Department of Pharmacognosy[1], Faculty of Pharmacy, Sana'a-University, Yemen; Helmholtz Centre for Infection Research[2], Research Group Microbial Drugs, Braunschweig, Germany; Department of Pharmaceutical and Medicinal Chemistry[3], Department of Pharmaceutical Biology[4], Institute of Pharmacy, Ernst-Moritz-Arndt-University, Greifswald, Germany

Antimicrobial and cytotoxic abietane diterpenoids from the roots of *Meriandera benghalensis* (Roxb.) Benth

R. A. A. Mothana[1], R. Jansen[2], R. Gruenert[3], P. J. Bednarski[3], U. Lindequist[4]

Received February 17, 2009, accepted March 20, 2009

Prof. Dr. Ramzi A.A. Mothana, Department of Pharmacognosy, Faculty of Pharmacy, Sana'a-University, P.O. Box 33039, Sana'a, Yemen
r_mothana@yahoo.com

Pharmazie 64: 613–615 (2009) *doi: 10.1691/ph.2009.9543*

Four abietane diterpenoids were isolated from the methanolic extract of the roots of *Meriandera benghalensis* and tested for their biological activity. Cryptotanshinone (**2**) and 17-hydroxycryptotanshinone (**4**) are known metabolites however the occurrence of tanshinone IIA (**1**) and przewaquinone A (**3**) from *Meriandera benghalensis* is reported for the first time. The four diterpenoids were identified by MS and one- and two dimensional NMR experiments. The isolated compounds were tested for their *in vitro* antiproliferative activity against three human cancer cell lines (a lung cancer (A-427), a urinary bladder cancer (5637) and a breast cancer (MCF-7) cell line) and for their antibacterial effect against three Gram-positive bacterial strains. All four abietanes showed potent cytotoxic effect against all cancer cell lines (IC_{50} between 1 and 8 μM) as well as antibacterial effect against the bacteria tested (MIC values between 33 and 70 μM).

1. Introduction

Meriandera benghalensis (Roxb.) Benth (Labiatae) is a much-branched aromatic shrub up to 2 m high, which grows on the high plateau in Yemen on rocky hills between 2000 and 2800 m (Wood 1997). The aerial part and the root of the plant are widely used in Yemeni traditional medicine. The infusion of the plant is mainly used as antiseptic agent for wounds and for the treatment of urinary tract infections. In a previous work (Mothana et al. 2009) we screened about 100 Yemeni plants for their cytotoxic, antimicrobial and antioxidant activities. *M. benghalensis* represented one of the most interesting plants regarding its *in vitro* anticancer and antimicrobial effects. From the aerial part of this plant 3 sesquiterpenoids have been isolated (Perales et al. 1983). Additionally, 4 abietane diterpenoids have been reported (Torre et al. 1992). However, biological studies on the plant have never been reported before. The present study extends our evaluation of cytotoxic and antimicrobial natural compounds from Yemeni medicinal plants. Thus, this paper reports the activity-guided isolation and the identification of four abietane diterpenoids as well as the evaluation of their *in vitro* anticancer and antimicrobial activities.

