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Identification and biological evaluation of grapefruit oil components as potential novel efflux pump modulators in methicillin-resistant *Staphylococcus aureus* bacterial strains

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) and MSSA strains were treated with: (a) grapefruit oil (GFO) components, isolated by chromatography and characterised by NMR and mass spectroscopy; (b) antimicrobial agents, or (c) a combination of both to evaluate (MIC determination) intrinsic antibacterial activity and to determine whether GFO components could modulate bacterial sensitivity to the anti-bacterial agents. Preliminary data suggested that the grapefruit component 4-{[(*E*)-5-(3,3-dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-7*H*-furo[3,2-*g*]chromen-7-one (**2**) enhances the susceptibility of test MRSA strains to agents, e.g., ethidium bromide and norfloxacin, to which these micro-organisms are normally resistant.

Keywords: Grapefruit oil; Efflux pump modulators; 4-{[(*E*)-5-(3,3-Dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-7*H*-furo[3,2-*g*]chromen-7-one (bergamottin epoxide); MRSA and MSSA strains

1. Introduction

The constant use of antibiotics in the hospital environment has selected bacterial populations that are resistant to many antibiotics. In particular, many strains of *Staphylococcus aureus* are developing increasing resistance to available antibacterial agents (methicillin-resistant *S. aureus*, MRSA) producing a serious problem in medical microbiology (Herwaldt, 1999).

Efflux pumps are widely involved in antibiotic resistance. Different pumps can efflux specifically a drug or group of drugs, such as the NorA system that transports quinolones (Poole, 2000), or TetA that transports tetracyclines (Levy, 2002), or they can efflux a large variety of molecules, such as certain efflux pumps of *Pseudomonas aeruginosa*, or the multidrug efflux pump found in mammalian cells, P-gp, or MsrA efflux pump specific for macrolides in *S. aureus* (Neyfakh et al., 1993).

Multidrug transporters can either be ATP hydrolysis dependent export mediated multidrug resistance such as P-gp, or driven by the proton-motive force (PMF) of the transmembrane electrochemical proton gradient ($\Delta\mu H^+$) (Paulsen et al., 1996). These proton-dependent multidrug transporters share no detectable sequence similarity with P-gp, but they do share transport of common substrates. The transmembrane electrochemical proton gradient ($\Delta\mu H^+$) is composed of a chemical gradient of hydrogen ions (ΔpH) and an electrical charge gradient

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($\Delta\Psi$). Either or both ΔpH and $\Delta\Psi$ components of the PMF are capable of driving drug efflux. PMF-dependent efflux proteins belong to one of three families of proteins: the major facilitator superfamily (MFS), the resistance nodulation cell division family (RND), and the small multidrug resistance family (SMR) (for review see Paulsen et al., 1996).

Several proteins belong to the 12 transmembrane segments (12TMS) subfamily of MFS, including the yeast and fungal multidrug efflux protein CaMDR1, two bacterial chloramphenicol resistance proteins Cm1A and Cm1B, Gram-negative bacteria tetracycline efflux proteins TetA-E, and gram-positive bacteria multidrug resistance efflux proteins Blt, Bmr, and NorA (Paulsen et al., 1996).

An "efflux pump inhibitor" can be defined as a potentiator or modulator which specifically interferes with the ability of an efflux pump to export antibiotics, but might also interfere with exporting normal (physiological) substrates of that pump. Such modulators can be used to enhance the activity of antibacterial agents whose clinical efficacy has been limited by the increasing prevalence of resistant strains. An example is the NorA membrane-associated multidrug efflux protein, which can decrease susceptibility to fluoroquinolones in *S. aureus*. NorA inhibition can increase fluoroquinolone killing activity and also the post-antibiotic effect following removal of the drug. However, studies with the alkaloid reserpine, the first identified inhibitor of NorA, indicate that reserpine is toxic to humans at the concentrations required to inhibit NorA. More durable and potent NorA inhibitor compounds are needed that can improve killing activity and prevent resistance (Schmitz et al., 1998).

Grapefruit oil (GFO) is a complex mixture of chemicals extracted from the peel of grapefruit with one of its major constituents being the monoterpene hydrocarbon, limonene. GFO contains some of the grapefruit juice (GFJ) components concentrated thousands of times and, owing to its strong fruity fragrance, GFO is often added as a flavour enhancer during commercial preparation of GFJ concentrate. The significance of furanocoumarins present in GFO on CYP3A4 inhibition has recently been reported (Schmiedlin-Ren et al., 1997). However, the effect of GFO and its compounds on efflux pump activity to date remains unexplored.

