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Phytochemistry 65 (2004) 331–335
PHYTOCHEMISTRY
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The anti-staphylococcal activity of *Angelica dahurica* (Bai Zhi)

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Received 11 September 2003; received in revised form 10 November 2003

Abstract

Bioassay-guided fractionation of a hexane extract prepared from the roots of the Chinese drug *Angelica dahurica* (Bai Zhi) led to the isolation of the polyacetylenic natural product falcarindiol (**1**). The absolute stereochemistry of this compound was confirmed by careful ^{1}H NMR analysis of its (*R*)- and (*S*)-Mosher ester derivatives as the 3(*R*), 8(*S*) isomer. Activity was tracked using a *Mycobacterium fortuitum* screening assay and the purified product was evaluated against multidrug-resistant and methicillin-resistant strains of *Staphylococcus aureus* (MRSA). The minimum inhibitory concentrations (MIC) of this metabolite ranged from 8 to 32 μg/ml highlighting the potential of the acetylene natural product class as antibiotic-lead compounds. These MIC values compare favourably with some of the newest agents in development for the treatment of MRSA infection and indicate that further evaluation of the antibiotic activity of acetylenes is warranted.

Keywords: Bai Zhi; *Angelica dahurica*; Apiaceae; Mosher's esters; Multidrug-resistance; *Staphylococcus aureus*; MDR; MRSA; Polyacetylenes

1. Introduction

Strains of *Staphylococcus aureus* express a series of multidrug resistance (MDR) efflux pumps such as Tet(K), Msr(A), Nor(A) and Qac(A) which confer resistance to a wide range of structurally unrelated antibiotics and antiseptics (Marshall and Piddock, 1997). These MDR pumps are part of an array of cytoplasmic membrane transport systems involved primarily in the uptake of essential nutrients, the excretion of toxic compounds and the maintenance of cellular homeostasis (Paulsen et al., 1996). In a continuing project to identify plant natural products with activity against MDR strains of *S. aureus*, extracts from the roots of *Angelica dahurica* (Apiaceae) were evaluated for in vitro antibacterial activity.

The plant is a perennial herb growing to 2.5 m with a hollow stem, large three-branched leaves and umbels bearing many white flower heads. It grows wild in thickets in China, Japan, Korea and Russia and the cultivated herb is mainly from central and eastern regions of China. The roots are known as Bai Zhi in traditional Chinese medicine, where they are classified as a sweat-inducing drug able to counter harmful external influences on the skin, such as cold, heat, dampness and dryness (Chevalier, 2001). Bai Zhi is also claimed to be effective in the treatment of acne, erythema, headache, toothache, sinusitis, colds and flu (Wagner, 1999).

2. Results and discussion

Antibacterial activity was associated with the hexane extract. Bioassay-guided fractionation by VLC and preparative thin-layer chromatography led to the isolation of the major active compound (**1**), which was a viscous pale yellow oil. Signals in the ^{13}C NMR spectrum for four quaternary carbons at δ_C 79.9, 78.3, 70.3 and 68.7 ppm were indicative of a polyacetylene natural product possessing two triple bonds. This was confirmed by a weak absorption in the IR spectrum at 2235

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doi:10.1016/j.phytochem.2003.11.010

cm^{-1}, which is also characteristic of an alkyne (Williams and Fleming, 1995). Further signals in the ^{1}H and ^{13}C NMR spectra included a *cis*-double bond, two methine protons bearing oxygen and three olefinic protons which were coupled to each other in a COSY spectrum. Two of these were part of an *exo*-cyclic methylene (δ_H 5.40 and 5.19). Negative ion mode APCI-MS revealed a peak at 259 $[M-H]^-$ and this with the NMR data indicated a molecular formula of $C_{17}H_{24}O_2$. These data were consistent with those published for the C_{17} polyacetylene falcarindiol (Fig. 1a), which is a common component of apiaceous plant roots (Furumi et al., 1998). The 3(*R*), 8(*S*) stereochemistry of falcarindiol was confirmed by Mosher's ester methodology (Rieser et al., 1992; Su et al., 2002; Ward and Rhee, 1991) and the (*S*)- and (*R*)-MTPA esters were prepared and chemical shifts in the H_2-1, H-2 and H-9/H-10 protons were measured for both derivatives (Fig. 1a). Using $\Delta_\delta = \delta_S - \delta_R$ methodology (Ohtani et al., 1991), for H_2-1 and H-2 protons a positive value for $\Delta_{\delta?}$ was observed for both sets of signals indicating that these groups should be place at R_1 in Fig. 1b and that R_2 would be the point of connection for the triple bond resulting in a 3(*R*) configuration for this stereogenic centre. For H-9 and H-10, Δ_δ was negative indicating that this *cis*-double bond should be placed at R_2 and that the absolute stereochemistry for C-8 corresponded to (*S*).