2. Investigations, results and discussion

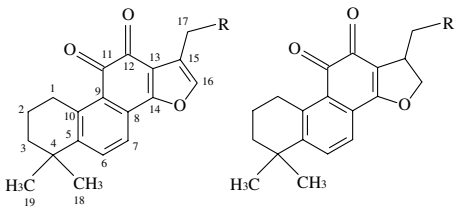
In our continuing search for bioactive compounds from Yemeni medicinal plants as part of research program (Alexander von Humboldt fellowship), the methanolic extract of the roots of *Meriandera benghalensis* showed a high cytotoxicity against human cancer cell lines (lung cancer (A-427), urinary bladder cancer (5637) and breast cancer (MCF-7) line) and antimicrobial activity against gram-positive bacteria. Consequently, this extract was selected for bioassay-guided fractionation on the basis of its cytotoxic and antibacterial effect. The methanolic extract of the roots of *Meriandera benghalensis* was partitioned between water and ethyl acetate and tested. Since both activities resided predominantly in the ethyl acetate layer, this extract was then subjected to column chromatography on columns of silica gel and Sehpadex LH-20 to afford four diterpenoid quinones with abietane skeleton. Compounds **2** and **4** showed physical and spectroscopic data identical with those found for cryptotanshinone (1 H, 13C NMR, MS, mp, Sairafianpour et al. 2001) and 17-hydroxycryptotanshinone (1 H, 13C NMR, MS, Luis and Grillo 1993) respectively, which were isolated from the same plant by Torre et al. (1992). Both compounds were also isolated from *Salvia munzii* and *Perovskia abrotanoides* (Luis and Grillo 1993; Sairafianpour et al. 2001). Compound **1** was obtained as red plates from methanol. In the positive ESI-MS, compound **1** showed $[M + H]$ peak at m/z 295 corresponding to the molecular formula $C_{19}H_{18}O_3$, in agreement with the ^{13}C NMR data, which indicated the presence of 19 carbon atoms. 19 carbon signals were also present in the ^{13}C NMR spectrum of compound **3**. However, compound **3** obtained as red needles, exhibited prominent $[M + H]^+$ and $[2\,M + NH_4]^+$ peaks at m/z 311 and 638 in its positive ESI-MS corresponding to the molecular formula $C_{19}H_{18}O_4$ (Table 1). Unlike compound **1**, the IR spectrum of compound **3** revealed a band at 3420 cm^{-1} indicating the presence of hydroxyl groups. The ^1H- and ^{13}C-NMR spectra of both compounds **1** and

Table 1: ^{1}H NMR spectral data of tanshinone IIA (1) and prezwaquinone A (3) in $CDCl_3$

No.	1	3
	δH m (J Hz)	δH m (J Hz)
1	3.19 t (6.4)	3.19 t (6.4)
2	1.79 m	1.80 m
3	1.65 m	1.66 m
4	–	–
5	–	–
6	7. 55 d (8.1)	7.57 d (8.1)
7	7.62 d (8.1)	7.65 d (8.1)
8	–	–
9	–	–
10	–	–
11	–	–
12	–	–
13	–	–
14	–	–
15	–	–
16	7.21 q (1.0)	7.38 s
17	2.26 d (1.5)	4.66 s
18	1.30 s	1.31 s
19	1.30 s	1.31 s

3 are very similar with only an observed difference. The difference confirmed the existence of a hydroxymethylene group (δ 4.66) in **3** instead of methyl group (δ 2.26) at the C-17 Position in compound **1** (Table 1).

After searching DNP with elemental composition and structural features recognized from NMR and IR spectra a comparison of NMR data with literature data identified compounds **1** and **3** as tanshinone IIA and prezwaquinone A, respectively, which have an additional double bond in position 15 in ring D. Previously **1** and **3** were isolated from different *Salvia* species e.g. *S. miltiorrhiza*, *S. prezewalskii* and *S. constanea* (Li et al. 1991; Onitsuka et al. 1983; Yang et al. 1996), but for the first time from *M. benghalensis*. The *Meriandera* abietanes **1–4** were found to be cytotoxic against all tested cancer cell lines with IC_{50} values between 1.1 and 8.7 μM (Table 2). Interestingly, the breast cancer cell line (MCF-7) was the most sensitive one among the tested cell lines (Table 2). When tetra- and tricyclic tanshinones were tested for their cytotoxicity, it has been observed that the presence of the D ring in tanshinones is obviously necessary for the high toxicity (Sairafianpour et al. 2001). Lee et al. (2008) reported that tanshinone IIA isolated from Danshen (*Salvia miltiorrhiza*) causes cytotoxicity of HepG2 cells (human hepatome cell line) through apoptosis without inducing changes in the GSH/GSSG ratio (an indicator of oxidative stress) despite the observed augmentation in GSH and GSSG, while cryptotanshinone showed lower efficacy in inducing apoptosis despite inducing in intracellular GSH/GSSG ratio in the HepG2 cells. Additionally, all four