The objectives of this current study were to evaluate the susceptibility of MSSA and MRSA strains to antibacterial agents in the presence of GFO isolates and to determine both intrinsic antibacterial activity and modulating effect of the GFO components.

2. Results and discussion

2.1. GFO isolation and characterisation

The GFO used in this preliminary study was a complex mixture of components. Purification of GFO by flash column chromatography resulted in the isolation of the 'oil' (presumed to be limonene and other similarly hydrophobic components) when eluting with petroleum ether. Three other major components (Fig. 1) were isolated by flash column chromatography: a coumarin derivative (**1**) eluted with petroleum ether–ethyl acetate 9:1 v/v, and an inseparable 2:1 mixture of a bergamottin epoxide derivative (**2**) and a coumarin epoxide derivative (**3**), eluted with petroleum ether–ethyl acetate 4:1 v/v. By TLC the oil was examined for the presence of quercetin and naringenin, two potential modulators of P-gp. However, these flavanoids were not found to be present in the oil, or the quantities too low to be detected by this method of analysis.

The individual components of GFO, including the inseparable mixture (**2** and **3**), were identified by ^{1}H

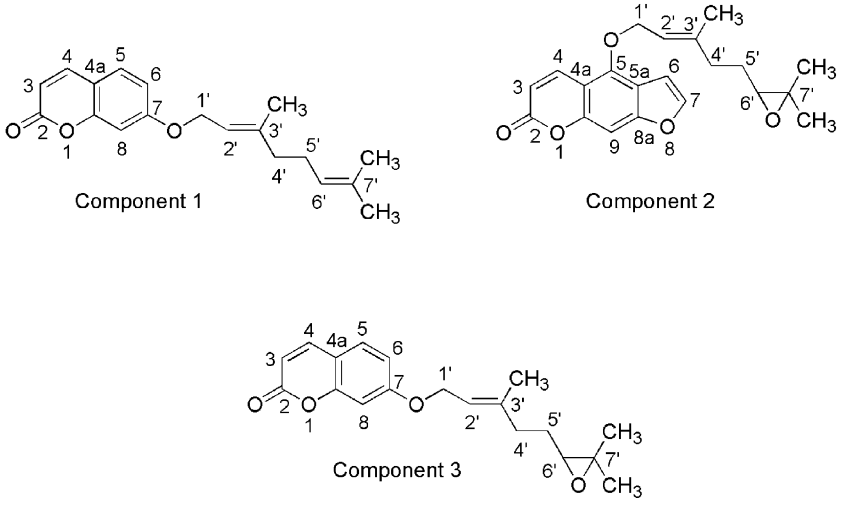

Fig. 1. Structures, with NMR numbering, of isolated GFO components (**1**), (**2**) and (**3**).

