Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 210–217
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*

Eileen C.J. Smith [a], Elizabeth M. Williamson [b], Neale Wareham [c], Glenn W. Kaatz [d], Simon Gibbons [a,*]

[a] *Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK*
[b] *Department of Pharmacy, University of Reading, Whiteknights, Reading, Berks RG6 6AJ, UK*
[c] *Stiefel International R&D, Whitebrook Park, 68 Lower Cookham Road, Maidenhead, Berkshire SL6 8XY, UK*
[d] *Department of Internal Medicine, Division of Infectious Diseases, School of Medicine, Wayne State University and the John D. Dingell Department of Veterans Affairs Medical Center, Detroit, MI 48201, USA*

Received 13 July 2006; received in revised form 29 September 2006
Available online 15 November 2006

Abstract

As part of an on-going project to characterize compounds from immature conifer cones with antibacterial or modulatory activity against multidrug-resistant (MDR) strains of *Staphylococcus aureus*, eight compounds were isolated from the cones of *Chamaecyparis lawsoniana*. The active compounds were mainly diterpenes, with minimum inhibitory concentrations ranging from 4 to 128 μg/ml against MDR effluxing *S. aureus* strains and two epidemic methicillin-resistant (EMRSA) clinical isolates. The compounds extracted were the diterpenes ferruginol, pisiferol and its epimer 5-epipisiferol, formosanoxide, *trans*-communic acid and torulosal, the sesquiterpene oplopanonyl acetate and the germacrane 4β-hydroxygermacra-1(10)-5-diene. Some of these compounds also exhibited modulatory activity in potentiating antibiotic activity against effluxing strains and ferruginol, used at a sub-inhibitory concentration, resulted in an 80-fold potentiation of oxacillin activity against strain EMRSA-15. An efflux inhibition assay using an *S. aureus* strain possessing the MDR NorA efflux pump resulted in 40% inhibition of ethidium bromide efflux at 10 μM ferruginol (2.86 μg/ml). We report the ^{1}H and ^{13}C NMR data for the *cis* A/B ring junction epimer of pisiferol which we have named 5-epipisiferol. We also unambiguously assign all ^{1}H and ^{13}C NMR resonances for *trans*-communic acid.

Keywords: *Chamaecyparis lawsoniana*; Diterpene; MRSA; Antibacterial; Multidrug-resistant; Efflux pump; Modulator; Ferruginol

1. Introduction

Multidrug-resistant (MDR) staphylococci have become a major health risk, in terms of both nosocomial and community-acquired infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been headline news in the UK for the past few years, resulting in considerable public awareness of the potentially lethal consequences of an MRSA infection. The latest figures released by the Office for National Statistics reveal that in England and Wales, the number of death certificates citing MRSA has risen from 669 in 2000 to 1,168 in 2004 (Office for National Statistics, 2006).

Some *S. aureus* strains exert their resistance by means of an efflux pump in the cell membrane, examples being TetK which effluxes certain tetracyclines and the MDR pump NorA which removes certain fluoroquinolones and other compounds including quaternary ammonium compounds (QACs). In this study, compounds were isolated from the immature cones of *Chamaecyparis lawsoniana* and assayed for anti-staphylococcal activity against a standard ATCC strain and five drug-resistant clinical isolates. These included a strain which not only has the TetK pump but is also an MRSA strain; two other effluxing strains, one

* Corresponding author. Tel.: +44 207 7535913; fax: +44 207 7535909.
E-mail address: simon.gibbons@pharmacy.ac.uk (S. Gibbons).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.10.001

with the MsrA macrolide pump, the other strain with NorA; and the epidemic MRSA strains EMRSA-15 and EMRSA-16 which account for the majority of MRSA bacteraemias in UK hospitals (Johnson et al., 2001).

Chamaecyparis lawsoniana (Murray) Parlatore, also known as Lawson's Cypress or Port-Orford cedar is a native tree of North America. It is found in coastal and mountainous regions of southwest Oregon and northern California (Vidaković, 1991). There is very little reported on the use of *Chamaecyparis* species in traditional medicine. The Southern Kwakiutl Indians of British Columbia used the leaves, branch tips and bark of *C. nootkatensis* to treat sores, arthritis and rheumatism (Turner, 1979), but the Salish people of British Columbia consider that illness could result from inhaling the strong odour of *Chamaecyparis* (Turner, 1988). However, all species have hard, aromatic wood which is highly prized and has been used by Native American peoples to make bows, canoe paddles and dishes (Turner, 1979). In Japan, the wood of *C. obtusa* is valued for use in the construction of important buildings such as temples and shrines and is also considered to have hygienic properties for use as counter tops in sushi bars (Koyama et al., 1997).

