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Bacterial resistance modifying agents from *Lycopus europaeus*

Simon Gibbons[a,*], Moyosoluwa Oluwatuyi[a], Nigel C. Veitch[b], Alexander I. Gray[c]

[a]*Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK*

[b]*Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK*

[c]*Department of Pharmaceutical Sciences, University of Strathclyde, 27 Taylor Street, Glasgow G2 0NR, UK*

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Abstract

As part of an ongoing project to identify plant natural products which modulate bacterial multidrug resistance (MDR), bioassay-guided isolation of an extract of *Lycopus europaeus* yielded two new isopimarane diterpenes, namely methyl-1α-acetoxy-7α 14α-dihydroxy-8,15-isopimaradien-18-oate (**1**) and methyl-1α,14α-diacetoxy-7α-hydroxy-8,15-isopimaradien-18-oate (**2**). The structures were established by spectroscopic methods. These compounds and several known diterpenes were tested for in vitro antibacterial and resistance modifying activity against strains of *Staphylococcus aureus* possessing the Tet(K), Msr(A), and Nor(A) multidrug resistance efflux mechanisms. At 512 μg/ml none of the compounds displayed any antibacterial activity but individually in combination with tetracycline and erythromycin, a two-fold potentiation of the activities of these antibiotics was observed against two strains of *S. aureus* that were highly resistant to these agents due to the presence of the multidrug efflux mechanisms Tet(K) (tetracycline resistance) and Msr(A) (macrolide resistance).

Keywords: Lamiaceae; *Lycopus europaeus*; *Staphylococcus aureus*; Multidrug resistance; MDR; Modulation; Isopimarane diterpenes

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the major causes of nosocomial infections and until recently, all strains of MRSA were susceptible to the glycopeptides, such as vancomycin; however, intermediate resistance to this antibiotic has been reported (Sieradzki et al., 1999). Whilst new anti-staphylococcal agents such as linezolid offer some respite, resistance to this agent has been reported in vancomycin-resistant *Enterococcus faecium* (Gonzales et al., 2001) and it is likely that this pattern will emerge in the staphylococci. MRSA strains produce 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 that modify bacterial resistance in MRSA, we screened an extract from *Lycopus europaeus* L. (Lamiaceae), which in combination with either tetracycline or erythromycin reduced the minimum inhibitory concentrations (MICs) of these antibiotics against two strains of *S. aureus* possessing multidrug efflux pumps. *L. europaeus*, commonly known as Gipsywort in Britain, is a native perennial of river and canal banks (Philips, 1977) and is known to have anti-thyreotropic and anti-gonadotropic activities, which are attributed to phenolic compounds (Bucar and Kartnig, 1995). Previous phytochemical studies on the constituents of this species have focused on isopimarane type diterpenoids (Jeremic et al., 1985) and straight chain aliphatic precursors of cyclic diterpenes (Hussein and Rodriguez, 2000).

This paper deals with the isolation and structure elucidation of two new and four known compounds and their bacterial resistance modifying activity.

* Corresponding author. Tel.: +44-207-7535913; fax: +44-207-7535909.

E-mail address: simon.gibbons@ulsop.ac.uk (S. Gibbons).

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Table 1
^1H (400MHz) and ^{13}C NMR (100 MHz) spectral data for **1** and **2** in $CDCl_3$

