Available online at www.sciencedirect.com

SCIENCE DIRECT

South African Journal of Botany 72 (2006) 349–352

SOUTH AFRICAN JOURNAL OF BOTANY

www.elsevier.com/locate/sajb

Antimycobacterial activity and possible mode of action of newly isolated neodiospyrin and other naphthoquinones from *Euclea natalensis*

F. van der Kooy *, J.J.M. Meyer, N. Lall

Department of Botany, University of Pretoria, Pretoria, 0002, South Africa

Received 19 September 2005; received in revised form 26 September 2005; accepted 8 March 2006

Abstract

The roots of *Euclea natalensis* are used by the indigenous people of southern Africa against various bacterial infections. Six naphthoquinones; diospyrin, isodiospyrin, mamegakinone, 7-methyljuglone, neodiospyrin and shinanolone were isolated from root extracts of this plant. In the case of neodiospyrin, this was a first time isolate. The activity of the six isolated compounds were tested against *Mycobacterium tuberculosis* using the radiometric BACTEC assay, providing a first report on the antimycobacterial activity of isodiospyrin, neodiospyrin and mamegakinone. The MIC values of diospyrin (8.0 μg/ml), isodiospyrin (10.0 μg/ml), 7-methyljuglone (0.5 μg/ml) and neodiospyrin (10.0 μg/ml) compared well to those of the known antimycobacterial drugs, ethambutol, isoniazid and rifampicin. A hypothesis on the structure-activity relationship for the naphthoquinones and a possible mode of action is discussed.

Keywords: Euclea; Mycobacterium; Naphthoquinones; Neodiospyrin

1. Introduction

Euclea natalensis is a shrub or small to medium size tree, which occurs in a variety of habitats, including coastal and inland forests as well as bushveld in southern Africa (Van Wyk and Van Wyk, 1997). The roots of *E. natalensis* are used by indigenous people of southern Africa for various bacterial infections (Watt and Breyer-Brandwijk, 1962). Powdered root bark of this species is used as an ingredient in medicines to treat urinary tract infections, venereal diseases and dysmenorrhoea. The custom of cleaning teeth and gums with chewed roots of *E. natalensis* is also practiced widely (Stander and Van Wyk, 1991). The Zulu of South Africa use the root bark to treat TB-related symptoms such as chest diseases, bronchitis, pleurisy and asthma (Watt and Breyer-Brandwijk, 1962).

Several secondary metabolites have been isolated from *E. natalensis* (van der Vijver and Gerritsma, 1974; Ferreira et al., 1977; Lall and Meyer, 2001). Nine of these compounds are naphthoquinones. Other compounds include dihydroxyursanoic acids (lactone derivatives), triterpenoids and one tetrahydroxy-flavanone arabinopyranoside. We report here on a newly isolated napthopquinone from root extracts and MIC values of six naphthoquinones against *Mycobacterium tuberculosis.*

2. Materials and methods

2.1. Plant material

E. natalensis A.DC. (Ebenaceae) root material was collected in the KwaZulu-Natal province of South Africa. The samples were similar to a voucher specimen (N.L.22), which was deposited at the H.G.W.J. Schweickerdt herbarium at the University of Pretoria.

2.2. Isolation of active compounds

The root material was air-dried at room temperature for one week and ground into a fine powder. The powdered root material (1.5 kg) was extracted with 10 L of chloroform for 48 h at room temperature. The solid residues were filtered off and re-extracted in 10 L chloroform. This procedure was repeated three times. Using a rotary-evaporator the extract was dried at 40 °C to yield 25 g of crude extract (yield 1.7%). A silica gel (Merck, 0.063–0.200 mm, 1.5 kg) column was prepared and the crude extract (25 g) was fractionated (Fig. 1) with a 9:1 hexane: ethyl

* Corresponding author.
E-mail address: fkooy@scientia.up.ac.za (F. van der Kooy).