Several species of *Chamaecyparis* have been shown to possess insecticidal activity. Termiticidal activity has been reported for the heartwood of *C. lawsoniana* (McDaniel, 1989) and seed extracts of this species exhibited juvenilising activity against the yellow mealworm beetle *Tenebrio molitor* (Jacobson et al., 1975). Antibacterial properties have been cited for *Chamaecyparis* (Johnson et al., 2001; Xiao et al., 2001;Yatagai and Nakatani, 1994) and Debiaggi et al. (1988) reported that an ethanolic extract of the leaves of *C. lawsoniana* had antiviral activity against *Herpes simplex* virus type 2. However, this is the first report of antibacterial compounds from the immature cones of *C. lawsoniana*. Our rationale for studying immature cones is that conifers invest considerable resources into cone production as, unlike angiosperms, the female gametophyte (cone) is formed with a food supply before fertilisation takes place. This is very wasteful of resources as some cones will not be fertilised, and together with the fact that some coniferous cones can take three years to ripen, it was considered likely that immature cones would contain protective compounds against microbial attack.

Anti-staphylococcal activity has been previously demonstrated for many of the isolated compounds (Politi et al., 2003;Xiao et al., 2001;Muhammad et al., 1995;Kobayashi et al., 1988), but here we report their activity against clinically relevant MDR and MRSA clinical isolates, and report for the first time the resistance modifying activity of some of these compounds against virulent *S. aureus* strains. Furthermore, we report the full ^{1}H and ^{13}C NMR data for 5-epipisiferol and *trans*-communic acid. 5-Epipisiferol has previously been synthesized (Matsumoto et al., 1983) and was also reported as 20-hydroxyferruginol (Son et al., 2005), but here we unambiguously assign the stereochemistry as the 5-epimer of pisiferol.

2. Results and discussion

Structure elucidation of the isolated compounds was conducted by extensive spectroscopic studies using 1D and 2D NMR and mass spectroscopy. The data were compared with and were in close agreement with the literature for ferruginol (Wenkert et al., 1976), pisiferol (Yatagai et al., 1978), formosanoxide (Hsu et al., 1995), 4β-hydroxygermacra-1(10)-5-diene (Cornwell et al., 2001;Feliciano et al., 1995), *trans*-communic acid (Yamamoto et al., 1997;Muhammad et al., 1995;Fang et al., 1989), torulosal (Su et al., 1994) and oplopanonyl acetate (De Bruyn et al., 1990). The ^{1}H and ^{13}C NMR data for 5-epipisiferol are presented in Table 1. The ^{1}H spectra for pisiferol and 5-epipisiferol revealed a large coupling for the H-5 methine to the axial H-6 proton ($J = 12.5$ and 12.0, respectively). In the *trans* A/B ring junction of pisiferol, proton H-5 was axial and relatively α with respect to ring A, but in 5-epipisiferol the position of this hydrogen was equatorial and relatively (β) with respect to ring A but axial with respect to the B ring. The relative stereochemistry of pisiferol was assigned by correlations seen in the NOESY spectrum (Fig. 1). A correlation between the H_2-20 methylene protons and the H_3-19 methyl group established that they were on the same face of the molecule and were assigned an axial (β) orientation as described in the literature (Yatagai et al., 1978). Further NOE signals between proton H-5 and the CH_3-18 methyl and between H-5 and proton H-6b and the axial proton of the H-7 methylene suggested an alpha orientation and a *trans* A/B ring junction. For 5-epipisiferol, the NOESY data indicated a *cis* A/B ring junction. This was deduced by through space interactions between the H-20b methylene proton and the H-5 proton which in turn had a correlation to the H_3-19 methyl indicating that these protons were all on the same face of the molecule. These groups were assigned a relative (β) configuration and we therefore describe the compound as 5-epipisiferol, an epimer of pisiferol.

All compounds were assessed for antibacterial activity in a minimum inhibitory concentration (MIC) assay (Table 2). The phenolic diterpenes ferruginol, pisiferol, 5-epipisiferol and the labdane diterpene *trans*-communic acid had the greatest activity at 4–16 μg/ml. The stereochemistry at the A/B ring junction of pisiferol and 5-epipisiferol (Fig. 1) did not affect activity as both epimers had MICs of 8–16 μg/ml against all strains. However, the presence of an ether bridge linking C-7 and C-20, seen in formosanoxide (Fig. 1), destroyed anti-staphylococcal activity, since this compound was inactive at 512 μg/ml against the two strains tested. 4β-hydroxygermacra-1(10)-5-diene displayed modest antibacterial activity at 128–256 μg/ml. The sesquiterpene oplopanonyl acetate was inactive at 128 μg/ml, as was torulosal, except against strain EMRSA-16 where its MIC was 128 μg/ml. It is interesting that nearly all the active compounds had a higher MIC against EMRSA-15 than for strain EMRSA-16. This was surprising, since the MIC for oxacillin against EMRSA-15 is only 32 μg/ml

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data for 5-epipisiferol and *trans*-communic acid in $CDCl_3$