The literature also describes 3(*S*), 8(*S*)-falcarindiol (Bernart et al., 1996; Kobaisy et al., 1997) which has a positive specific rotation ($[\alpha]_D^{25} + 144.8$), whereas our isolate has a negative value. Whilst optical rotation is useful in characterizing known compounds, the presence of two chiral centres in a molecule could complicate the comparison with literature data. This investigation proves that the laevorotatory isolated falcarindiol corresponds to the 3(*R*), 8(*S*) diasteroisomer, whilst the alternative laevoisomer would represent the enantiomeric form of the previously described dextrorotatory natural product [i.e. 3(*R*), 8(*R*)]. Given the ease of preparation of Mosher's esters, the present application exemplifies the potential of this approach to confirm the stereochemical identity of novel and known natural products. This is particularly important where bioactivity studies are conducted and changes in absolute stereochemistry could dramatically alter biological activity. The use of a solid phase carbodiimide resin ensured that if the Mosher's acid chlorides, which are usually sufficiently reactive, are hydrolysed to the acids by the presence of extraneous amounts of moisture in the reaction solvent (i.e. dichloromethane or pyridine), the esters are still formed by the coupling action of the carbodiimide. These resins can then be conveniently removed by filtration.

Falcarindiol displayed low minimum inhibitory concentrations against a panel of methicillin-resistant and multidrug resistant (MDR) strains of *Staphylococcus aureus* (Table 1). Two of these strains (XU212 and RN4220) possess the TetK and MsrA transporters that export tetracycline and macrolide antibiotics respectively. Strain SA-1199B possesses the NorA MDR efflux mechanism, the major drug pump in this pathogen. Activity was also demonstrated against epidemic MRSA strain EMRSA-15 which was a clinical isolate initially isolated from hospitals in the Midlands and south-east of England (Richardson and Reith, 1993). Interestingly, falcarindiol was more active against the MDR strains than against the non-resistant ATCC strain. Whilst the anti-staphylococcal activity of 3(*S*), 8(*S*)-falcarindiol has been reported before (Kobaisy et al., 1997), this is the first report of the 3(*R*), 8(*S*) isomer possessing activity against MDR strains of this species. This result supports a recent study (Tegos et al., 2002), which demonstrated that certain plant natural products are more active against MDR than sensitive strains. Whilst falcarindiol has been shown to be mildly cytotoxic and has anti-inflammatory properties (Bernart et al., 1996; Liu et al., 1998) the activity exhibited here against MDR and methicillin-resistant *Staphylococcus aureus* indicates that the acetylene class of natural products should be further investigated as antibiotic leads. This is certainly worthwhile given the burden of drug-resistant mycobacterial and staphylococcal species

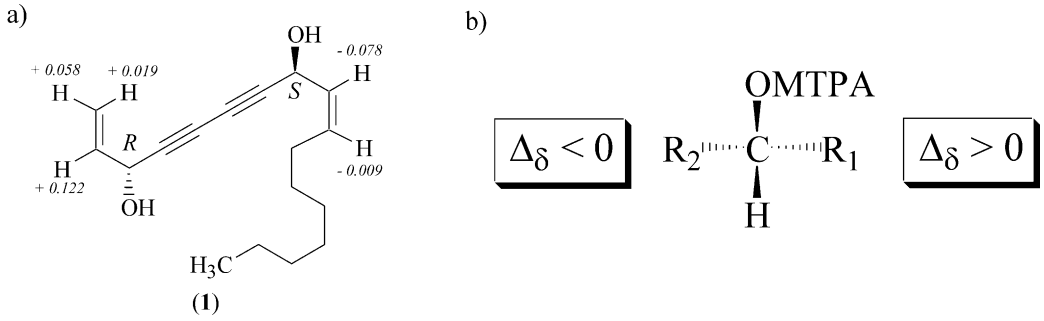

Fig. 1. (a) Structure of falcarindiol (**1**) depicting its 3-(*R*), 8-(*S*) absolute stereochemistry. The figures in italics are for Δ_δ where $\Delta_\delta = \delta_S - \delta_R$. δ_S and δ_R are the chemical shift values for a particular group of the (*S*) and (*R*) Mosher's esters of falcarindiol. (b) The Mosher's ester was (*R*)- or (*S*)-α-methoxy-α-trifluoromethylphenyl acetate (MTPA). When Δ_δ for a group of diagnostic resonances is positive then it must be placed at R_1. When Δ_δ for a group of diagnostic resonances is negative then the group must be placed at R_2.

