were very unstable in this organic solvent. The structural differences between the compounds do not give any clues as to way it is unstable. The substituents on the compounds, a methyl group and hydroxyl group are both *ortho*- and *para* directing activators. A methyl substituent is weakly electron donating through the inductive effect. The hydroxyl substituent is weakly electron withdrawing through the inductive effect, but also strongly electron donating through the resonance effect. Dimethyl sulfoxide is a widely used solvent. It oxidises primary and secondary alcohols to the corresponding ketone or aldehyde groups in the Swern oxidation process. DMSO must however first be activated by an acid chloride. It is therefore unsure why only these three compounds are unstable.

The BACTEC vial, which is used in the determination of the MIC against *M. tuberculosis*, contains ^{14}C-labelled palmitic acid. In addition, the Middlebrook 7H12 agar contains various salts and micro-elements. It also contains catalase and pyridoxine. Catalase is an important detoxifying agent for aerobic bacteria, but unfortunately will also react with NQ's. Pyridoxine has a similar function and this might be the explanation for the disappearance of some of the NQ,s. This will in effect create a two-fold dilemma. The bacteria generate toxic hydrogen peroxide, which the catalase detoxifies. If the NQ's block this enzyme, the bacteria can inhibit further growth and replication by their normal metabolism. The other consideration is that the compounds might be far more active than suspected. If the catalase or pyridoxine inactivate most of the 7-methyljuglone**,** then the few molecules that are able to reach the bacterial cell, might kill it immediately. In effect the MIC should then be considerably lower, unless the breakdown products are also antimycobacterial.

The analysis of the 7H9 Middlebrook broth with 5% glycerol and 20% DMSO appeared to be stable for at least four days. The stock solutions were prepared and administered daily for 4 days. A potential problem of precipitation did occur. If the sample is not shaken thoroughly before administration, the dose might be too low or too high, depending on how deep the syringe was in the mixture. If it was subtracted from the top part a low concentration would have been administered. At the bottom a far higher concentration would have been administered. This might have caused toxic affects in the mice.

The toxicity bioassay medium also showed that the compounds are broken down quite quickly. No proof could be found that the NQ's might react to the ingredients in MEM. It is therefore possible that due to the hydrophobic nature of the compounds it might precipitate. The microtitre plates are not shaken during the bioassay and this might cause the compounds to precipitate onto the cells and in effect have a dramatic increase in concentration.

The media used for the mode of action studies, 7H9 Middlebrook broth with OADC growth supplement, had a similar effect as the BACTEC analysis. The compounds however only disappeared after 24 hours. The mode of action studies (Chapter 8) were therefore only continued for 12-14 hours after the addition of the compounds.

In conclusion it can be said that in three of the solvents some of the NQ's broke down quite rapidly. The 7H9 Middlebrook broth that was used for the mice work showed that the

compounds were stable for the required 4 days. The solution did turn brownish after day 5 and continued to darken with time. Eventually the compounds did also break down in this solvent. These experiments emphasise the importance of stability studies. The MIC of our lead compound in the BACTEC experiments might therefore be a result of inactivation by catalase, but it might also be far more active than suspected. The toxicity bioassay showed that the compounds might be less toxic due to precipitation or that one of the breakdown products is the toxic principle.

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University of Pretoria etd – Van der Kooy, F (2007)