5-Epipisiferol			*trans*-Communic acid		
Position	^{1}H (*J* in Hz)	^{13}C	Position	^{1}H (*J* in Hz)	^{13}C
1	1.52 *m*	41.8	1	1.05 *m*	38.0
	1.84 *m*			2.16 *m*	
2	1.44 *m*	18.7	2	1.52 *m*	19.1
3	1.24 *m*	42.4	3	1.14 *dd* (13.5, 4.0)	39.2
	1.41 *d* (2.5)			1.86 *m*	
4		34.4	4		44.1
5	2.64 *dd* (12.0, 2.5)	58.0	5	1.34 *dd* (12.0, 2.5)	56.2
6	1.26 *m*	24.3	6	1.96 *m*	25.8
	1.99 *m*		7	1.91 *m*	38.5
7	2.66 *m*	35.4		2.39 *m*	
	2.73 *m*		8		147.9
8		133.0	9	1.76 *s*	56.4
9		135.5	10		40.3
10		71.6	11	2.13 *m*	23.3
11	6.66 *s*	118.8		2.36 *m*	
12 (OH)	5.99 *s*	151.6	12	5.39 *t* (6.5)	133.9
13		133.4	13		133.4
14	6.90 *s*	126.6	14	6.31 *dd* (17.5,11.0)	141.6
15	3.17 *q*	26.6	15	4.86 *d* (11.0)	109.9
16	1.21[a] *d* (7.0)	22.5[a]		5.03 *d* (17.5)	
17	1.19[a] *d* (7.0)	22.8[a]	16	1.73 *s*	11.8
18	0.91 *s*	21.7	17	4.45 *s*	107.7
19	0.88 *s*	32.2		4.82 *s*	
20	2.55 *d* (14.0)	51.0	18	1.23 *s*	29.0
	3.00 *d* (14.0)		19		182.5
			20	0.64 *s*	12.8

[a] Interchangeable values.

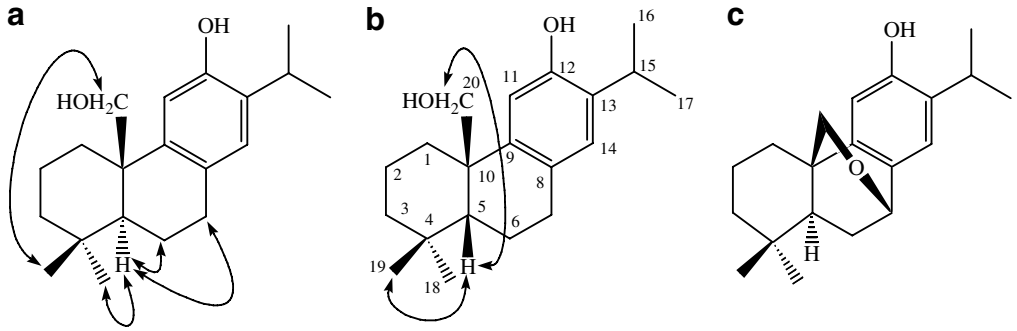

Fig. 1. Pisiferol (a), 5-epipisiferol (b) (selected NOEs) and formosanoxide (c).

compared with 512 μg/ml against strain 16. EMRSA-16 is resistant to norfloxacin whereas strain 15 is sensitive, and it also has a two fold greater resistance to erythromycin. It is possible that acquisition of resistance to many antibiotics has resulted in a loss in fitness, making EMRSA-16 more susceptible to some compounds or types of compounds than EMRSA-15. Another possibility is that EMRSA-15 may possess an as yet uncharacterized mechanism of resistance, or some efflux of compounds may occur, accounting for the higher MICs seen for this strain compared with EMRSA-16.

There was no significant variation in the anti-staphylococcal activity of a compound between the six strains. For example, there were no instances where a compound had a high activity against the standard ATCC 25923 strain, but considerably reduced or even no activity against the resistant strains. If a compound had activity against one *S. aureus* strain, it was active against all strains tested. With the exception of ferruginol (Fig. 2), which had a four-fold higher activity against EMRSA-15 than strain 16, none of the compounds had more than a twofold difference in activity between strains. This was a surprising result which may reflect that the active compounds were mainly diterpenes, some with a very similar structure and with the same functional groups and possibly the same mode of action. However, *trans*-communic acid (Fig. 2), which had good activity compared with the standard antibiotics, is a labdane diterpene and does not possess a phenolic hydroxyl group as found in the active abietanes. Our previous work on isopimaric acid (Smith et al., 2005) isolated

Table 2
MICs (μg/ml) of isolated compounds and standard antibiotics against a standard ATCC strain and five clinical isolates of *S. aureus* (resistance mechanism)

Compound	ATCC 25923	XU212 (TetK)/ (*mecA*)	SA1199B (NorA)	RN4220 (MsrA)	EMRSA-15 (*mecA*)	EMRSA-16 (*mecA*)
Ferruginol	8	8	4	8	16	4
Formosanoxide	>512	>512	–	–	–	–
4β-Hydroxygermacra-1(10)-5-diene	128	128	256	128	256	128
Oplopanonyl acetate	>128	>128	>128	>128	>128	>128
Pisiferol	16	16	16	8	8	8
5-Epipisiferol	8	16	8	16	16	8
Torulosal	>128	>128	>128	>128	>128	128
trans-Communic acid	16	16	8	8	16	8
Tetracycline	0.25	128	0.25	0.25	0.125	0.125
Norfloxacin	1	16	32	2	0.5	128
Erythromycin	0.25	4,096	0.25	128	2,048	4,096
Oxacillin	0.125	128	0.25	0.25	32	512

