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Antimicrobial constituents of *Scrophularia deserti*

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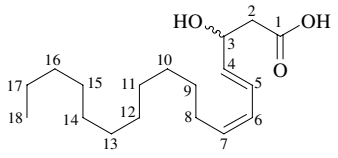
Abstract

A study of the chemistry and antibacterial activity of *Scrophularia deserti* led to the isolation of eight compounds, including the metabolite 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid (**1**). The known compounds ajugoside (**2**), scropolioside B (**3**), 6-*O*-α-L-rhamnopyranosylcatalpol (**4**), buddlejoside A_8 (**5**), scrospioside A (**6**), laterioside (**7**) and 3*R*-1-octan-3-yl-3-*O*-β-D-glucopyranoside (**8**) were also isolated. Compounds **1**–**3** exhibited moderate antibacterial activity against strains of multidrug and methicillin-resistant *Staphylococcus aureus* (MRSA) and a panel of rapidly growing mycobacteria with minimum inhibitory concentration (MIC) values ranging from 32 to 128 μg/ml.

Keywords: *Scrophularia*; Antimicrobial; Iridoids; *Staphylococcus aureus*; Multidrug-resistance; Mycobacterium

1. Introduction

The Scrophulariaceae, also known as the figwort family, comprise approximately 5100 species belonging to 268 genera (Mabberley, 1997). Phytochemically this family is a rich source of iridoid glycosides, especially from the genera *Buddleja*, *Scrophularia* and *Verbascum* (Ahmed et al., 2003; Miyase et al., 1991; Seifert et al., 1989). *Scrophularia deserti* Del. is the most common figwort found in Kuwait and is fairly abundant in areas where limestone underlies the sand (Shuaib, 1995). This plant can mainly be found growing in the Saharo-Arabian and adjacent Irano-Turanian territories, including Egypt, Palestine, Jordan, Syria, Iraq, Saudi Arabia, Bahrain, Iran as well as in Kuwait (Daoud and Al-Rawi, 1985). *S. deserti* is used in traditional medicine as an antipyretic, a remedy for kidney diseases and for tumours and lung cancer (Ahmed et al., 2003). In an investigation to evaluate antibacterial plant natural products, extracts of the whole plant of *S. deserti* were studied. This paper details the characterisation of a new hydroxylated unsaturated fatty acid (**1**), which exhibited moderate antibacterial activity. A further six known compounds all belonging to the iridoid glycoside natural product class were isolated, two of which (**2** and **3**) were shown to possess anti-staphylococcal activity. These compounds were evaluated against a panel of methicillin and multidrug-resistant *Staphylococcus aureus* strains and rapidly growing mycobacteria.



(**1**)

2. Results and discussion

Compound **1** was isolated as a colourless oil from the hexane extract following vacuum liquid chromatography

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Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) spectral data and ^{1}H and ^{13}C long-range correlations of compound **1** recorded in $CDCl_3$

Position	^{1}H	^{13}C	^{2}J	^{3}J
1	–	179.1		
2	1.52 *m*	37.2	C-3	
3	4.17 *dd* (13.0, 6.5)	72.9	C-2, C-4	C-5
4	5.67 *dd* (15.5, 7.0)	135.7	C-3	C-2, C-6
5	6.49 *dd* (15.5, 11.0)	125.8	C-6	C-3, C-7
6	5.97 *t* (11.0)	127.8	C-5	C-4, C-8
7	5.44 *dt* (11.0, 8.0)	132.8	C-8	C-5, C-9
8	2.18 *m*	27.6	C-7	C-6
9	1.39 *m*	25.1		C-7
10–13	1.31 *m*	28.9		
14	1.31 *m*	28.8		
15	1.31 *m*	29.3		
16	1.31 *m*	31.8		
17	1.31 *m*	22.6		
18	0.88 *t* (7.0)	14.0	C-17	C-16

(VLC) and multiple development preparative thin layer chromatography (p-TLC). HRCIMS revealed the M^+ ion at $m/z = 296.2324$ (calc. for $C_{18}H_{32}O_3$: 296.2352). The ^{1}H and ^{13}C NMR data (Table 1) indicated the presence of four olefinic protons, an oxymethine group, a carbonyl carbon, a methylene envelope, two downfield methylene groups and finally a methyl group.