Table 1
Minimum inhibitory concentrations (MIC) of falcarindiol and standard antibiotics in μg/ml

Bacterium	Falcarindiol	Norfloxacin	Tetracycline	Erythromycin	Ethambutol	Isoniazid
S. aureus SA-1199B (NorA)	8	32	32	2	[a]	[a]
S. aureus XU-212 (TetK)	16	8	256	[a]	[a]	[a]
S. aureus RN-4220 (MsrA)	16	64	64	64	[a]	[a]
S. aureus EMRSA-15 (MecA)	32	2	0.25	[a]	[a]	[a]
S. aureus ATCC 25923	32	0.5	0.5	0.25	[a]	[a]
M. fortuitum ATCC 6841	8	[a]	[a]	[a]	8	0.5

Resistance mechanism of staphylococcal strains in parentheses.
[a] Not tested.

which are difficult to treat and eradicate in both the clinical and community setting (Levy, 1998).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Bellingham and Stanley ADP 200 polarimeter. IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded in $CDCl_3$ on a Bruker AVANCE 500 spectrometer. Chemical shifts values (δ) are reported in parts per million (ppm) relative to NMR solvent $CDCl_3$ ($\delta_H = 7.20$, $\delta_C = 77.0$). Coupling constants (J values) are given in Hertz. ^{1}H–^{1}H COSY, HMBC and HMQC experiments were recorded with gradient enhancements using sine shaped gradient pulses. APCI mass spectra were recorded on a Finnigan Navigator instrument. Vacuum liquid chromatography on Merck Silica gel 60 $PF_{254+366}$ was used for fractionation and isolation. TLC was performed using Kieselgel 60 F_{254} (Merck) pre-coated plates and spots were visualized by spraying with vanillin-sulphuric acid spray followed by heating.

3.2. Plant material

Air-dried roots of the plant were obtained from Kingham Herbs (Batch no. 863402001) and a voucher specimen (SG-2003-1) has been deposited at the Centre.

3.3. Bacterial strains

S. aureus RN4220 containing plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein, was provided by J. Cove (Ross et al., 1989). EMRSA-15 which possesses the *mecA* gene was provided by Dr. Paul Stapleton, ULSOP. Strain XU-212, which possesses the TetK tetracycline efflux protein, was provided by E. Udo (Gibbons and Udo, 2000). SA-1199B, which overexpresses the *norA* gene encoding the NorA MDR efflux protein was provided by G. Kaatz (Kaatz et al., 1993). *S. aureus* strains were cultured on nutrient agar (Oxoid) and *Mycobacterium fortuitum* was cultured on Columbia agar (Oxoid) supplemented with 5% defibrinated horse blood (Oxoid) and incubated for 24 and 72 h, respectively at 37 °C prior to MIC determination.

3.4. Minimum inhibitory concentration (MIC)

Tetracycline, norfloxacin, and erythromycin were obtained from Sigma Chemical Co. Mueller-Hinton broth (MHB; Oxoid) was adjusted to contain 20 and 10 mg/l of Ca^{2+} and Mg^{2+}, respectively. An inoculum density of 5×10^5 cfu of each of the test organisms was prepared in normal saline (9 g/l) by comparison with a MacFarland standard. MHB (125 μl) was dispensed into 10 wells of a 96-well microtitre plate (Nunc, 0.3 ml volume per well). Tetracycline and erythromycin were dissolved in MHB to give stock solutions. A stock solution of norfloxacin was prepared by dissolving the antibiotic in DMSO (Sigma) and dilution in MHB to give a final concentration of 0.625%. A DMSO control was included in all assays.

Antibiotics were serially diluted into each of the wells followed by the addition of the appropriate bacterial inoculum. The plate was incubated at 37 °C for 72 h (for *Mycobacterium fortuitum*) and 18 h (for *Staphylococcus aureus*) and the MIC recorded as the lowest concentration at which no growth was observed. This was facilitated by the addition of 20 μl of a 5 mg/ml methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) to each of the wells and incubation for 20 min. A blue colouration indicated bacterial growth.