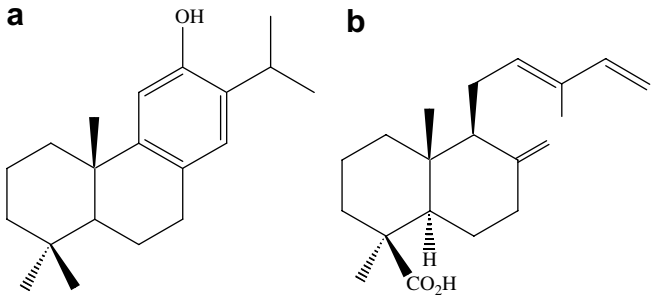

Fig. 2. Ferruginol (a) and *trans*-communic acid (b).

from the cones of *Pinus nigra*, and commercially obtained abietic acid, although not as active (MICs 32–64 μg/ml), still displayed the same trend, that if they were active against one strain, they were active against all strains at approximately the same MIC value. This suggests there may be a common mode of action for each compound against all strains, for example membrane perturbation is often suggested as the antibacterial action for phenolic diterpenes. However, research by Clarkson et al. (2003) demonstrated that ferruginol did not affect erythrocyte morphology even at high concentration (100 μg/ml), therefore the mode of antibacterial action of these diterpenes is unlikely to be merely membrane lysis or disruption. It is possible that these compounds have a mode of action which clinically relevant isolates have not previously encountered.

Oplopanonyl acetate and torulosal were tested in combination with the MDR inhibitor reserpine against the effluxing strains XU212 and SA1199B. This was conducted to assess whether these inactive compounds had antibacterial activity when the efflux pump was inhibited, as seen with several compounds tested by Tegos et al. (2002). However, the presence of an efflux inhibitor had no effect and both compounds remained inactive at 128 μg/ml.

4β-Hydroxygermacra-1(10)-5-diene and compounds with no anti-staphylococcal activity in the MIC assay were tested in the modulation assay at 10 μg/ml and active compounds were assayed at half MIC. Ferruginol was the most active compound in the modulation assays (Table 3) and displayed a similar activity to the control reserpine against the effluxing strain XU212 possessing the TetK pump, causing a fourfold reduction in the MIC of tetracycline. Against strain SA1199B which has the NorA pump, a twofold potentiation of norfloxacin activity was observed in the presence of ferruginol, whereas reserpine caused an eightfold increase in norfloxacin activity, reducing its MIC from 32 to 4 μg/ml. The plant alkaloid reserpine inhibits the NorA and TetK pumps of *S. aureus* (Markham et al., 1999). However, unlike reserpine, ferruginol also potentiated the activity of erythromycin against a strain possessing the MsrA efflux pump. No inhibitor of this pump has so far been reported.

Ferruginol showed excellent potentiation of oxacillin activity against the epidemic MRSA strain EMRSA-15,

Table 3
MICs (μg/ml) of standard antibiotics in the presence and absence of ferruginol and 5-epipisiferol

Compound	XU212 tetracycline	XU212 oxacillin	SA1199B norfloxacin	RN4220 erythromycin	EMRSA-15 oxacillin	EMRSA-16 oxacillin
Ferruginol	128 (**32**)	256 (**32**)	32 (**16**)	128 (**32**)	32 (**0.40**)	–[a]
5-Epipisiferol	128 (**32**)	–	–[a]	–[a]	–[a]	–[a]
Reserpine	128 (**32**)	256 (**256**)	32 (**4**)	128 (**128**)	–	–
Epicatechin gallate	–	256 (**0.16**)	–	–	32 (**0.32**)	256 (**0.32**)

Figures in bold denote MICs in the presence of the test compound. Reserpine was assayed @ 20 μg/ml; epicatechin gallate @ 10 and 4 μg/ml against EMRSA-15 and EMRSA-16, respectively.

[a] A concentration of half MIC inhibited the growth control; a concentration of one quarter MIC was inactive.

comparable with the activity for the epicatechin gallate control against the same strain. An 80-fold reduction in the MIC for oxacillin was achieved, restoring oxacillin sensitivity in this resistant strain. This potentiation of antibiotic activity by ferruginol against MRSA and effluxing strains of *S. aureus* has not been previously reported. However, similar activity has been demonstrated against mycobacteria. Mossa et al. (2004) reported the antimycobacterial activity of ferruginol at 5 μg/ml and its fourfold potentiation of isonaizid activity at half MIC against several species of mycobacteria.

5-Epipisiferol also potentiated the activity of tetracycline, causing a fourfold reduction in its MIC against strain XU212. This is an interesting result since pisiferol was inactive in the modulation assays, which suggests that the stereochemistry at the A/B ring junction is important for modulatory activity but does not affect the antibacterial activity of the epimers.