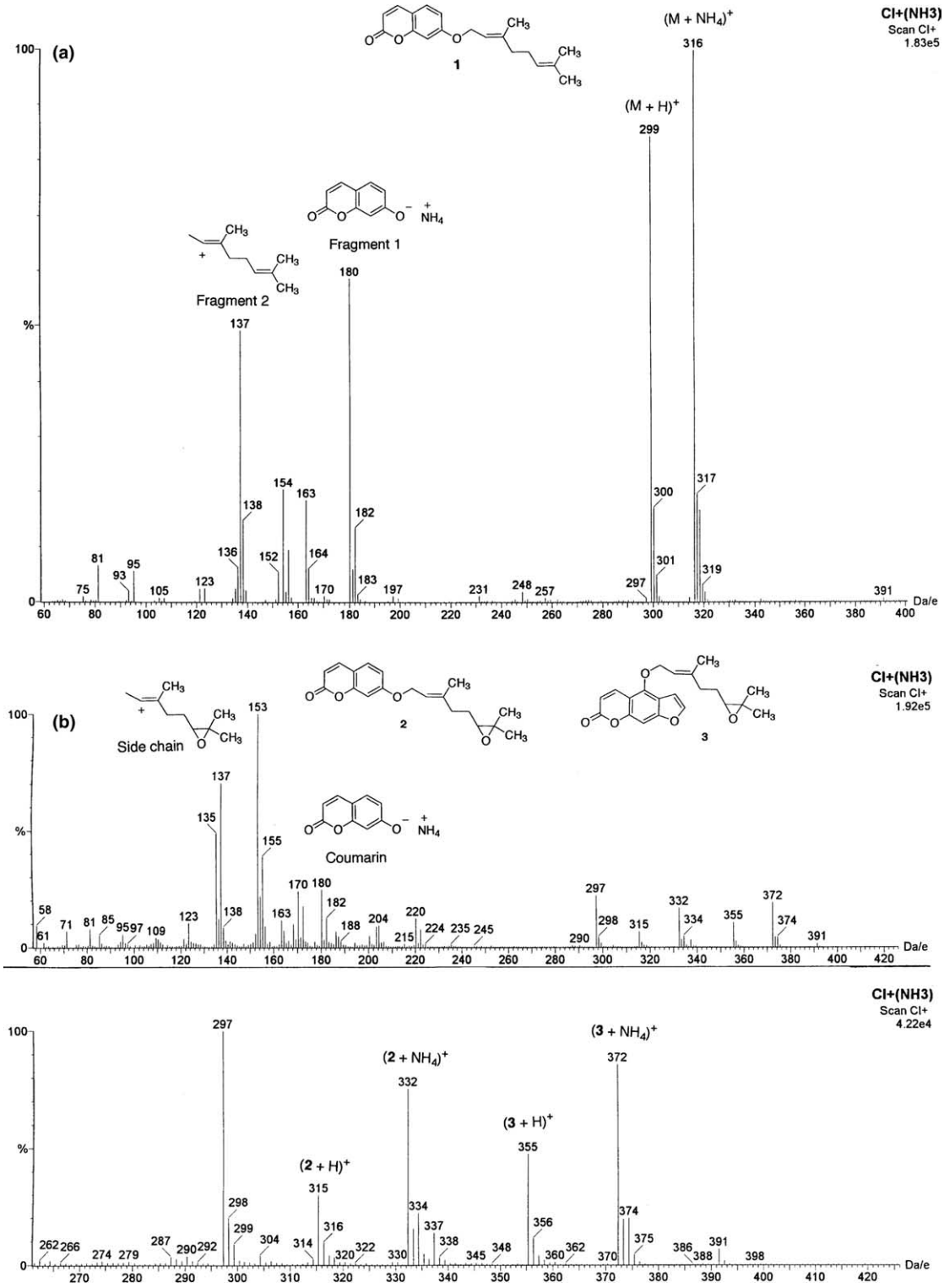

Fig. 2. Mass spectroscopy data for: (a) coumarin (**1**); (b) the inseparable mixture of (**2**) and (**3**).

and ^{13}C NMR and by mass spectroscopy with further confirmation achieved by comparison with synthesised components. Fig. 1 shows the structures (with NMR numbering) of components **1**, **2** and **3** isolated from GFO.

Fig. 2 shows the mass spectrometry for component **1** (Fig. 2(a)) and the inseparable mixture of components **2** and **3** (Fig. 2(b)). Component **1** was identified as the 7-*O*-geranyl-coumarin, 7-{[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy}-2*H*-2-chromenone. Component **2** was identified