Position	**1**		**2**	
	^1H (*J* in Hz)	^{13}C	^1H (*J* in Hz)	^{13}C
1	4.90 *t* (3.0)	72.9	4.84 *t* (2.8)	72.8
2	1.88 *m*	21.7	1.85 *m*	21.7
3	1.45 *m*	30.0	1.37 *m*	29.9
4		46.5		46.5
5	2.84 *dd* (13.2, 1.8)	35.0	2.80 *dd* (12.8, 1.6)	34.8
6	1.45, 1.83 *m*	29.6	1.37, 1.65 *m*	29.5
7	4.16 *br d* (3.5)	68.2	3.87 *br s*	66.8
8		130.2		128.4
9		142.3		144.6
10		42.0		42.3
11	1.82, 1.95 *m*	20.0	1.82, 1.95 *m*	20.1
12	1.45, 1.72 *m*	30.7	1.46, 1.66 *m*	31.4
13		39.9		39.3
14	3.87 *br s*	78.3	5.31 *s*	79.0
15	6.00 *dd* (18.5, 10.6)	142.2	5.90 *dd* (18.0, 11.2)	141.5
16	5.10 *dd* (10.6, 1.2)	114.6	4.98 *dd* (11.2, 0.8)	
	5.15 *dd* (18.5, 1.2)		4.99 *dd* (18.0, 0.8)	113.8
17	1.02 *s*	23.2	0.89 *s*	23.4
18		178.1		178.1
19	1.24 *s*	16.6	1.17 *s*	16.6
20	1.05 *s*	18.8	0.99 *s*	18.8
OAc	2.08 *s*	21.3, 170.7	2.02, 2.00 *s*	21.3, 21.3, 171.1, 170.7
OMe	3.71 *s*	52.2	3.64 *s*	52.2
OH	3.00 *br s*, 3.16 *br s*		2.11 *br s*	

2. Results and discussion

By VLC and reverse phase preparative HPLC compound (**1**) was isolated as a colourless crystalline solid. Accurate FAB-MS indicated a molecular formula of $C_{23}H_{34}O_6$ and characteristic signals in the ^1H NMR spectrum for one acetyl group (δ_H 2.08, s) and one methoxyl substituent (δ_H 3.71, s) suggested that (**1**) was a diterpene. Further signals in the ^1H and ^{13}C spectra (Table 1) for three tertiary methyl singlets [δ_H 1.02 (3H-17), 1.24 (3H-19) and 1.05 (3H-20)], an olefin (δ_H 6.00, H-15, dd, $J = 18.5$, 10.6 Hz), an exocyclic methylene (δ_H 5.10 and 5.15, 2H) and two additional olefinic carbons (δ_C 130.2, C-8, 142.3, C-9; Table 1) were typical of isopimarane diterpenes which have been previously isolated from this species (Hussein et al., 1999; Hussein and Rodriguez, 2000).

HMBC (Table 2) and COSY spectra were acquired to identify long-range ^1H–^{13}C and ^1H–^1H connectivities respectively. In the HMBC spectrum, methyl H-19 exhibited a ^2J correlation to C-4 and ^3J correlations to C-3, C-5 and to the carbonyl carbon of C-18 (δ_C 178.1). The methoxyl protons at δ_H 3.71 also showed a ^3J correlation to the C-18 carbonyl, indicating the presence of a methyl ester at C-18, which is a common feature of *Lycopus* diterpenes (Hussein et al., 1999). The proton attached to C-5 (δ_H 2.84, 1H, dd, $J = 13.2$, 1.8 Hz) showed a COSY correlation to H_2-6 (δ_H 1.45, 1.83, 2H, m) which further correlated to an oxymethine proton (H-7, 4.16, br d, $J = 3.5$ Hz) which in the HMBC spectrum, showed correlations to two olefinic carbons (C-8 and C-9). This C-9 carbon was correlated with the C-20 methyl, which exhibited additional correlations to C-10 (^2J), C-5 and C-1 (^3J). A proton attached to the carbon at C-1 was deshielded with respect to H-7 (δ_H 4.90, t, $J = 3.0$ Hz) and exhibited a ^3J correlation to a carbonyl carbon of the acetate group indicating that the acetate group should be placed at C-1. Further signals in the HMBC, COSY and DEPT-135 spectra of **1** showed correlations from H-1 to C-3 (CH_2) and H-1 to a methylene of C-2 indicating the presence of methylene groups at C-2 and C-3 and completing the A and B rings of the diterpene nucleus.