Some compounds which had antibacterial activity at low MICs, when tested in the modulation assay at half MIC, inhibited the growth control but were inactive at one quarter MIC. This reflects the intrinsic twofold variability in results achieved for MIC assays. The results suggest that there is a narrow window of modulatory activity for these compounds. This may be partly due to the fact that compounds which were active in the modulation assay also had antibacterial activity and, when used at half MIC, there might still be some antibacterial activity. This was in comparison with reserpine which has no anti-staphylococcal activity even at 512 μg/ml and its potentiation of tetracycline and norfloxacin activity would not be due to additive effects.

An efflux inhibition assay (Fig. 3) supported the modulation results for ferruginol, showing that the presence of ferruginol resulted in a reduction in efflux of ethidium bromide (EtBr) in SA1199B, a strain which overexpresses the NorA pump (Kaatz et al., 1993). Ferruginol (10 μM) resulted in a 40% inhibition of efflux, with 50% inhibition occurring at around 17 μM. These two values correspond to 2.86 μg/ml and 4.86 μg/ml, respectively. Fifty per cent inhibition of efflux occurred at the MIC value for ferruginol against this strain and, as the antibacterial activity of ferruginol would have an effect at this concentration, the experiment was stopped at 30 μM ferruginol. The results suggest that ferruginol is likely to be a weak efflux pump inhibitor. In the modulation assay, ferruginol at half its MIC value (2 μg/ml), resulted in a twofold reduction in the MIC of norfloxacin against SA1199B. Reserpine reduced the MIC of norfloxacin from 32 to 4 μg/ml which suggested that the pump was not completely inhibited and that there was still some residual efflux occurring. The presence of other efflux pumps for which norfloxacin and EtBr are substrates, but which are not inhibited by reserpine or affected by ferruginol could also be responsible for some efflux of compounds (Kaatz et al., 2000).

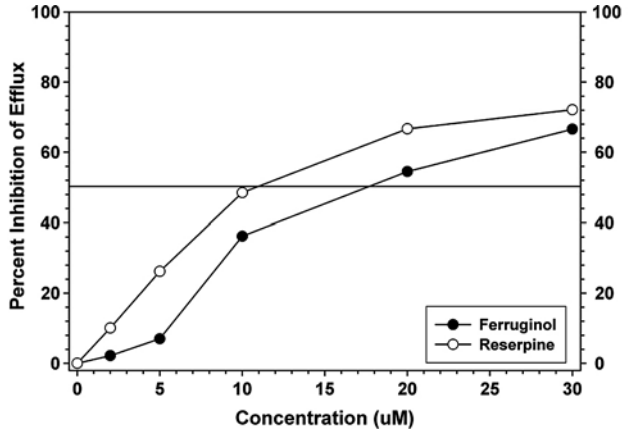

Fig. 3. Ethidium efflux inhibition assay against SA1199B, which overexpresses the NorA efflux pump.

Work by Shiota et al. (1999) on the polyphenols epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) showed that ECG reduced the MIC of oxacillin against MRSA strains by 250–500-fold. Epigallocatechin gallate (EGCG), which differs from ECG only by an extra hydroxyl group on the B-ring, had much lower activity, resulting in a 4–64-fold reduction in oxacillin MIC. It is interesting that an extra phenolic hydroxyl on EGCG should have such an effect on modulatory activity, suggesting that either the position of the hydroxyl groups is important or that the increase in hydrophilicity afforded by the extra hydroxyl was sufficient to affect the activity. Research carried out by our group (Gibbons et al., 2004a) on these two compounds showed that in the antibacterial MIC assays, conversely, EGCG has a 2–16-fold greater anti-staphylococcal activity than ECG which suggests that potentiation of antibiotic activity is by a different mechanism from antibacterial activity. An efflux inhibition assay also revealed the surprising result that at low concentration, ECG increases efflux of EtBr, but at high concentration inhibits efflux. Ferruginol demonstrated good modulatory activity in restoring oxacillin sensitivity in strain EMRSA-15, but against the MRSA isolate XU212 which is also an effluxing strain possessing the TetK pump, the potentiation of oxacillin activity was modest in comparison. Here, ferruginol only resulted in an eightfold reduction in the MIC of oxacillin from 256 to 32 μg/ml, which is not sufficient to restore oxacillin sensitivity (MIC $\geqslant$ 4 μg/ml). Against SA1199B (NorA) and RN4220 (MsrA), ferruginol only caused a two and fourfold potentiation of the activity of norfloxacin and erythromycin, respectively. One possibility for this lower activity is that ferruginol may be a substrate for the efflux pumps; however, it is likely that the modulatory mode of action against effluxing strains differs from that against MRSA strains. The efflux pump inhibitor reserpine had no effect on oxacillin activity against strain XU212, which suggests that oxacillin is not a substrate for the TetK pump. However, ECG reduced the MIC of oxacillin from 256 to 0.16 μg/ml. Shiota et al. (1999) suggested that ECG acts on the penicillin binding protein PBP2′ encoded by the *mecA* gene. This conclusion was also reached by