(**1**) R = H
(**3**) R = OH

(**2**) R = H
(**4**) R = OH

compounds showed a high antibacterial activity against all Gram-positive bacterial strains tested (Table 2). Przewaquinone A (**3**) and 17-hydroxycryptotanshinone (**4**), both featuring a hydroxyl group in position 17, exhibited superior antibacterial activity. Lee et al. (1999) reported that cryptotanshinone and dihydrotanshinone I isolated from *Salvia miltiorrhiza*, generated superoxide radicals in *Bacillus subtilis* lysates. It was suggested that these superoxide radicals are important for the antibacterial action of these tanshinones.

3. Experimental

3.1. General procedures

UV-spectra in methanol were determined using a Shimadzu UV-1601PC spectrometer. The IR spectrum were recorded in Shimadzu FTIR-8300 spectrometer in KBr. The mass spectrometric analyses were obtained on a PE SCIEX API 2000 LC/MS/MS (Applied Biosystems). ^{1}H and ^{13}C NMR spectra were recorded on a AM-600 spectrometer (Bruker) in $CDCl_3$.

3.2. Plant material

The plant was collected around Sana'a-Yemen in July 2005 and identified at the Pharmacognosy Department, Faculty of Pharmacy, Sana'a University. A voucher specimen was deposited at the Pharmacognosy Department, Faculty of Pharmacy, Sana'a University.

3.3. Extraction and isolation

The air-dried and powdered roots of *Meriandera benghalensis* (150 g) were defatted with hexane (500 ml × 2) by stirring at room temperature for 10 h. After evaporation of the solvent the residue was dried over the night and then extracted with 1000 ml methanol (CH_3OH) in a Soxhlet apparatus for 12 h. The obtained methanolic extract (12.2 g) was filtered and evaporated in a rotary evaporator at 40 °C. The obtained nearly dried methanolic extract was redissolved in 200 ml of water and shaken several times with 200 ml of ethyl acetate. The combined ethylacetate phase was dried over Na_2SO_4, filtered and evaporated to dryness to give a residue of 3.5 g. Each phase was tested for its antimicrobial and cytotoxic activity. Consequently, it was shown that both activities resided predominantly in the ethylacetate phase. Thus the ethyl acetate soluble part was subjected to column chromatography on a pre-packed silica gel column (35 mm i.d. × 350 mm) to give 15 fractions. The elution was performed with a gradient of petroleum ether:ethyl acetate (10 : 1), to pure ethyl acetate, finally with methanol. TLC analysis of the fractions with anisaldehyde/sulfuric acid and heating at 100 °C, allowed the constitution of 15 fractions. Fraction 2, 5, 7 and 12 were found to be the most active. Fraction 2 (95 mg) was rechromatographed on a silica gel column with petroleum ether : ethyl acetate (7 : 1) to give compound **1**, which was further purified using preparative silica gel TLC to afford 10 mg of compound **1** (red plates from methanol). Fraction 5 (350 mg) was subjected to a silica gel column chromatography using

Table 2: IC_{50} values (μM) for cell growth inhibition of the isolated diterpenes on three human cancer cell lines and MIC values (μM) on three Gram-positive bacterial strains

Isolated comounds	IC_{50} values (μM)			MIC in (μM)		
	5637	A-427	MCF-7	*S. aureus*	*B. subtilis*	*M. flavus*
Tanshinone IIA (**1**)	6.74 ± 0.92	8.69 ± 2.54	1.88 ± 0.67	35.28	70.57	17.64
Cryptotanshinone (**2**)	3.19 ± 1.70	4.83 ± 0.19	1.54 ± 0.83	35.05	70.10	17.52
Przewaquinone A (**3**)	2.49 ± 1.75	5.58 ± 2.05	1.08 ± 0.58	16.73	33.46	16.73
17-Hydroxycryptotanshinone (**4**)	5.96 ± 5.52	8.19 ± 1.65	3.44 ± 2.91	16.62	33.25	16.62
Etoposide	0.54 ± 0.30	0.13 ± 0.10	0.50 ± 0.19			