0254-6299/$ - see front matter © 2006 SAAB. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.sajb.2005.09.009

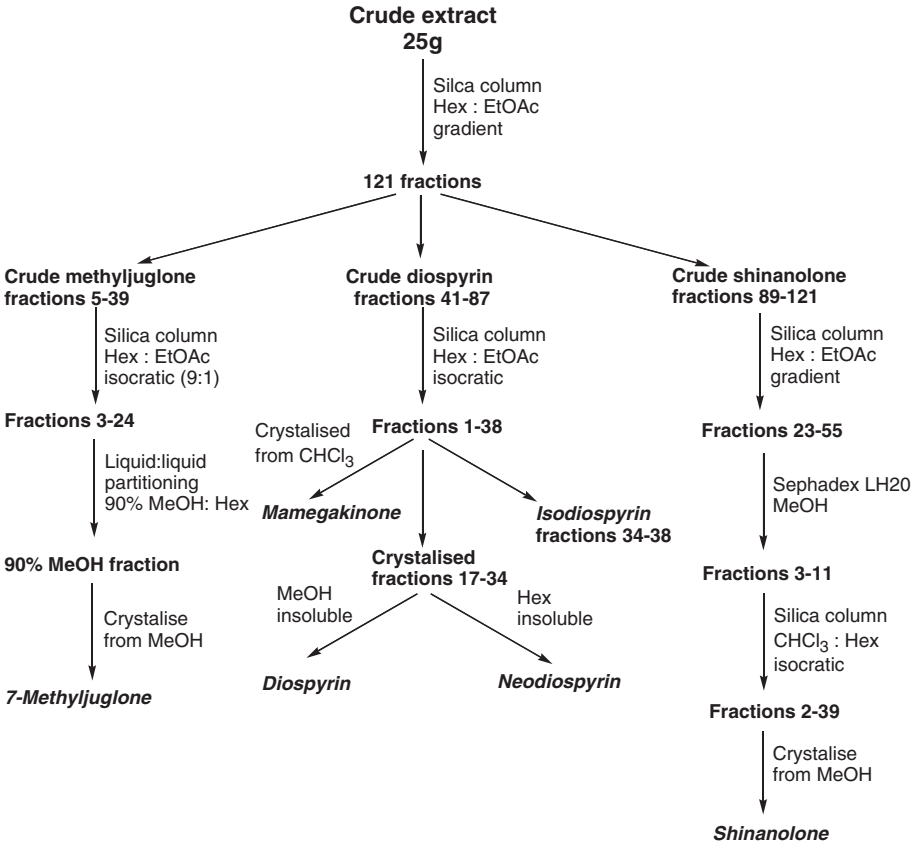

Fig. 1. The chromatographic steps followed to isolate the six naphthoquinones.

acetate (1.5 L) mobile phase. This was followed with an 8:2 ratio (1.5 L) until a 1:1 ratio was reached. The eluting fractions (121) were spotted on a TLC plate (Merck, Kieselgel F256, 20 × 20 cm) and subjected to UV scrutiny. According to the TLC profiles fractions 5–39 (11.0 g), 41–87 (1.5 g) and 89–121 (1.7 g) were pooled. These fractions were re-chromatographed with silica gel and Sephadex LH-20 (100 g) to yield six compounds (Fig. 2). The yields of the six isolated compounds were: diospyrin (460 mg), isodiospyrin (69 mg), mamegakinone (153 mg), 7-methyljuglone (265 mg), neodiospyrin (55 mg), shinanolone (490 mg).

2.3. Antimycobacterial bioassay

A susceptible strain of *M. tuberculosis* H37Rv, obtained from the American Type Culture Collection (Rockville, MD, USA), was used to investigate the activity of the purified naphthoquinones. The 7H12 Middlebrook TB medium (Middlebrook et al., 1977) used during these studies consisted of an enriched Middlebrook 7H9 broth base supplemented with bovine serum albumin, catalase, casein hydrolysate and ^{14}C-labelled substrate (palmitic acid) as a source of carbon. Growth of the mycobacterium leads to the consumption of the carbon source, with subsequent release of labelled $^{14}CO_2$. This labelled CO_2 moves into the atmosphere above the medium in the sealed vial and the BACTEC TB 460 instrument detects the amount of $^{14}CO_2$ and records it as a growth index (GI) on a scale of 0–999 (Heifets and Good, 1994).

For the 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, dissolved in dimethylsulfoxide (DMSO), were added into the vials to give a final concentration of 1% DMSO. The positive drug controls ethambutol, izoniazid and rifampicin and a 1% DMSO control were used.