Nicolson et al. (1999) who suggested that modulators of methicillin activity act by inhibition of *de novo* synthesis of PBP2′. If this is the case, then the mode of action in potentiating antibiotic activity against effluxing strains and MRSA strains must be different. It has recently been reported (Tian, 2006) that ECG inhibits animal fatty acid synthase and this enzyme could also be a target of ECG activity in bacteria. It is possible that the modest increases in antibiotic activity against effluxing strains were due to an additive effect as the compounds are antibacterial, although used at sub-inhibitory concentrations. The efflux inhibition experiments on ferruginol however, do indicate that this compound is a weak inhibitor of efflux.

In searching the literature for NMR data on *trans*-communic acid, there were inconsistencies in some of the ^{13}C data published (Yamamoto et al., 1997;Muhammad et al., 1995;Fang et al., 1989). Here we have revised the ^{13}C NMR data for *trans*-communic acid based on extensive 1D and 2D data (Table 1). This data is in close agreement to that reported by Muhammad et al., differing only in the assignment of the signals for C-1 and C-3 which we have revised.

3. Experimental

3.1. General experimental procedures

GC–MS analysis was carried out using an Agilent 6890 GC coupled to an Agilent 5973 mass selective detector. An HP-5 ms capillary column of 30 m length with a diameter of 250 μm was used with a non-polar stationary phase of 5% phenylmethylsiloxane and a film thickness of 0.25 μm. Samples were introduced into the system using split injection with a split ratio of between 5:1 and 10:1 and an injector temperature of 250 °C. Helium was used as the carrier gas at an average linear velocity of 50 cm/s. The initial oven temperature was 50 °C and the temperature was increased after 5 min at a rate of 5 °C to a maximum of 300 °C. The MS was run in EI mode.

NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Chemical shift values (δ) are reported in parts per million (ppm) relative to an appropriate internal solvent standard and coupling constants (J values) are given in Hertz.

3.2. Plant material

Immature cones of *C. lawsoniana* were identified and supplied by Dr Caroline Priestley. A voucher specimen (ECJS/001) was placed in the herbarium at the Centre for Pharmacognosy and Phytotherapy.

3.3. Extraction and isolation

Four hundred grams of immature cones were chopped and placed in a Soxhlet apparatus. Sequential extraction started with hexane, followed by $CHCl_3$, acetone and, finally MeOH. Vacuum liquid chromatography (VLC) on 8 g of hexane extract yielded 12 fractions. Elution commenced with 100% hexane going to 100% EtOAc in 10% increments, finishing with an EtOAc/MeoH 50:50 wash. MIC assays identified the most active fraction (4) and solid phase extraction (SPE) was performed on 450 mg of this fraction. A Phenomenex Strata Sl-1 silica column (10 g/60 ml giga tubes) was used and elution commenced with 100% petroleum spirit, followed by a gradient of 5% increments of Et_2O to 70:30 petroleum spirit/Et_2O. The Et_2O increments were then increased to 40%, 60% and 80%, finally eluting with 100% Et_2O. PTLC was performed on 50 mg of fraction 3, using four analytical silica plates (petroleum spirit/toluene 80:20, 1 development; petroleum spirit/Et_2O 90:10, 2 developments) to give 25 mg ferruginol. SPE fractions 7–11 were run on three analytical silica plates (hexane/Et_2O/EtOAc 80:15:5, 2 developments), which yielded *trans*-communic acid (5.5 mg). Two hundred milligrams of VLC fraction 6 were loaded onto a LH-20 sephadex column (200 mg per column run) and eluted with $CHCl_3$, followed by CH_2Cl_2, CH_2Cl_2/MeOH 50:50, finishing with 100% MeOH. PTLC (hexane/Et_2O/EtOAc 80:15:5, 3 developments) on pooled fractions 12–17 gave oplopanonyl acetate (15.1 mg) and torulosal (10.3 mg). Using the same solvent system (4 developments), fractions 36–42 yielded pisiferol (6.2 mg), formosanoxide (4.2 mg) and 5-epipisiferol (14.9 mg). VLC fraction 3 (390 mg) was run on PTLC to yield 6.2 mg 4β-hydroxygermacra-1(10)-5-diene.

3.4. Antibacterial assay and modulation assay

A standard *S. aureus* strain ATCC 25923 and a clinical isolate (XU212), which possesses the TetK efflux pump and is also an MRSA strain, were obtained from E. Udo (Gibbons and Udo, 2000). Strain RN4220 which has the MsrA macrolide efflux pump was provided by J. Cove (Ross et al., 1989). EMRSA-15 (Richardson and Reith, 1993) and EMRSA-16 (Cox et al., 1995) were obtained from Paul Stapleton. Glenn Kaatz provided strain SA1199B which over-expresses the NorA MDR efflux pump (Kaatz et al., 1993). Minimum inhibitory concentration (MIC) and modulation assays were carried out as previously described (Smith et al., 2005).