Chapter 6

Toxicity of naphthoquinones

6.1 Introduction **89**

6.2 Materials and methods **89**

6.2.1 Materials **89**

6.2.1.1 Culturing of vero monkey kidney cells **89**

6.2.1.2 Toxicity of 7-methyljuglone and diospyrin in mice **90**

6.2.1.3 Toxicity of 7-methyljuglone in *Musca domestica* **90**

6.2.2 Methods **90**

6.2.2.1 Determination of the IC50 of naphthoquinones on vero cells **90**

6.2.2.2 Toxicity of 7-methyljuglone and diospyrin in mice **91**

6.2.2.3 Toxicity of 7-methyljuglone in *Musca domestica* **91**

6.3 Results **91**

6.3.1 Determination of the IC50 of naphthoquinones on vero cells **91**

6.3.2 Toxicity of 7-methyljuglone and diospyrin in mice **92**

6.3.3 Toxicity of 7-methyljuglone in *Musca domestica* **93**

6.4 Discussion and conclusions **95**

6.5 References **98**

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 6

Toxicity of naphthoquinones

6.1 Introduction

Toxicology is the subject concerned with the study of the noxious effects of chemical substances on living systems. The amount of foreign chemicals (xenobiotics) to which humans are exposed has been growing rapidly during the past century. These include drugs, pesticides, environmental pollutants, food additives and industrial chemicals (Timbrell, 1996). Various research papers have been published on the biological activity/toxicity of diospyrin and 7-methyljuglone. In the literature review (Chapter 2), various references on the biological activity of naphthoquinones are given. In this chapter the compounds and some derivatives were tested on a vero cell line and also in mice. The toxicity of 7-methyljuglone was in addition also tested on *Musca domestica* to establish a possible mode of action of these compounds in diverse organisms.

6.2 Materials and methods

6.2.1 Materials

6.2.1.1 Culturing of vero monkey kidney cells

Vero cells were cultured in minimal essential medium (Eagle) containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 μg/ml penicillin, 10 μg/ml streptomycin, 0.25 μg/ml fungizone and 10 % fetal bovine serum at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were subcultured in a 1:6 ratio every second to third day after trypsinization of confluent cultures. On day 0, confluent cultures were trypsinized and diluted in complete MEM to a concentration of 1×10^5 cells/ml. In the outer wells of a 96-well plate(s), 200 μl of medium was dispensed. All inner wells received 100 μl (1×10^4 cells) of the cell suspension. The plate(s) was incubated overnight at 37 °C in a humidified atmosphere with 5 % CO_2 (Zheng *et al.,* 2001).

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6.2.1.2 Toxicity of 7-methyljuglone and diospyrin in mice

The *in vitro* mice experiments were conducted at the Medical Research Counsel, Pretoria, South Africa. These tests had to be performed by specially trained personnel (Dr. Kobus Venter) and had to be approved by the ethics committee of the MRC. A pilot study was conducted to investigate the toxicity of naphthoquinones *in vivo* in mice. The experiment constituted 140 mice (inbred female BALB/c mice - six to eight weeks of age) divided into six groups.

6.2.1.3 Toxicity of 7-methyljuglone in *Musca domestica*

Approximately 2000 fly larvae (*Musca domestica*) were obtained from the South African Bureau of Standards. Milk powder and sugar, for their feed, were obtained from a local vendor.

6.2.2 Methods

6.2.2.1 Determination of the IC50 of naphthoquinones on vero cells

Stock solutions of twelve pure NQ's **(**8-chloro-7-methyljuglone **(1),** dichlon **(2),** 2,3-dimethoxynaphthoquinone (**3),** diospyrin **(4),** 2-hydroxynaphthoquinone **(5),** isodiospyrin **(6),** juglone **(7),** lapachol **(8),** menadione **(9)** 7-methyljuglone **(10),** neodiospyrin **(11),** plumbagin **(12)** Figure 7.1**)** were prepared in DMSO at 20 mg/ml after which it was immediately diluted 200 times in complete MEM to 100 μg/ml. As a positive control rifampicin was used. To establish the IC50 value for the compounds, the solutions were then serially diluted to obtain eight different concentrations.