The HMBC spectrum showed a ^{2}J correlation between a downfield methylene (δ_H 1.52 *m*, H_2-2) towards the carbonyl carbon (δ_C 179.1, C-1) of the carboxyl group. These methylene protons also showed a COSY correlation to the oxymethine proton, placing this oxymethine at C-3 (δ_H 4.17 *dd*, $J = 13.0$, 6.5 Hz, δ_C 72.9) and β to the carbonyl carbon of the carboxyl group. This oxymethine proton in turn showed a COSY coupling to an olefinic proton (δ_H 5.67 *dd*, $J = 15.5$, 7.0 Hz, H-4). Furthermore, H-4 coupled to the olefinic proton, H-5 (δ_H 6.49 *dd*, $J = 15.5$, 11.0 Hz) and the large coupling constant shown between H-4 and H-5 ($J = 15.5$ Hz) indicated that these protons were *trans*-orientated. The H-5 olefin also coupled to a third olefinic proton H-6 (δ_H 5.97 *t*, $J = 11.0$ Hz) which in turn coupled to a final olefinic hydrogen, H-7 (δ_H 5.44 *dt*, $J = 11.0$, 8.0 Hz). As H-6 appeared as a triplet in the ^{1}H spectrum, with a smaller coupling constant ($J = 11.0$ Hz), this suggested that H-6 and H-7 were *cis*-orientated. H-7, gave a COSY and HMBC correlation to a methylene group (δ_H 2.18 *m*, H_2-8). In the COSY spectrum this then coupled to the methylene envelope at δ_H 1.31 ppm, indicating the beginning of the alkyl chain. The alkyl chain was found to consist of 10 methylene groups and a terminal methyl group based on the result of mass spectrometry for this compound. This terminal methyl group coupled into the methylene envelope in the COSY spectrum. An attempt was made to assign the absolute stereochemistry at the C-3 position for compound **1** using Mosher's ester methodology, but unfortunately this was not possible due to the unstable nature of the compound. The optical rotation for **1** was zero and it is likely that **1** is racemic. This would make sense if **1** is biosynthesized through a non-enzymatic hydrolysis of a 2–3 double bond where attack by a water molecule from both faces of the olefin would lead to a racemate. However, 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid (**1**) is new and the full NMR data are reported here for the first time (Table 1).

Compound **2** was isolated from the chloroform extract as a colourless oil. The 1D and 2D NMR data enabled the structure of this compound to be elucidated as the known iridoid glucoside, ajugoside (Guiso et al., 1974, Bianco et al., 1981). The first report of ajugoside, by Guiso et al. (1974), was represented with the hydroxyl at position 6 in an α-orientation. However, subsequent analysis of the ^{13}C NMR data of iridoid glucosides (Chaudhuri et al., 1980), including ajugol and myoporoside and their acyl-derivatives (Damtoft et al., 1982), indicated that the configuration of the hydroxyl at position 6 should in fact be reversed to a β-orientation.

Compound **3** was isolated as a white amorphous solid from the chloroform extract. By comparison of the ^{1}H and ^{13}C NMR data with the literature, the structure of **3** was confirmed as scropolioside B (Calis et al., 1988).

6-*O*-α-L-rhamnopyranosylcatalpol (**4**), buddlejoside A_8 (**5**), scrospioside A (**6**), laterioside (**7**) and 3*R*-1-octan-3-yl-3-*O*-β-D-glucopyranoside (**8**) were also isolated and characterised by direct comparison with the literature (Hosny and Rosazza, 1998, Seifert et al., 1989, Miyase et al., 1991, Pachaly et al., 1994, Pardo et al., 1998, Yamamura et al., 1998).

Three of the eight compounds isolated from this plant exhibited antimicrobial activity. A panel of *S. aureus* strains, including one possessing the NorA multidrug-resistance efflux pump was tested along with a panel of rapidly growing mycobacteria. The unsaturated and hydroxylated fatty acid, 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid (**1**), exerted an anti-staphylococcal and antimycobacterial activity against all the strains tested with MIC values ranging from 32 to 128 μg/ml (Table 2).

The antibacterial activity of unsaturated fatty acids, such as 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid (**1**), against both *S. aureus* (Knapp and Melly, 1986, Kabara et al., 1972) and also mycobacteria (Saito et al., 1983) has long been known. However, it has only recently been deciphered that these compounds exert their antibacterial effect by inhibiting an enzyme or enzymes of Type II fatty acid synthesis (FAS) (Zheng et al., 2005). The enzyme FabI, an enoyl-acyl carrier protein reductase catalysing the final step in chain elongation, has been identified as a target for bacterial inhibition (Zheng et al., 2005, Heath et al., 2001, Payne et al., 2001). A series of hydroxylated unsaturated fatty acids have been reported as potent acetyl CoA carboxylase inhibitors (Watanebe et al., 1999) further indicating that these compounds interfere with fatty acid synthesis. Type II FAS differs from that found in mammalian cells (Type I FAS) therefore the differences between the two systems allows these enzymes to be used as a potential target for drug development. Further work to identify the

Table 2
MICs of **1**, **2** and **3** and standard antibiotics

Bacteria	**1**	**2**	**3**	Norfloxacin	Ethambutol
Staphylococcus aureus 1199B (NorA)	64	32	ξ	32	–
Staphylococcus aureus EMRSA-15	128	ξ	ξ	2	–
Staphylococcus aureus ATCC 25923	128	128	128	0.5	–
Mycobacterium fortuitum ATCC 6841	32	ξ	ξ	–	4
Mycobacterium phlei ATCC 11758	32	ξ	ξ	–	2
Mycobacterium aurum Pasteur Institute 104482	32	ξ	ξ	–	1
Mycobacterium smegmatis ATCC 14468	32	ξ	ξ	–	0.5

ξ = not active at 128 μg/ml; –, not tested.

actual enzyme being inhibited within FAS II needs to be performed.