3.5. Efflux inhibition assay

Ferruginol was assayed at varying concentrations against SA1199B (NorA) for potential to reduce the efflux of ethidium bromide (EtBr) which is a substrate for the NorA MDR pump. EtBr fluorescence will decrease over time as it is effluxed from the cell, the potential of a compound to inhibit efflux can be determined by the strength of the fluorescent signal which remains in the cell. The efflux inhibition was carried out as previously described (Kaatz et al., 2000).

Acknowledgements

We thank Stiefel International R&D Ltd. for the award of a studentship and post-doctoral funding to E. Smith. The Engineering and Physical Sciences Research Council is thanked for a multi project equipment grant (Grant No. GR/R47646/01).

References

Clarkson, C., Campbell, W.E., Smith, P., 2003. In vitro antiplasmodial activity of abietane and totarane diterpenes isolated from *Harpagophytum procumbens* (Devil's Claw). Planta Medica 69, 720–724.

Cornwell, C.P., Reddy, N., Leach, D.N., Wyllie, S.G., 2001. Germacradienols in the essential oils of the Myrtaceae. Flavour and Fragrance Journal 16, 263–273.

Cox, R.A., Conquest, C., Mallaghan, C., Maples, R.R., 1995. A major outbreak of methicillin-resistant *Staphylococcus aureus* caused by a new phage type (EMRSA-16). Journal of Hospital Infection 29, 87–106.

Debiaggi, M., Pagani, L., Cereda, P.M., Landini, P., Romero, E., 1988. Antiviral activity of *Chamaecyparis lawsoniana* extract: study with *Herpes simplex* virus type 2. Microbiologica 11, 55–61.

De Bruyn, A., De Pooter, H.L., De Buyck, L., Jans, A.W.H., Budesinsky, M., Sedmera, P., 1990. Identification of oplopanonyl acetate isolated from *Chamaecyparis pisifera*. Magnetic Resonance in Chemistry 28 (12), 1030–1039.

Fang, J.M., Hsu, K.C., Cheng, Y.S., 1989. Terpenoids from leaves of *Calocedrus formosana*. Phytochemistry 28 (4), 1173–1175.

Gibbons, S., Moser, E., Kaatz, G.W., 2004a. Catechin gallates inhibit multidrug resistance (MDR) in *Staphylococcus aureus*. Planta Medica 70, 1240–1242.

Gibbons, S., Udo, E.E., 2000. The effect of reserpine, a modulator of multidrug efflux pumps, on the *in vitro* activity of tetracycline against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) possessing the *tet*(K) determinant. Phytotherapy Research 14, 139–140.

Hsu, K.C., Fang, J.M., Cheng, Y.S., 1995. Diterpenes from pericarps of *Chamaecyparis formosensis*. Journal of Natural Products 58 (10), 1592–1595.

Jacobson, M., Redfern, R.E., Mills, G.D., 1975. Naturally occurring insect growth regulators part 2 screening of insect and plant extracts as insect juvenile hormone mimics. Lloydia 38 (6), 455–472.

Johnson, A.P., Aucken, H.M., Cavendish, S., Ganner, M., Wale, M.C.J., Warner, M., Livermore, D.M., Cookson, B.D., 2001. Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). Journal of Antimicrobial Chemotherapy 48, 143–144.

Kaatz, G.W., Seo, S.M., O'Brien, L., Wahiduzzaman, M., Foster, T.J., 2000. Evidence for the existence of a multidrug efflux transporter distinct from NorA in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 44 (5), 1404–1406.

Kaatz, G.W., Seo, S.M., Ruble, C.A., 1993. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 37 (5), 1086–1094.

Kobayashi, K., Nishino, C., Fukushima, M., Shiobara, Y., Kodama, M., 1988. Antibacterial activity of pisiferic acid and its derivatives against gram-negative and gram-positive bacteria. Agricultural and Biological Chemistry 52 (1), 77–83.

Koyama, S., Yamaguchi, Y., Tanaka, S., Motoyoshiya, J., 1997. A new substance (Yoshixol) with an interesting antibiotic mechanism from wood oil of Japanese traditional tree (Kiso-Hinoki), *Chamaecyparis obtusa.*. General Pharmacology 28 (5), 797–804.

Markham, P.N., Westhaus, E., Klyachko, K., Johnson, M.E., Neyfakh, A.A., 1999. Multiple novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 43, 2404–2408.

Matsumoto, T., Endo, Y., Okimoto, M., 1983. The synthesis of (+)-pisiferol and (+)-pisiferal. Bulletin of the Chemical Society of Japan 56, 2018–2022.

McDaniel, C.A., 1989. Major termiticidal components of heartwood of Port-Orford cedar, *Chamaecyparis lawsoniana* (A. Murr.). Parl. Material und Organismen 24 (1), 1–16.

Mossa, J.S., El-Feraly, F.S., Muhammad, I., 2004. Antimycobacterial constitutents from *Juniperus procera, Ferula communis* and *Plumbago zeylanica* and their *in vitro* synergistic activity with isonicotinic acid hydrazide. Phytotherapy Research 18, 934–937.