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 compounds were tested in triplicate.

3. Results

3.1. Isolated compounds

Diospyrin, 7-methyljuglone and shinanolone were identified by means of TLC and HPLC in the presence of reference

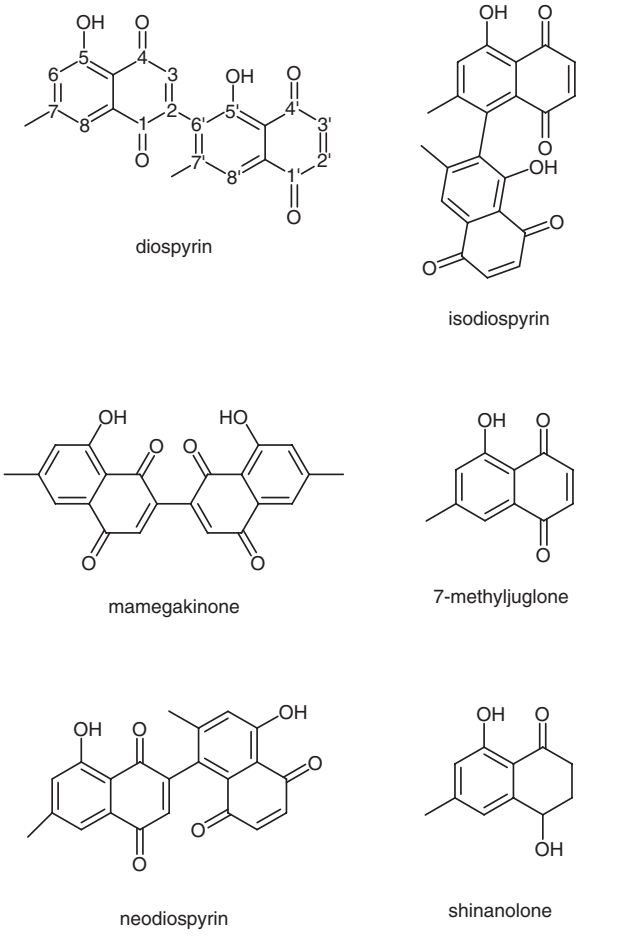

Fig. 2. Chemical structures of the six naphthoquinones isolated. The numbering system is indicated for diospyrin.

samples. ^{1}H NMR were also performed on these three compounds and the results compared to published data (Lillie and Musgrave, 1977). Isodiospyrin, mamegakinone and neodiospyrin were identified by means of ^{1}H NMR and compared to published results (Lillie and Musgrave, 1977; Kumari et al., 1982). The ^{1}H NMR chemical shifts of the isolated naphthoquinones are shown in Table 1.

Table 2
Minimum inhibitory concentrations (MIC) on the H37Rv *Mycobacterium tuberculosis* strain, of isolated naphthoquinones and the antimycobacterial drugs, isoniazid, rifampicin and ethambutol

Compound	MIC (μg/ml)	Lowest concentration tested (μg/ml)
Isoniazid	0.062	0.016
Rifampicin	0.125	0.063
Ethambutol	1.250	0.625
Diospyrin	8.000	5.000
Isodiospyrin	10.000	5.000
Mamegakinone	100.000	50.000
7-methyljuglone	0.500	0.250
Neodiospyrin	10.000	5.000
Shinanolone	100.000	10.000

3.2. Antimycobacterial activity

The MIC values of the isolated naphthoquinones are given in Table 2. The activity of 7-methyljuglone (MIC = 0.5 μg/ml) was found to be comparable to rifampicin and better than ethambutol. The naphthoquinones; diospyrin, isodiospyrin and neodiospyrin showed good activity with mamegakinone and shinanolone showing the least activity at 100 μg/ml.