3.5. Isolation of falcarindiol

The chopped dry roots (500 g) were ground and extracted sequentially in a Soxhlet apparatus with hexane (2.5 l) and dichloromethane (2.5 l) which when concentrated under vacuum gave 6.57 g and 2.05 g of extracts respectively. These were then screened for antibacterial

activity using an in vitro assay and the hexane extract was found to be active. 6.57 g of this were subjected to vacuum liquid chromatography (VLC) on silica gel by eluting with 200 ml hexane, and increasing polarity by 10% increments with EtOAc and finally a wash with methanol to yield 12 fractions.

Activity was traced to compound **1** which was isolated from VLC fraction 5 (hexane–EtOAc, 6:4) by PTLC using EtOAc–hexane (3:7, two developments) as a solvent system. Purity was monitored by TLC (EtOAc–hexane, 1:1) and **1** gave a black colouration on TLC with vanillin-sulphuric acid at R_f 0.47.

3.6. Preparation of Mosher's esters

Falcarindiol (18 mg) was dried under vacuum for 1 h and then two portions (4 mg each) were dissolved in dry CH_2Cl_2 (2 ml) containing 2 mg of dimethylaminopyridine (Sigma Chemical Co.). Eight equivalents of PS-carbodiimide resin (Argonaut Technologies Inc.) were added to each portion of falcarindiol. Six equivalents of either the (*S*) or the (*R*) isomer of α-methoxy-α-trifluoromethylphenylacetyl chloride (Sigma Chemical Co.) were added to a portion of falcarindiol which resulted in the formation of the (*R*) and (*S*) Mosher's esters of falcarindiol respectively. The reaction mixtures were stirred for 24 h, filtered to remove the PS-carbodiimide resin, concentrated under nitrogen and then redissolved in $CDCl_3$ (0.75 ml) and loaded into separate NMR tubes. NMR spectra were then recorded at 500 MHz and differences in the signals of protons that neighboured the chiral centres noted (Fig. 1a). The bulk effect on chemical shifts induced by esterification with the chiral reagents was calculated using $\Delta_\delta = \delta_S - \delta_R$ where δ_S and δ_R are the shifts (in ppm) of diagnostic protons neighbouring the chiral centres in falcarindiol of the (*S*) and (*R*) Mosher's esters, respectively. The positive and negative magnitudes of Δ_δ were interpreted using the model adapted by Kakisawa and collaborators (Ohtani et al., 1991) (Fig. 1b).

3.7. 3(R), 8(S)-falcarindiol (1)

Pale yellow oil; $[\alpha]_D^{25}$ −130 (MeOH, *c* 0.02); IR ν_{max} (thin film) cm^{-1}: 3393, 2925, 2855, 2235, 1735, 1718, 1457, 1375, 1228, 1216, 1034, 724; ^1H NMR ($CDCl_3$) δ: 0.82 (*t*, 6.9 Hz, 3H, H_3-17), 1.21 (*m*, 8H), 1.31 (*m*, 2H), 2.04 (*q*, 7.2 Hz, H_2-11), 4.88 (*d*, 5.4 Hz, H-3), 5.14 (*bd*, 8.2 Hz, H-8), 5.19 (*bd*, 10.1 Hz, H-1b), 5.41 (*bd*, 17.0 Hz, H-1a), 5.46 (*bd*, 8.8 Hz, H-9), 5.56 (*dt*, 11.0 and 7.6 Hz, H-10) and 5.87 (*ddd*, 17.0, 10.1 and 5.3 Hz, H-2); ^{13}C NMR ($CDCl_3$) δ: 14.1 (C-17), 22.6 (C-16), 27.7 (C-11), 29.1 (C-14), 29.1 (C-13), 29.2 (C-12), 31.8 (C-15), 58.6 (C-8), 63.4 (C-3), 68.7 (C-6), 70.2 (C-5), 78.2 (C-7), 79.8 (C-4), 117.3 (C-1), 127.6 (C-9), 134.6 (C-10), 135.8 (C-2); APCI-MS: *m*/*z* 259 $[M-H]^-$.

Acknowledgements

The EPSRC and the School of Pharmacy are thanked for the award of studentships to M. Oluwatuyi and M. Stavri, respectively. The Royal Society and Academia Mexicana de Ciencias are thanked for funding the visit of R. Pereda-Miranda to the University of London School of Pharmacy (Grant No. 15823).

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