Compound **2** exhibited weak anti-staphylococcal activity against *S. aureus* ATCC 25923 (Table 2) which has been reported previously (Ezer et al., 1995) and compound **3** was also shown to exert a similar effect against this strain. Interestingly, compound **2** exhibited a 4-fold greater anti-staphylococcal activity against the multidrug-resistant strain *S. aureus* 1199B, which codes for the NorA efflux pump transporter. Further work to identify more active iridoids and to elucidate the molecular target of this natural product class would appear to be worthwhile.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard. Coupling constants (J values) are given in Hertz. Mass spectra were recorded on Finnigan Mat 95. IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer and UV recordings were made on a Perkin–Elmer Lambda 15 UV/VIS spectrophotometer.

3.2. Plant material

A collection of the plant sample was made at Wadi Al-Batin, in north-western Kuwait, bordering Iraq, in February 1999. A voucher specimen (KTM 4226, collected by K.T. Mathew and S. Gibbons on 19/2/1999) is deposited at the Kuwait University Herbarium (KTUH).

3.3. Extraction and isolation

The whole plant was air-dried for 3 days and ground to a fine powder. The powdered plant material (450 g) was sequentially extracted in a Soxhlet apparatus with hexane, chloroform and methanol (3 l each). Vacuum liquid chromatography (VLC) of the hexane extract (5.1 g) on silica gel using a step-gradient of 10% EtOAc in hexane followed by a methanol wash yielded 12 fractions. Fraction 7 (310 mg) was then subjected to LH-20 Sephadex column chromatography, eluting with dichloromethane followed by a methanol wash to give four fractions. Sephadex fraction 4 (58 mg) was then loaded onto four reverse phase TLC plates and developed twice with a 60:40 ACN-H_2O + AcOH (two drops) system to yield 15 mg of compound **1** (R_F: 0.21). VLC of the chloroform extract (10.5 g) using the same method described above again gave 12 fractions. Fraction 12 (1.5 g) was then fractioned further by LH-20 Sephadex column chromatography again using the same method as above to give 11 fractions. Solid phase extraction (C-18, SPE) of Sephadex fraction 10 (500 mg) using a step-gradient of 10% methanol in water yielded 11 fractions. SPE fractions 3 and 4 were combined (72 mg) and compound **2** was isolated by p-TLC using a 95:15 EtOAc–methanol + AcOH (two drops) solvent system. This afforded 27 mg of compound **2** (R_F: 0.32). Applying the same SPE protocol to Sephadex fraction 9 (425 mg) gave 11 fractions. Multiple development p-TLC in the normal phase mode (silica) of SPE fraction 9 (26 mg) using a 95:10 EtOAc–methanol + AcOH (two drops) (two developments) solvent system resulted in the isolation of compound **3** (10 mg; R_F: 0.44).

3.4. Antibacterial assay

S. aureus strain ATCC 25923 was a gift from E. Udo (Kuwait University, Kuwait), *S. aureus* strain SA-1199B, which overexpresses the *norA* gene encoding the NorA MDR efflux protein was a generous gift from G. Kaatz (Kaatz et al., 1993). Strain EMRSA-15 was provided by P. Stapleton. *Mycobacterium fortuitum* ATCC 6841, *Mycobacterium smegmatis* ATCC 14468, *Mycobacterium phlei* ATCC 11758 and *Mycobacterium aurum* Pasteur Institute 104482 were obtained from NTCC. The strains of *S. aureus* were cultured on nutrient agar and incubated for 24 h at 37 °C prior to MIC determination whilst the panel of rapidly growing mycobacteria were cultured on Columbia Blood agar (Oxoid) supplemented with 7% defibrinated Horse blood (Oxoid). *M. fortuitum*, *M. phlei* and *M. smegmatis* were incubated for 72 h and *M. aurum* for 120 h prior to MIC determination. Bacterial inocula equivalent to 5×10^5 cfu/ml were prepared in normal saline using the 0.5 McFarland turbidity standard followed by dilution. The MIC was recorded as the lowest concentration at which no bacterial growth was observed (Gibbons

and Udo, 2000). Norfloxacin was used as a positive control against all *S. aureus* strains and ethambutol was used as a positive control against all the mycobacterial strains. Growth and sterile controls were also performed.

3.5. 3(ζ)-Hydroxy-octadeca-4(E),6(Z)-dienoic acid (1)

Colourless oil. $[\alpha]_D^{24}$ 0° (*c* 0.2, $CHCl_3$). UV ($CHCl_3$): λ_{max}: ($\log \varepsilon$) 275 (2.90), 243 (3.56) nm. IR (film) υ_{max}: 3359, 2928, 2855, 1709, 1412, 1248, 985 cm^{-1}; ^1H NMR and ^{13}C NMR ($CDCl_3$): see Table 1; HR-CIMS (*m*/*z*): 296.2324 $[M]^+$ (calc. for $C_{18}H_{32}O_3$, 296.2352).

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