Methyl-17 displayed a correlation to a quaternary carbon (C-13, ^2J) and ^3J correlations to a methylene

Table 2
Long-range ^1H-^{13}C connectivities detected in HMBC experiments for **1** and **2**

Position	**1** Correlated C-atom HMBC (H→C)		**2** Correlated C-atom HMBC (H→C)	
	^2J	^3J	^2J	^3J
1	C-10	C-3, C-5, C=O		C-3, C-5
2	C-1	C-4		
3				
5	C-4, C-10	C-3, C-7, C-9, C-18, C-19, C-20	C-4, C-10	C-3, C-7, C-9, C-18, C-19, C-20
6	C-7	C-10	C-7	C-4, C-10
7	C-8	C-5, C-9		
11	C-9, C-12	C-8, C-13		C-8, C-10
12	C-11, C-13	C-15, C-17	C-13	C-9, C-14, C-15, C-17
14	C-8, C-13	C-9/C-15, C-17	C-8, C-13	C-9, C-12, C-15, C-17, C=O
15	C-13	C-12, C-14, C-17	C-13	C-14, C-17
16	C-15	C-13	C-15	C-13
17	C-13	C-12, C-14, C-15	C-13	C-12, C-14, C-15
19	C-4	C-3, C-5, C-18	C-4	C-3, C-5, C-18
20	C-10	C-1, C-5, C-9	C-10	C-1, C-5, C-9
OAc	C=O		C=O	
OMe		C-18		C-18

(C-12), an oxygenated methine carbon of C-14 ($\delta_C = 78.3$) and to an olefinic carbon (C-15, 142.2) whose attached proton coupled to the exocyclic methylene group in the COSY spectrum. Couplings between C-12 methylene and the allylic C-11 methylene in the COSY spectrum and ^2J and ^3J correlations between H_2-11 and C-9 and C-8 in the HMBC spectrum completed the isopimarane skeleton. From the molecular formula and the ^1H and ^{13}C chemical shifts of C-7 and C-14, hydroxyl groups must be placed at these positions. This was supported by the presence of two broad singlets (δ 3.00, 3.16) which are attributed to hydroxyl protons.

The relative stereochemistry of (**1**) was established by inspection of ^1H and NOESY spectra. The magnitude of the observed coupling constants for H-1 ($J = 3.0$ Hz) and H-7 ($J = 3.2$ Hz) indicated an equatorial (β) orientation for these protons and therefore that the acetate and hydroxyl groups at these positions be axial (α). In the NOESY spectrum a through-space interaction between H-7 and H-14 indicated that they were on the same face of the molecule (Fig. 1) and that the hydroxyl group at C-14 should also be axial (α). This was supported by a NOESY correlation between the proton at H-14 and methyl-17 indicating that they are on the same face and are both β. This is in agreement with the stereochemistry of related compounds isolated from this plant (Hussein et al., 1999). Compound (**1**) is therefore assigned as methyl-1α-acetoxy-7α,14α-dihydroxy-8,15-isopimaradien-18-oate.

By accurate FAB-MS, compound (**2**) solved for a molecular formula of $C_{25}H_{36}O_7$ and signals in the ^1H and ^{13}C NMR spectra were almost identical to those of (**1**) (Table 1) but with the presence of an additional acetate group. With respect to compound (**1**), H-14 (δ_H 5.31) was downfield shifted by 1.44 ppm indicating that esterification by the acetate had taken place at this position. This was corroborated by examination of the HMBC spectrum, which showed a correlation between H-14 and the carbonyl group of the acetate. Full HMBC analysis again led to the placement of acetate at C-1, and hydroxyl at C-7 of the isopimarane nucleus. The stereochemistry of (**2**) was identical to that of (**1**) with H-1 displaying a small coupling to H_2-2 (δ_H 4.84, $J = 2.8$ Hz), implying an equatorial (α) orientation for H-1. H-7 was a broad singlet and did not display any discernable coupling to H_2-6. As with compound (**1**), a NOESY experiment again showed correlations between H-7 and H-14 and between H-14 and methyl-17 indicating that compound (**2**) is methyl-1α,14α-diacetoxy-7α-hydroxy-8,15-isopimaradien-18-oate.