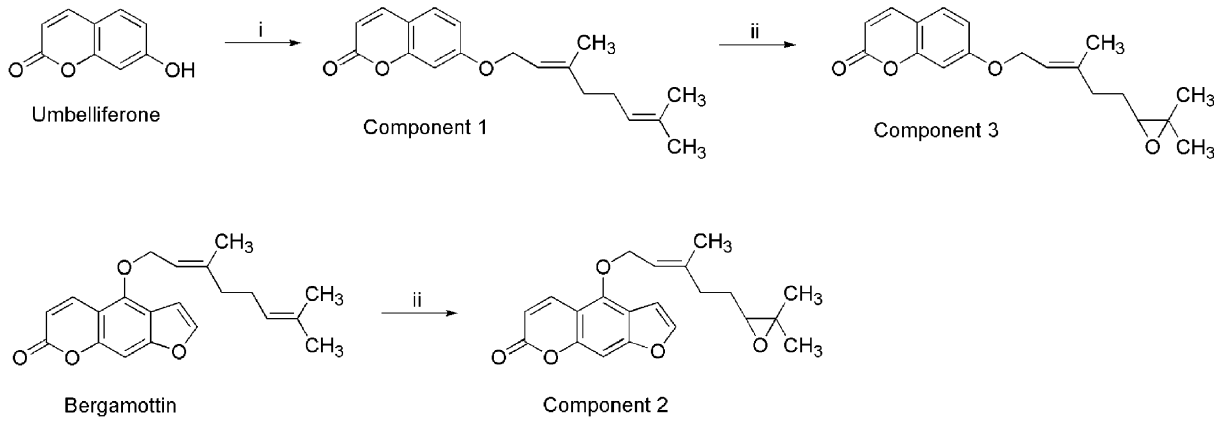

Fig. 3. (i) Geranyl bromide, K_2CO_3, acetone, reflux, 3 h. (ii) *m*-CPBA, CH_2Cl_2, 2 h.

as bergamottin epoxide, 4-{[(*E*)-5-(3,3-dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-7*H*-furo[3,2-*g*]chromen-7-one. Component **3** was identified as 7-{[(*E*)-5-(3,3-dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-2*H*-2-chromenone.

The structures of component **2** and **3** were further confirmed by spectroscopic comparison with synthesised compounds (Fig. 3).

2.2. MRSA and MSSA strains

MRSA strains were chosen for evaluation because they are considered to be a significant medical problem. MRSA are believed to contain the NorA transporter, which mediates transport of ethidium and norfloxacin and is dependent on the PMF and driven by ΔpH.

The toxicity of the compounds alone was first examined. None of the three isolates (components **1**, **2** and **3**) was growth-inhibitory up to concentrations of 100 μM, i.e., 30, 35.7 and 31.4 mg/L, respectively, and for GF oil up to 0.1% v/v, whereas the efflux energy blocker carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was inhibitory at a concentration of 0.75 mg/L.

Studies were performed with ethidium bromide, which is known to be effluxed by the MRSA strains used in this study, thus making MRSA strains resistant to this dye whereas MSSA strains used in this study were not affected. In the MSSA strains the CCCP, GF oil or the individual GF components did not modulate ethidium bromide MIC (2 μg/ml for all treatment combinations). The combination of bergamottin epoxide (**2**) and ethidium bromide in different MRSA strains resulted in reduction of the MIC by 6-fold, whereas the same combination did not affect the toxicity in MSSA, suggested the effect of **2** was specific.

The results showed that the GFO isolate bergamottin epoxide (**2**) reduced the MICs in MRSA strains 9543 and 7 by 6-fold compared with CCCP which caused a 25-fold reduction. Bergamottin epoxide (**2**) displayed comparable modulating activity to the de-energising agent CCCP in the clinical MRSA isolate 50325 with a 6-fold reduction in MIC when combined with ethidium bromide (Table 1).

Enhancement of ethidium bromide toxicity would not be beneficial from a clinical point of view therefore enhancement of antibiotic killing activity using norfloxacin was determined. Results obtained with this antibiotic showed no enhancement of susceptibility in MSSA strains whereas a 20-fold reduction in MIC was obtained with a combination of norfloxacin and either of the GFO isolates (**2**) or (**3**) (Table 2).

Compared with grapefruit isolates **2** and **3**, which decreased the MIC of norfloxacin 20-fold in the resistant *S. aureus* strains (Table 2), other NorA inhibitors (10

Table 1
MICs in MRSA strains to ethidium bromide in the presence and absence of modulators

Ethidium bromide with	MRSA strains 9543 and 7		MRSA strain 50325	
	MIC (μg/ml)	Relative MICs[b]	MIC (μg/ml)	Relative MICs[b]
–[a]	100	1	25	1
CCCP (0.75 mg/L)	4	0.04	4	0.16
GF oil (0.1%)	100	1	25	1
1 (30 mg/L)	50	0.5	6	0.32
2 (35.7 mg/L)	17.5	0.175	4	0.16
3 (31.4 mg/L)	50	0.5	8	0.32

[a] Ethidium bromide alone.
[b] In relation to ethidium bromide alone.