A 100 μl solution of the pure compound dilutions were dispensed into cell-containing wells in triplicate of the microtitre plate. The final concentrations of pure compounds in the wells were 0.78, 1.56, 3.13, 6.25, 12.50, 25.00 50.00 and 100.00 μg/ml. Control wells received a final concentration of 0.25 % DMSO in complete medium. Plates were then returned to 37 °C in a humidified atmosphere with 5 % CO_2 for another 3 days. On day 4, 5 μl of XTT reagent was added to the wells and incubation commenced for another 1-4 hrs. The optical densities of the wells were then measured at 450 nm (690 nm reference wavelength) with an Eliza plate reader.

University of Pretoria etd – Van der Kooy, F (2007)

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6.2.2.2 Toxicity of 7-methyljuglone and diospyrin in mice

Inbred female mice were treated with the NQ's for five days per week over four weeks. All agents were administered by oral gavage at a dose of 6.25, 12.50 and 25.00 mg/kg of body mass for each of **(4)** and **(10).** Izoniazid was administered at similar concentrations.

6.2.2.3 Toxicity of 7-methyljuglone in *Musca domestica*

Twenty flies were placed in 1 L glass containers, which were covered by mesh cloth. In the containers a bowl of water, sugar and milk powder (mixed with **(10)**) was placed. The concentration of **(10)** mixed with the milk powder were 0.1, 1.0, 10.0 and 20.0 mg/g. Two control groups were used. One group received only the water, sugar and milk powder (without the compound) while the other group received only water (to determine how long the flies would survive without food). The flies were routinely monitored for a period of 2 weeks.

In another experiment 200 flies were placed in a container and the milk powder containing different concentrations of **(10)** was placed at random in the container. It was noted by hourly observation (during the day) which concentration was visited the most and the amount of milk powder that had been consumed after a 2 week period calculated.

6.3 Results

6.3.1 Determination of the IC50 of naphthoquinones on vero cells

(4) and **(10)** did not show cytotoxicity to vero cells at concentrations below their MIC (0.5 mg/ml and 8.0 mg/ml, respectively) *in vitro* (Table 6.1). Most of the compounds showed rather high toxicity when compared to their activity (the activity results are presented in Chapter 7). It does however give valuable information about the active site of the molecule. This will be discussed in the next chapter.

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Table 6.1. The cytotoxicity of naphthoquinones on vero cells in nM/ml and µg/ml

Sample	IC_{50}[a] nM/ml	IC_{50}[a] µg/ml
(1)	11.01	2.45 ± 0.10
(2)	128.20	25.00 ± 0.26
(3)	257.73	50.00 ± 2.43
(4)	41.44	17.78 ± 1.69
(5)	287.36	50.00 ± 2.12
(6)	80.21	6.25 ± 1.83
(7)	6.90	3.13 ± 0.46
(8)	413.20	100.00 ± 4.23
(9)	37.97	3.13 ± 1.70
(10)	6.54	1.23 ± 0.36
(11)	86.04	12.5 ± 3.71
(12)	16.49	3.13 ± 0.10
Rifampicin (control)	102.99	84.76 ± 3.45

[a]50% inhibitory concentration

6.3.2 Toxicity of 7-methyljuglone and diospyrin in mice

The preliminary toxicity results showed that **(10)** is toxic to mice at a dose of 25 mg/kg and that **(4)** is not harmful at this concentration. This can be seen in Fig 6.1. The mice treated with **(10)** showed a marked decrease in body mass compared to the control. After treatment stopped the mice recovered most of their body mass and none of these mice died. It can be concluded from the preliminary *in vivo* pilot toxicity study that **(4)** was not cytotoxic at a dose of 25 mg/kg (Figure 6.1) and **(10)** at a dose of 6.25 mg/kg.