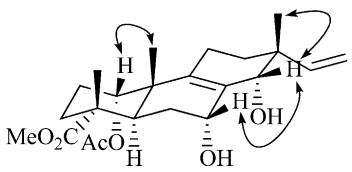
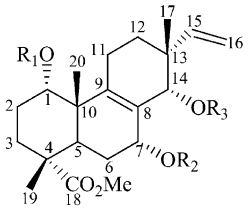
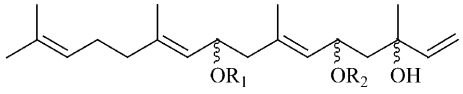


Fig. 1. Selected NOE connectivities for compound **1**.

Four additional compounds (**3**–**6**) were also isolated from the hexane extract and were identical to those isolated previously from *L. europaeus* on comparison of their ^1H and ^{13}C data with that published (Hussein et al., 1999; Hussein and Rodriguez, 2000). Compound **6** has previously been isolated from *Geigeria* species and data is in close agreement with that published (Bohlmann et al., 1982).



1 R_1 = Ac, R_2 = R_3 = H
2 R_1 = R_3 = Ac, R_2 = H
3 R_1 = R_2 = R_3 = Ac
4 R_1 = R_2 = Ac, R_3 = H



5 R_1 = R_2 = H
6 R_1 = R_2 = Ac

Compounds **1**–**6** showed no antibacterial activity at 512 μg/ml against any of the MDR strains of *S. aureus*. When each of the diterpenes were incorporated into the growth medium at low concentration (10 μg/ml), a two fold reduction in the MICs of tetracycline and erythromycin was observed against two strains which possessed the tetracycline Tet(K) and macrolide Msr(A) MDR efflux transporters respectively (Table 3). Surprisingly, there were no differences in activities of these diterpenes, despite the obvious structural differences between the groups **1**–**4** and **5** and **6**. These compounds modulated the activities of the antibiotics by halving the concentration of antibiotic needed to inhibit the growth of the drug resistant bacteria. Interestingly, no resistance

Table 3
MICs of test strains in the absence and presence of **1**–**6**[a] and reserpine

Strain (MDR efflux protein)	Tetracycline	Erythromycin	Norfloxacin[b]
IS-58 (TetK)	128, 64, **32**[c]	–	–
RN4220 (MsrA)	–	256, 128, **256**	–
SA-1199B (NorA)	–	–	32, 32, **2**

[a] 10 μg/ml. All MICs were determined in duplicate.
[b] MICs in the absence and presence of **1**–**6** are expressed in μg/ml.
[c] Figures in bold denote MICs in the presence of reserpine at 20 μg/ml.

modifying activity was observed against a strain possessing the Nor(A) efflux system, the major MDR transporter in *S. aureus*. The MDR inhibitor reserpine (Beck et al., 1988) caused a four-fold reduction in the MIC of tetracycline against the tetracycline-resistant strain and an eight-fold reduction in the MIC of norfloxacin against the fluoroquinolone (Nor(A)) resistant strain. Reserpine did not however affect the MIC of erythromycin against the erythromycin-resistant (Msr(A) producing) strain.

Previously discovered modulators of MDR in *S. aureus* have been described for strains possessing the Nor(A) efflux mechanism. These compounds are from many structural classes, including natural products such as flavonolignans, flavones and porphyrins (Stermitz et al., 2000a, b) and synthetic naphthylamides (Markham et al., 1999). A common feature of all of these resistance modifying agents is however, a high degree of lipophilicity which is also apparent in compounds **1**–**6**. This property is presumably important for interaction with the efflux proteins which are membrane bound. Further work is underway to find more potent and broad spectrum alternatives to counter the wide array of multidrug efflux mechanisms produced by *S. aureus*.