Table 2
MICs of MRSA strains to norfloxacin in the presence and absence of the modulators

Norfloxacin combined with	MRSA strains			
	16565 MIC (μg/ml)	9543 MIC (μg/ml)	5 MIC (μg/ml)	7 MIC (μg/ml)
–[a]	>100	>100	>100	>100
CCCP (0.75 mg/L)	1	1	1	1
2 (35.7 mg/L)	5–10	5–10	5–10	5–10
3 (30 mg/L)	5–10	5–10	5–10	5–10

[a] Norfloxacin alone.

mg/L) had reduced modulating activity; reserpine decreased the MIC of norfloxacin 16-fold, omeprazole decreased the MIC 8-fold, verapamil produced only a 2-fold decrease and cyclosporin A had no activity. It is interesting to note that the NorA inhibitors used in that study decreased the MIC even in the wild type (Aeschlimann et al., 1999).

This is a significant result and demonstrates that the MIC of norfloxacin, and potentially other fluoroquinolones, could be significantly reduced by the addition of the epoxides **2** and **3**.

Preliminary evidence has been obtained to demonstrate that GFO fractions, in particular bergamottin epoxide (**2**), increased the activity of ethidium bromide and norfloxacin against MRSA strains but not against the MSSA strains. A possible reason for this enhanced effect is a reduction in efflux in MRSA strains. The GFO components identified and synthesised in this present report, namely the bergamottin and coumarin epoxides (**2** and **3**), have also been characterised by their ability to maintain and restore the intracellular accumulation of P-gp substrates within MDR positive cell lines (Abulrob et al., 2001). Therefore they potentially have a broad application for the enhancement of therapeutic agents used for both bacterial infections and MDR solid tumours.

3. Experimental

3.1. General

GFO was obtained from Alexander Essentials (London, UK); bergamottin was obtained from Indofine Chemical company (USA); 6′,7′-dihyrdoxorubicinybergamottin was prepared according to a literature procedure (Dryer and Huey, 1973). Ethidium bromide and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemicals, Poole, Dorset, UK. All other chemicals used were of the highest grade available. ^{1}H and ^{13}C NMR experiments were performed on a Bruker Avance DPX300 spectrometer operating at 300 and 75 MHz, respectively. Mass spectroscopy (CI technique) was performed at the EPSRC National Mass Spectrometry Service Centre, University of Wales Swansea, UK. Flash column chromatography employed silica gel 60 (230–400 mesh, Merck), thin layer chromatography (TLC) employed precoated silica plates (Kielsel gel 60 F_{254}, BDH Chemicals, Poole, UK), with visualisation by UV and by charring with vanillin stain. All *S. aureus* strains were obtained from St. Thomas Hospital (London).

3.2. Isolation and structural confirmation of active components from GFO

Purification and isolation of the components from GFO was achieved by flash column chromatography using petroleum ether–ethyl acetate as the mobile phase, and monitoring by TLC. The individual components of GFO were identified by ^{1}H and ^{13}C NMR and by mass spectroscopy with comparison to the literature. Confirmation was also achieved by spectroscopic comparison to synthesised components.

7-{[(2E)-3,7-Dimethyl-2,6-octadienyl]oxy}-2H-2-chromenone (***1***): ^{1}H NMR ($CDCl_3$) δ 7.69 (1H, *d*, $J_{4,3}$ = 9.5 Hz, H-4), 7.41 (1H, *d*, $J_{5,6}$ = 8.5 Hz, H-5), 6.90 (1H, *dd*, $J_{6,8}$ = 2.4 Hz, $J_{6,5}$ = 8.5 Hz, H-6), 6.87 (1H, *d*, $J_{8,6}$ = 2.3 Hz, H-8), 6.29 (1H, *d*, $J_{3,4}$ = 9.5 Hz, H-3), 5.52 (1H, *dt*, *J* = 1.0 Hz, $J_{2',1'}$ = 6.5 Hz, H-2′), 5.13 (1H, *m*, H-6′), 4.65 (2H, *d*, $J_{1',2'}$ = 6.5 Hz, H-1′), 2.26 (4H, *m*, H-4′ and H-5′), 1.81 (3H, *s*, vinyl CH_3), 1.72 (3H, *s*, CH_3), 1.65 (3H, *s*, CH_3); ^{13}C NMR ($CDCl_3$) δ 162.57 (C, C-7), 161.77 (C=O, C-2), 152.28 (C, C-8a), 143.91 (CH, C-4), 142.79 (C, C-3′), 132.38 (C, C-7′), 129.11 (CH, C-5), 124.03 (CH, C-6′), 118.82 (CH, C-2′), 113.67 (CH, C-6), 113.36 (CH, C-3), 112.84 (C, C-4a), 102.00 (CH, C-8), 65.91 (CH_2, C-1′), 39.94 (CH_2, C-4′ or C-5′), 30.13 (CH_2, C-4′ or C-5′), 29.79 and 18.14 (2× CH_3, $C(CH_3)_2$), 17.20 (CH_3, vinyl CH_3); CIMS *m/z* 316 $(M + NH_4)^+$, 299 $(M + H)^+$, 180 (fragment 1)$^+$, 137 (fragment 2)$^+$ (see Fig. 2(a)).