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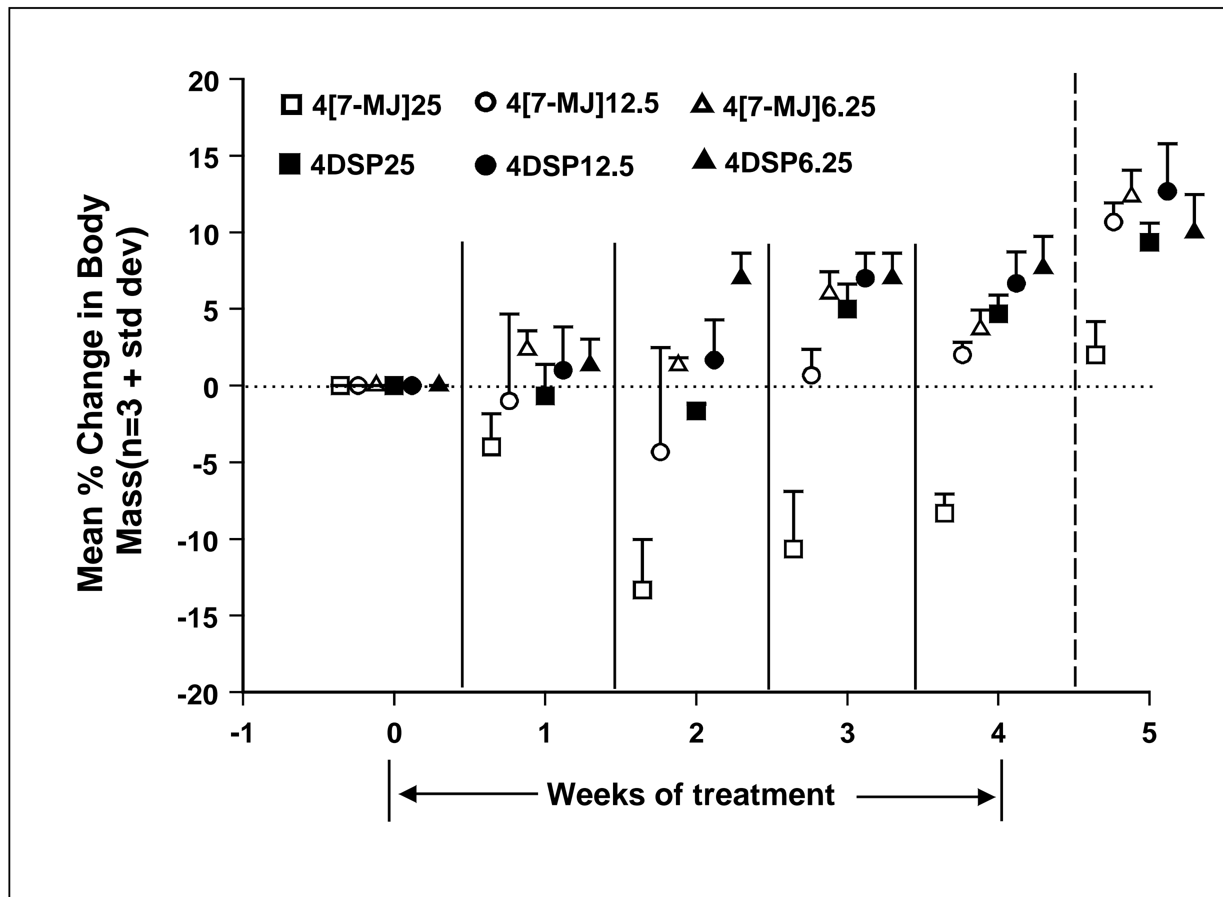

Fig 6.1. Tolerance of (10) (7-MJ) and (4) (DSP) was tested by oral administration (once daily, five times a week for four weeks) at 25.00, 12.50 and 6.25 mg/kg in uninfected mice. Only mice treated with (10) at 25.00 mg/kg displayed significant ($p<0.05$) loss in body mass during weeks 2 to 4. The izoniazid control group did not show any significant changes in body mass (not shown in figure).