4. Discussion

During this study neodiospyrin was newly isolated from *E. natalensis*. The minimum inhibitory concentration (MIC) of only three of the naphthoquinones (diospyrin, 7-methyljuglone and shinanolone) has previously been reported for a drug-sensitive strain of *Mycobacterium tuberculosis* (Weigenand et al., 2004; Lall et al., 2005). The results showed that the naphthoquinones tested have good activity against *M. tuberculosis* when compared to the positive drug controls. The MICs of the naphthoquinones indicate that the ketone groups on C1 and C4 are important for antimycobacterial activity. This can be seen by comparing the activity of 7-methyljuglone (MIC = 0.5 μg/ml) with shinanolone (MIC = 100.0 μg/ml). The ketone group on carbon 1 of shinanolone is reduced to the corresponding hydroxyl group. The lack of aromaticity between carbons 1 and 4 might also play a role in

Table 1
The proton chemical shifts of the isolated naphthoquinones as analysed on a 300 MHz spectrometer in $CDCl_3$

Compound	Ring	Chemical shifts						
		H-1	H-2	H-3	H-6	7-Me	H-8	5-OH
Diospyrin	A			6.88	7.11 bd j = ~1	2.43	7.49 bd j = ~1	11.85
	B		6.93	6.93		2.29	7.54	12.11
Isodiospyrin	A		6.70 d j = 10.4	6.90 d j = 10.4	7.28	2.01	7.59	12.41
	B		6.93	6.92		2.0		12.03
Mamegakinone	A		6.96		7.11 bd j = ~1	2.45	7.48 bd j = ~1	11.70
7-methyljuglone	A		6.89	6.89	7.06	2.42	7.43	11.83
Neodiospyrin	A		6.76 d j = 10.4	6.92 d j = 10.4	7.25	2.28		12.27
	B		6.60		7.09 bd j = ~1	2.45	7.52 bd j = ~1	11.73
Shinanolone	A	4.80 dd j = 3.7, 7.3	2.11 mp (equa.) 2.25 mp (axial)	2.57 mp (equa.) 2.87 mp (axial)	6.71	2.28	6.82	12.37

The ring system refers to the naphthalene molecule in the naphthoquinones. d = duplet, bd = broad duplet, mp = multiplet, dd = dublet-of-dublet, j = coupling constant in Hz.

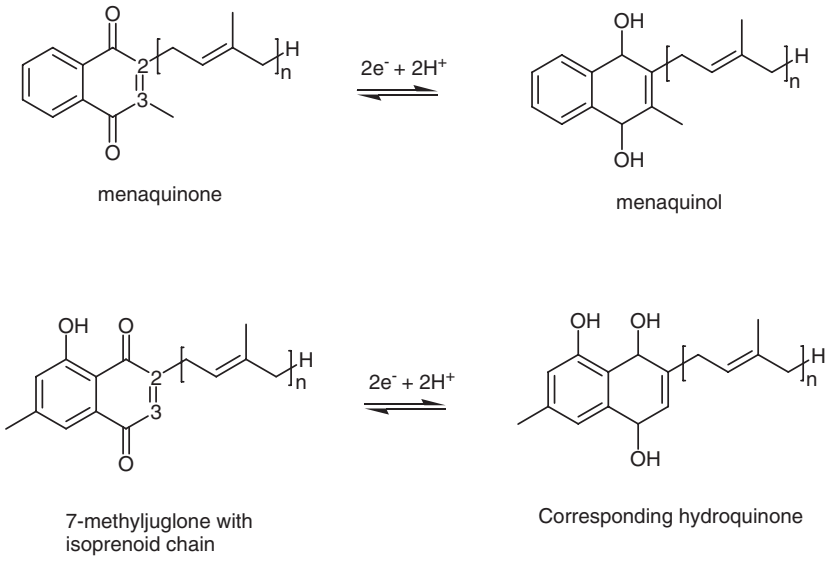

Fig. 3. The incorporation of 7-methyljuglone into the electron transport chain might cause the electron flow to stop or decrease. Alternatively it might competitively bind to the Men enzymes with the result that the isoprene side-chain cannot be attached to carbon 2.

decreasing the activity. The decrease of activity for the dimeric molecules of 7-methyljuglone might be explained by the larger size of these molecules and that the ketone groups are hindered to take part in chemical reactions by the three dimensional shape of the molecule itself. Mamegakinone is completely blocked in this region, which might be the reason for its low antimycobacterial activity. The shape of the most active molecule, 7-methyljuglone, is very similar to menaquinone, which is part of the mycobacterial electron transport system.