4-{[(E)-5-(3,3-Dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-7H-furo[3,2-g]chromen-7-one, bergamottin epoxide (***2***): ^{1}H NMR ($CDCl_3$) δ 8.21 (1H, *d*, $J_{4,3}$ = 9.8 Hz, H-4), 7.65 (1H, *d*, $J_{7,6}$ = 2.4 Hz, H-7), 7.21 (1H, *s*, H-9), 7.01 (1H, *dd*, *J* = 0.9 Hz, $J_{6,7}$ = 2.4 Hz, H-6), 6.33 (1H, *d*, $J_{3,4}$ = 9.8 Hz, H-3), 5.65 (1H, ψt, *J* = 1.2 Hz, $J_{2',1'}$ = 6.8 Hz, H-2′), 5.01 (2H, *d*, $J_{1',2'}$ = 6.8 Hz, H-1′), 2.76 (1H, *dd*, *J* = 5.5, 6.8 Hz, H-6′), 2.28 (2H,

m, H-4′ or H-5′), 1.78 (3H, *s*, vinyl CH_3), 1.71 (2H, *m*, H-4′ or H-5′), 1.36 (3H, *s*, CH_3), 1.33 (3H, *s*, CH_3); ^{13}C NMR ($CDCl_3$) δ 161.68 (C=O, C-2), 158.53 (C, C-8a), 156.25 (C, C-9a), 145.38 (CH, C-7), 142.52 (C, C-5), 141.86 (C, C-3′), 139.97 (CH, C-4), 119.86 (CH, C-2′), 113.00 (CH, C-3), 112.90 (C, C-5a), 107.85 (C, C-4a), 105.45 (CH, C-6), 94.64 (CH, C-9), 69.99 (CH_2, C-1′), 64.20 (CH, C-6′), 58.79 (C, C-7′), 36.70 (CH_2, C-4′ or C-5′), 27.53 (CH_2, C-4′ or C-5′), 25.26 and 19.19 (2× CH_3, $C(CH_3)_2$), 17.13 (CH_3, vinyl CH_3); CIMS *m*/*z* 372 $(M + NH_4)^+$, 355 $(M + H)^+$, 153 (side chain)$^+$ (see Fig. 2(b)).

7-{[(E)-5-(3,3-Dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-2H-2-chromenone (***3***): ^1H NMR ($CDCl_3$) δ 7.68 (1H, *d*, $J_{4,3} = 9.5$ Hz, H-4), 7.41 (1H, *d*, $J_{5,6} = 8.5$ Hz, H-5), 6.88 (1H, *dd*, $J_{6,8} = 2.4$ Hz, $J_{6,5} = 8.5$ Hz, H-6), 6.85 (1H, *d*, $J_{8,6} = 2.3$ Hz, H-8), 6.28 (1H, *d*, $J_{3,4} = 9.5$ Hz, H-3), 5.56 (1H, *t*, $J_{2',1'} = 6.5$ Hz, H-2′), 4.65 (2H, *d*, $J_{1',2'} = 6.5$ Hz, H-1′), 2.76 (1H, *t*, $J_{6',5'} = 6.2$ Hz, H-6′), 2.29 (2H, *m*, H-5′), 1.82 (3H, *s*, vinyl CH_3), 1.74 (2H, *q*, $J_{4',5'} = 7.3$ Hz, H-4′), 1.34 (3H, *s*, CH_3), 1.31 (3H, *s*, CH_3); ^{13}C NMR ($CDCl_3$) δ 162.46 (C, C-7), 161.70 (C=O, C-2), 156.26 (C, C-8a), 143.89 (CH, C-4), 141.87 (C, C-3′), 129.16 (CH, C-5), 119.45 (CH, C-2′), 113.59 (CH, C-6), 113.42 (CH, C-3), 112.90 (C, C-4a), 101.96 (CH, C-8), 65.75 (CH_2, C-1′), 64.28 (CH, C-6′), 58.83 (C, C-7′), 36.65 (CH_2, C- C-5′), 27.49 (CH_2, C-4′), 25.25 and 19.18 (2× CH_3, $C(CH_3)_2$), 17.20 (CH_3, vinyl CH_3); CIMS *m*/*z* 332 $(M + NH_4)^+$, 315 $(M + H)^+$, 314 $(M)^+$, 180 (coumarin)$^+$, 153 (side chain)$^+$ (see Fig. 2(b)).