6.3.3. Toxicity of 7-methyljuglone in *Musca domestica*

Table 6.2 gives the results after 2 weeks of monitoring. It is clear from the results that the flies consumed the most milk powder in the control group, K1. The milk powder treated with the lowest concentration of **(10)** was consumed the second most. The total amount of milk powder

University of Pretoria etd – Van der Kooy, F (2007)

consumed decreased further with an increase in concentration of **(10).** The amount of flies that died at the different concentrations of **(10)** might indicate a slight toxicity, but this is not conclusive as the variation in the results are very high. The results indicated that the compound might be acting as an antifeedant, as suggested by Tokunaga (2004).

Table 6.2. The amount of milk powder consumed by the flies after a two week period. The number of dead flies is also indicated. K1=milkpowder without (10), K2= no milkpowder.

Sample	[7-methyl juglone] (mg/g)	Amount consumed (mg)	Dead flies (2 weeks)	Average consumed (mg)	Average number of dead flies
1	20.0	38.3	0	77.8 ± 37.1	1.30 ± 1.53
2	20.0	111.8	3		
3	20.0	83.4	1		
4	10.0	102.1	2	87.1 ± 19.7	2.00 ± 1.00
5	10.0	94.4	3		
6	10.0	64.7	1		
7	1.0	60.0	0	114.8 ± 75.0	0.33 ± 0.56
8	1.0	84.0	0		
9	1.0	200.3	1		
10	0.1	206.7	0	155.7 ± 50.7	0.00
11	0.1	105.4	0		
12	0.1	155.1	0		
K1	-------	252.4	0	252.4	0.00
K2	-------	--------	all	0.0	15.00

The results of the experiment that contained milk powder mixed with **(10)** which was placed at random in one container, are given in Table 6.3. The results clearly indicate that the flies prefer the milk powder with no addition of **(10).** The lowest concentrations are also visited more than the higher concentrations. This might indicate that **(10)** acts as an antifeedant.

University of Pretoria etd – Van der Kooy, F (2007)

Table 6.3: Amount of fly visits (observed at random) and the amount of milk powder consumed at different concentrations of (10) mixed with milk powder.

[(10)] in milk powder in mg/g	Milk powder consumed in mg	Total amount of flies that visited
0.0	300.5 ± 22.5 (96%)	22.5 ± 4.5
0.1	238.7 ± 53.5 (80%)	26.0 ± 20.0
1.0	193.4 ± 20.0 (63%)	22.0 ± 9.0
10.0	129.1 ± 17.0 (43%)	1.5 ± 0.5
20.0	123.5 ± 48.5 (41%)	3.0 ± 0.0

6.4. Discussion and conclusions

The toxicity against vero cells gave a clear indication that these naphthoquinones are toxic to cells. The solubility and stability of these compounds in MEM must however be taken into consideration. These compounds have a log P value of approximately 3, which means that they are hydrophobic (**(4)** log P = 4.5, **(10)** Log P = 2.2). This might indicate that the compounds might have precipitated and was absorbed by the cells at a higher dose than was actually meant.

Khambay *et al*. (2003) reported on the toxicity of structurally similar naphthoquinones to *M. domestica*. The MIC for two derivatives of dunnione ranged from 3-4 μg/ml against *M. domestica*. Fig. 6.1 illustrates the chemical structures of the three compounds. The mechanism of action proposed by him involves the inhibition of complex 1 in the electron transport chain (Khambay *et al*., 2003). Our fly experiments indicated that **(10)** is not toxic to *Musca domestica*. It does however influence the amount of milk powder consumed. This probably means that the flies are repelled by the compound. There are reports that the structurally similar compound plumbagin, shows high activity against flies. Most flies died after a topical addition of 10 μg of plumbagin on the flies (Saxena *et al.,* 1996). This compound occurs in the well known Venus fly trap (*Dionaea muscipula*) and its role there has not yet been defined.