petroleum ether : ethyl acetate (4 : 1) as a solvent to afford compound **2** (red-orange powder, 150 mg). Fraction 7 (80 mg) was subjected to sephadex LH20 column and DCM : MeOH (4 : 1) as a solvent to afford compound **3** (red plates from methanol, 16 mg). Fraction 12 (118 mg) was separated by silica gel column chromatography with petroleum ether:ethyl acetate (1 : 1) as a eluent to give compound **4** (orange-red needles from methanol, 20 mg).

Tanshinone IIA (**1**): red plates (MeOH); mp 214–216 °C; UV (MeOH) λ_{max} (log ε): 225 (4.31), 268 (4.41), 350 (3.31), 452 (3.5). IR (KBr) $\nu = 3112$, 1690, 1532, 1476, 1165, 840, 713 cm^{-1}; ESI-MS m/z 295 [M+H] (294.3 calculated for $C_{19}H_{18}O_3$).

Prezwaquinone A (**3**): red crystals (MeOH), mp 172–174 °C; UV (MeOH) λ_{max} (log ε): 222 (4.30), 266 (4.40), 352 (3.29), 448 (3.48). IR (KBr) $\nu = 3420$, 2928, 1621, 1546, 1420, 1294, 1160, 935, 842, 710 cm^{-1}; 1H and 13 C NMR, see Table 1; ESI-MS m/z 311 and 638 [M + H] and [2M + H_2O] (310.3 calculated for $C_{19}H_{18}O_4$).

3.4. Broth micro-dilution assay for minimum inhibitory concentrations (MIC)

The broth micro-dilution method described by Mann and Markham (1998) with modifications was used to determine the MIC of extracts against three Gram-positive bacteria namely *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6059) and *Micrococuss flavus* (SBUG 16). Using sterile round-bottom 96-well plates, duplicate twofold serial dilutions of extract (100 µl/well) were prepared in the appropriate broth containing 5% (v/v) DMSO to produce a concentration range of 2000 to 15.6 µg of extract/ml. Two-fold dilutions of ampicillin were used as a positive control. 100 µl of a bacterial cell suspension (prepared in the appropriate broth) corresponding to 1×10^6 CFU/ml was added to all wells except those in column 10, 11 and 12 which served as saline, extract and media sterility controls, respectively. Controls for bacterial growth without plant extract were also included on each plate. The final concentration of bacteria in the assay was 5×10^5 CFU/ml. The final concentration of extracts was 1000 to 7.8 µg/ml. Plates were then incubated at 37 °C for 18 h. After incubation, the MIC of each extract was determined as the lowest concentration at which no growth was observed in the duplicate wells. p-Iodonitro-tetrazolium violet solution (20 µl, 0.04%, w/v) (Sigma, USA) was then added to the wells. The plates were incubated for a further 30 min, and estimated visually for any change in color from yellow to pink indicating reduction of the dye due to bacterial growth. The highest dilution (lowest concentration) that remained yellow corresponded to the MIC. Experiments were performed in duplicate.

3.5. Determination of cytotoxicity on human cancer cell lines

For the estimation of the *in vitro* cytotoxic potency of the investigated extracts, an established microtiter plate assay was used with 3 human cancer cell lines: a lung cancer (A-427), a urinary bladder cancer (5637) and a breast cancer (MCF-7) cell line. Cytotoxicity determinations are based on cellular staining with crystal violet and were performed as previously described in detail (Bracht et al. 2006). Briefly, a volume of 100 µl of a cell suspension was seeded into 96-well microliter plates at a density of 1000 cells/well. Twenty-four hours later, cells were treated with the plant extracts at five dilutions and exposed continuously to the extracts for the next 96 h. At the end of the exposure time, the medium was removed and the cells were fixed with glutaraldehyde solution. The cells were then stained with crystal violet and the optical density (OD) was measured at $\lambda = 570$ nm with a plate reader. Etoposide was used as a positive control. The percent growth values were calculated by the following equation:

$$\text{Growth } (\%) = OD_T - OD_{c,0}/OD_c - OD_{c,0} \times 100$$

where OD_T is the mean absorbance of the treated cells, OD_c is the mean absorbance of the controls, $OD_{c,0}$ is the mean absorbance at the time the extract was added. The IC_{50} values were estimated by a linear least-squares regression of the growth values versus the logarithm of the extract concentration; only concentrations that yielded growth values between 10% and 90% were used in the calculation.

Acknowledgements: The authors wish to thank the Alexander von Humboldt Foundation for the fellowship awards (George-Foster-Scholarship) enabling the stay of Prof. Dr. Ramzi Mothana at Ernst-Moritz-Arndt University Greifswald to perform this part of research project.

References

Bracht K, Boubakari, Gruenert R, Bednarski PJ (2006) Correlations between the activities of 19 antitumor agents and the intracellular GSH concentrations in a panel of 14 human cancer cell lines: Comparisons with the NCI data. Anti-Cancer Drugs 17: 41–51.

Lee D-S, Lee S-H, Noh J-G, Hong S-D (1999) Antibacterial activities of cryptotanshinone and dihydrotanshinone I from a medicinal herb, *Salvia miltiorrhiza* Bunge. Biosci Biotechnol Biochem 63: 2236–2239.

Lee WYW, Chiu LCM, Yeung JHK (2008) Cytotoxicity of major tanshinones isolated from Danshen (*Salvia miltiorrhiza*) on HepG2 cells in relation to glutathione perturbation. Food Chem Toxicol 46: 328–338.

Li Bo, Niu Fandi, Lin Zhongwen, Zhang Hongjie, Wang Dezu, Sun Handong (1991) Diterpenoids from the roots of *Salvia przewalskii*. Phytochemistry 30: 3815–3817.

Luis JG, Grillo TA (1993) New diterpenes from *Salvia munzii*: Chemical and biogenetic aspects. Tetrahedron 49: 6277–6284.

Mann CM, Markham JL (1998) A new method for determining the minimum inhibitory concentration of essential oils. J Appl Microbiol 84: 538–544.

Mothana RAA, Gruenert R, Bednarski PJ, Lindequist U (2009) Evaluation of the in vitro anticancer, antimicrobial and antioxidant activities of some Yemeni plants used in folk medicine. Pharmazie 64: 260–268.

Onitsuka M, Fujiu M, Shinma N, Maruyama HB (1983) New platelet aggregation inhibitors from Tan-Shen; radix of *Salvia miltiorrhiza* Bunge. Chem Pharm Bull 31: 1670–1675.

Perales A, Martinez-Ripoll M, Fayos J, Savona G, Bruno M, Rodriguez B (1983) Sesquiterpenoid constituents of *Meriandra benghalensis* (Labiatae). X-ray structure analysis. J Org Chem 48: 5318–5321.

Sairafianpour M, Christensen J, Staerk D, Budnik BA, Kharazmi A, Bagherzadeh K, Jaroszewski JW (2001) Leishmanicidal, antiplasmodial and cytotoxic activity of novel diterpenoid 1,2-quinones from *Perovskia abrotanoides*: New source of tanshinones. J Nat Prod 64: 1398–1403.

Torre MC, Bruno M, Rodriguez B, Savona G 1992. Abietane and 20-norabietane diterpenoids from the root of *Meriandra benghalensis*. Phytochemistry 31: 3953–3955.

Wood JRI (1997) A handbook of the Yemen flora. Royal botanic garden Kew, Whitstable Litho Printers Ltd, UK, pp. 253.

Yang M-H, Blunden G, Xu Y-X, Nagy G, Mathe I (1996) Diterpenoids from *Salvia* species. Pharm Sci 2: 69–71