3. Experimental section

3.1. General experimental procedures

Melting points were determined on a Gallenkamp apparatus. Optical rotations were measured on a Bellingham and Stanley ADP 200 polarimeter. UV spectra were recorded on a Perkin-Elmer UV/vis spectrophotometer and IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in $CDCl_3$ on Bruker DRX, AVANCE and AMX 400 spectrometers. Chemical shift values (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS $\delta = 0$) as internal standard and coupling constants (*J* values) are given in Hertz. ^{1}H–^{1}H COSY, HMBC and HSQC experiments were recorded with gradient enhancements using sine shaped gradient pulses. FAB Mass spectra were recorded on a VG ZAB-SE instrument. Vacuum liquid chromatography on Merck Si gel 60 $PF_{254+366}$ and preparative HPLC using Waters Delta 600 were used for fractionation and isolation. TLC was carried out on Kieselgel 60 F_{254} (Merck) pre-coated plates and spots were visualized by spraying with Vanillin-Sulphuric acid followed by heating.

3.2. Plant material

Plant material used in this study was collected in September 2000 from Wilstone, Hertfordshire, UK on the verge of the Aylesbury arm of the Grand Union Canal and a voucher specimen SG01/09/2000 has been deposited at the Centre for Pharmacognosy and Phytotherapy at the University of London School of Pharmacy.

3.3. Extraction and isolation

Air-dried and powdered herb (450 g) was exhaustively extracted in a Soxhlet apparatus with a series of solvents (3 l) in order of increasing polarity (hexane, chloroform, ethyl acetate, acetone and methanol). Extracts were dried under vacuum in a rotary evaporator. Vacuum liquid chromatography (VLC) was carried out on the hexane extract (23.6 g) with silica gel using a step gradient system with 10% increments from 100% hexane to 100% EtOAc and finally 10% methanol in EtOAc yielding 12 fractions. Preparative reverse phase HPLC (on two coupled 40 × 100 mm 6 μm Nova-Pak HR C_{18} columns) using a gradient system from 100% water to 100% acetonitrile over 30 min was carried out on VLC fractions 8 (862.1 mg) and 10 (272.4 mg) yielding compounds **1** (57.2 mg) and **2** (11.4 mg) respectively.

3.4. 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). Strain IS-58, 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). All strains were cultured on nutrient agar (Oxoid) and incubated for 24 h at 37 °C prior to MIC determination.

3.5. 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 18 h

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.

In the case of the modulation assay, diterpenes and reserpine were dissolved in DMSO and diluted into MHB to give final concentrations of 10 and 20 μg/ml, respectively. This medium was then used in the minimum inhibitory concentration assay.

3.6. Methyl-1α-acetoxy-7α,14α-dihydroxy-8,15-isopimaradien-18-oate (*1*)

Colourless crystals; mp 150–153 °C; $[\alpha]_D^{25}$ +0.08 ($CHCl_3$; *c* 0.12); UV λ_{max} (MeOH) nm (log ε): 217 (2.22); IR ν_{max} (thin film) cm^{-1}: 2950,1723, 1645, 1434, 1372, 1241, 1028, 754; ^1H NMR and ^{13}C NMR ($CDCl_3$): see Table 1; HRFAB-MS (*m*/*z*): 429.2268 $[M+Na]^+$ (calcd. for $C_{23}H_{34}O_6Na$, 429.2253).

3.7. Methyl-1α,14α-diacetoxy-7α-hydroxy-8,15-isopimaradien-18-oate (*2*)

Pale yellow oil; $[\alpha]_D^{25}$−0.01 ($CHCl_3$; *c* 0.8); UV λ_{max} (MeOH) nm (log ε): 215 (2.16); IR ν_{max} (thin film) cm^{-1}: 3409, 2931, 1723, 1641, 1433, 1372, 1234; 1145, 1028, 965, 751; ^1H NMR and ^{13}C NMR ($CDCl_3$): see Table 1; HRFAB-MS (*m*/*z*): 581.1538 $[M+Cs]^+$ (calcd. for $C_{25}H_{36}O_7Cs$, 581.1515).

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