3.3. Synthesis of active components from GFO

The chemical scheme for the synthesis of the individual major components (components **1**, **2** and **3**) isolated from GFO is shown in Fig. 3.

Preparation of 4-{[(E)-5-(3,3-dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-7H-furo[3,2-g]chromen-7-one (component ***2***): To a solution of bergamottin (15 mg, 0.044 mmol) in dry dichloromethane (1 mL) was added *meta*-chloro-peroxybenzoic acid (8.2 mg, 0.047 mmol) and the reaction stirred at room temperature under nitrogen overnight. The reaction mixture was diluted with dichloromethane (2 mL), washed with aqueous $NaHCO_3$ (2 mL), then the dichloromethane solution dried ($MgSO_4$) and concentrated under reduced pressure. Purification was undertaken by column chromatography (petroleum ether–ethyl acetate 4:1 v/v), collection and evaporation of the fractions containing pure material, gave the product (**2**) as a yellow oil. Yield: 11.8 mg (75%).

Preparation of 7-{[(E)-5-(3,3-dimethyl-2-oxiranyl)-3-methyl-2-pentenyl]oxy}-2H-2-chromenone (***3***): To a solution of 7-{[(2*E*)-3,7-dimethyl-2,6-octadienyl]oxy}-2*H*-2-chromenone (**1**) (50 mg, 0.17 mmol in dry dichloromethane – 2 mL), readily prepared by the reaction of umbelliferone and geranyl bromide in anhydrous acetone in the presence of potassium carbonate as the base, was added *meta*-chloro-peroxybenzoic acid (31 mg, 0.18 mmol) and the reaction stirred at room temperature under nitrogen for 2 h. The reaction mixture was diluted with dichloromethane (3 mL), washed with aqueous $NaHCO_3$ (3 mL) then the dichloromethane solution dried ($MgSO_4$) and concentrated under reduced pressure to give a pale yellow waxy solid, crude yield: 53 mg. Purification was undertaken by column chromatography (petroleum ether–ethyl acetate 4:1 v/v), and collection and evaporation of the fractions containing pure material, gave the product (**3**) as a pale yellow solid. Yield: 28 mg (53%).

3.4. Bacterial strains

The bacterial strains consisted of *S. aureus* (Oxford – NCTC 6571) and ATCC 83254, both of which were antibiotic-sensitive. MRSA clinical isolates strains 5, 7, 9543, 50325 and 16565 were obtained from appropriate sources. Their antibiotic susceptibility and resistance were described earlier (Suller and Russell, 1999, 2000).

3.5. MIC determination

MIC determinations were undertaken for the test compound alone, antimicrobial agent alone and a combination of the two to determine intrinsic antibacterial activity. The agar plates were autoclaved and allowed to cool under sterile conditions. Following this the chemical agents were aseptically incorporated into molten agar at about 40 °C and plates allowed to dry. The MIC for the combination of ethidium bromide and each of the agents was determined and compared with the combination of CCCP (0.75 mg/L) and ethidium bromide. The range of ethidium bromide concentration used was 0–100 mg/L. CCCP was dissolved in absolute ethanol with a final experimental concentration of 1% v/v. This final concentration of ethanol had no deleterious effect on bacterial growth.

Bacterial strains were grown overnight in Tryptone Soya Broth in a shaking water bath operating at 100 oscillations/min at 37 °C. One microliter of an overnight culture (diluted 10-fold), equivalent to ca. 5×10^4 cfu/spot was used to inoculate the surface of a Nutrient agar (Oxoid) plate using a Denley multipoint inoculator (Denley, Billinghurst, UK). Plates were incubated at 37 °C for 24 h and the MIC taken as the lowest concentration that inhibited growth.

Acknowledgements

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