University of Pretoria etd – Van der Kooy, F (2007)

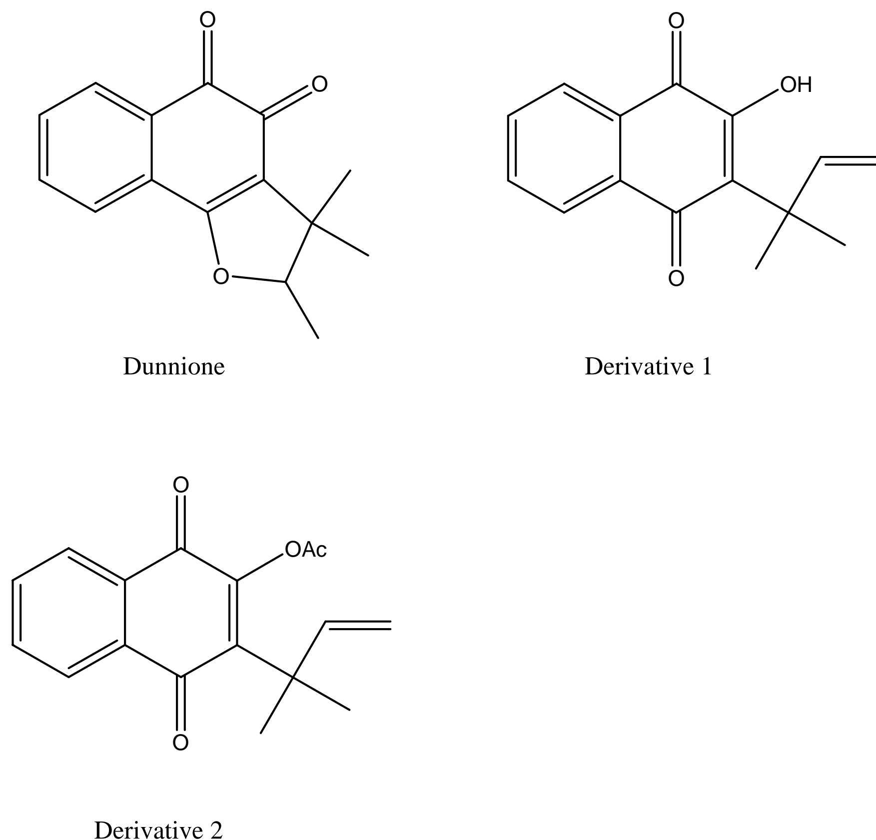

Fig. 6.1: Structures of naphthoquinones which show activity against *M. domestica*

After 2 weeks the flies exposed to the highest concentration did not die at a higher rate than those treated with lower concentrations, or the control group. This could clearly be seen when the flies were given a choice between the different concentrations. It was clearly visible that the most flies visited the control milk powder and the lowest concentration of **(10)** mixed with milk powder.

The toxicity results of all three experiments indicated that the naphthoquinones are toxic to some extent. The conclusions that can be drawn from the vero cells toxicity experiments, is that the cells were probably exposed to a higher concentration than that was meant, due to solubility problems. To overcome this problem will be a rather difficult exercise. Various lipophilic carriers must be tested on cell lines to ensure that it is not toxic to cells at a rather high dosage. The fly experiments indicated that it is not as such toxic to *M. domestica,* but might act as a repellent.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

This is rather counter-intuitive as *D. capensis* plants might produce high amounts of the compound to attract insects by means of the bright colour (orange-red) of the leaves. Does the plant use this compound to kill only specific types of insects (that reacts to the colour attraction) and to repel others that might damage the leaf lamina? To solve this question experiments have to be set up that test various different kinds of insects. This will give answers to the use of this compound to the plant. The toxicity of **(4)** and **(10)** in mice showed that the compounds were toxic at the doses administered. The insolubility of the compounds in the carrier is however a cause for concern. The compounds did start to precipitate out of solution after a couple of hours.

University of Pretoria etd – Van der Kooy, F (2007)

University of Pretoria etd – Van der Kooy, F (2007)

6.5. References

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