Mycobacterium contains the natural redox cycler menaquinone, which mediate electron transfer between different membrane-bound enzymes of the respiratory chain (Garbe, 2004). Most bacteria (including *E. coli*) and mammals make use of ubiquinone to fulfill this function. *E. coli* does however make use of both forms. According to Truglio et al. (2003) *M. tuberculosis* lacks ubiquinone and makes only use of menaquinone in the electron transport chain. It is therefore an attractive drug target because it lacks a human homologue. Due to structural similarities between 7-methyljuglone and menaquinone it is feasible to postulate that 7-methyljuglone can interact with enzymes in the *mycobacterial* electron transport chain (Fig. 3). Due to the difference in the redox potential of the incorporated 7-methyljuglone, the electron flow might be slowed or stopped. It is also possible that 7-methyljuglone binds to the *Men* enzymes responsible for the formation of menaquinone and inhibits the addition of the hydrophobic side-chain. This will influence ATP production and result in a detrimental effect on the organism.

Acknowledgements

We thank Mr. Eric Palmer, Department of Chemistry for the NMR analysis, Benedict Bapela for the MIC determinations at the MRC and the National Research Foundation of South Africa for financial support.

References

Ferreira, A.M., Alves, A.C., Costa, M.A.C., Paul, M.I., 1977. Naphthoquinone dimers and trimers from *Euclea natalensis*. Phytochemistry 12, 433–435.

Garbe, T.R., 2004. Co-induction of methyltransferase RvO560c by naphthoquinones and fibric acids suggests attenuation of isoprenoid quinone action in *Mycobacterium tuberculosis*. Canadian Journal of Microbiology 50, 771–778.

Heifets, L.B., Good, R.C., 1994. Current laboratory methods for the diagnoses of tuberculosis. In: Bloom, B.R. (Ed.), Tuberculosis: Pathogenesis, Prevention and Control. ASM Press, Washington DC. ISBN: 1-55581-072-1, pp. 85–108.

Kumari, L.K., Babu, M.H., Pardhasaradhi, M., 1982. Synthesis of neodiospyrin and fixation of aryl-quinone linkage in its structure. Indian Journal of Chemistry 21B, 619–621.

Lall, N., Meyer, J.J.M., 2001. Inhibition of drug-sensitive and drug-resistant strains of Mycobacterium tuberculosis by diospyrin, isolated from *Euclea natalensis*. Journal of Ethnopharmacology 78, 213–216.

Lall, N., Meyer, J.J.M., Wang, Y., Bapela, N.B., van Rensburg, C.E.J., Fourie, B., Franzblau, S.G., 2005. Characterisation of intracellular activity of antitubercular constituents from the roots of *Euclea natalensis*. Pharmaceutical Biology 43, 353–357.

Lillie, T.J., Musgrave, O.C., 1977. Ebenaceae extractives: Part 7. Use of hydroxy proton shifts of juglone derivatives in structure elucidation. Journal of the Chemical Society. Perkin Transactions 355–359.

Middlebrook, G., Reggiards, Z., Tigertt, W.D., 1977. Automable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. American Review of Respiratory Diseases 115, 1067–1069.

Stander, I., Van Wyk, C.W., 1991. Toothbrushing with the root of *Euclea natalensis*. Journal de Biologie Buccale 19, 167–172.

Truglio, J.J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P.J., Kisker, C., 2003. Crystal structure of *Mycobacterium tuberculosis* MenB, a key enzyme in Vitamin K_2 biosynthesis. Journal of Biological Chemistry 278, 42352–42360.

Van der Vijver, L.M., Gerritsma, K.W., 1974. Naphthoquinones of Euclea and Diospyros species. Phytochemistry 13, 2322–2323.

Van Wyk, B.-E., Van Wyk, P., 1997. Field guide to trees of Southern Africa. Struik, McKenzie Street, Cape Town. ISBN: 1-86825-922-6, pp. 184–185.

Watt, J.M., Breyer-Brandwijk, M.G., 1962. The medicinal and poisonous plants of southern and eastern Africa, 2nd edition. Livingstone, Londen. ISBN: B-0000E-GKU-O, p. 390.

Weigenand, O., Hussein, A.A., Lall, N., Meyer, J.J.M., 2004. Antibacterial activity of naphthoquinones and triterpenoids from *Euclea natalensis* root bark. Journal of Natural Products 67, 1936–1938.