Muhammad, I., Mossa, J.S., Al Yahya, M.A., Ramadan, A.F., El-Feraly, F.S., 1995. Further antibacterial diterpenes from the bark and leaves of *Juniperus procera* Hochstex Endl. Phytotherapy Research 9, 584–588.

Nicolson, K., Evans, G., O'Toole, P.W., 1999. Potentiation of methicillin activity against methicillin-resistant *Staphylococcus aureus* by diterpenes. FEMS Microbiology Letters 179, 233–239.

Office for National Statistics, 2006. Report: Deaths involving MRSA: England and Wales 2000–2004. Health Statistics Quarterly 29, 63–68.

Politi, M., Braca, A., De Tommasi, N., Morelli, I., Manunta, A., Battinelli, L., Mazzanti, G., 2003. Antimicrobial diterpenes from the seeds of *Cephalotaxus harringtonia* var. *drupacea*. Planta Medica 69, 468–470.

Richardson, J.F., Reith, S., 1993. Characterisation of a strain of methicillin-resistant *Staphylococcus aureus* (EMRSA-15) by conventional and molecular methods. Journal of Hospital Infection 25, 45–52.

Ross, J.I., Farrell, A.M., Eady, E.A., Cove, J.H., Cunliffe, W.J., 1989. Characterisation and molecular cloning of the novel macrolide streptogramin B resistance determinant from *Staphylococcus epidermidis*. Journal of Antimicrobial Chemotherapy 24, 851–862.

San Feliciano, A., Medarde, M., Gordaliza, M., Lucas, M.J., 1995. Structure elucidation of germacrane alcohols from *Juniperus communis* subsp. *Hemisphaerica*. Journal of Natural Products 58 (7), 1059–1064.

Shiota, S., Shimizu, M., Mizushima, T., Ito, H., Hatano, T., Yoshida, T., Tsuchiya, T., 1999. Marked reduction in the minimum inhibitory concentration (MIC) of beta-lactams in methicillin-resistant *Staphylococcus aureus* produced by epicatechin gallate, an ingredient of green tea (*Camellia sinensis*). Biological & Pharmaceutical Bulletin 22, 1388–1390.

Smith, E., Williamson, E., Zloh, M., Gibbons, S., 2005. Isopimaric acid from *Pinus nigra* shows activity against multidrug-resistant and EMRSA strains of *Staphylococcus aureus*. Phytotherapy Research 19, 538–542.

Son, K.H., Oh, H.M., Choi, S.K., Han, D.C., Kwon, B.M., 2005. Antitumor abietane diterpenes from the cones of *Sequoia sempervirens*. Bioorganic & Medicinal Chemistry Letters 15, 2019–2021.

Su, W.C., Fang, J.M., Cheng, Y.S., 1994. Labdanes from *Cryptomeria japonica*. Phytochemistry 37 (4), 1109–1114.

Tegos, G., Stermitz, F.R., Lomovskaya, O., Lewis, K., 2002. Multi-drug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrobial Agents and Chemotherapy 46, 3133–3141.

Tian, W.X., 2006. Inhibition of fatty acid synthase by polyphenols. Current Medicinal Chemistry 13 (8), 967–977.

Turner, N.J., 1988. Ethnobotany of coniferous trees in Thompson and Lillooet Interior Salish of British Columbia. Economic Botany 42 (2), 177–194.

Turner, N.J., 1979. The ethnobotany of the Southern Kwakiutl Indians of British Columbia. Economic Botany 27, 257–310.

Vidaković, M., 1991. Conifers Morphology and Variation. Grafički Zavod Hrvatske, Zagreb, Croatia.

Wenkert, E., Buckwalter, B.L., Burfitt, I.R., Gašic, M.J., Gottlieb, H.E., Hagaman, E.W., Schell, F.M., Wovkulich, P.M., 1976. Carbon-13 nuclear magnetic resonance spectroscopy of naturally occurring substances. In: Levy, George C. (Ed.), Topics in Carbon-13 NMR Spectroscopy. John Wiley & Sons Inc., pp. 98–100 (Chapter 2).

Xiao, D., Kuroyanagi, M., Itani, T., Matsuura, H., Udayama, M., Murakami, M., Umehara, K., Kawahara, N., 2001. Studies on constituents from *Chamaecyparis pisifera* and antibacterial activity of diterpenes. Chemical and Pharmaceutical Bulletin 49 (11), 1479–1481.

Yamamoto, H., Asano, N., Sawano, C., Sone, T., Gasha, T., Ono, Y., 1997. Diterpenes isolated from the resin of the resinous stem canker of Japanese Cypress, *Chamaecyparis obtusa*. Mokuzai Gakkaishi 43, 558–565.

Yatagai, M., Nakatani, N., 1994. Antimite, antifly, antioxidative and antibacterial activities of pisiferic acid and its congeners. Mokuzai Gakkaishi 40, 1355–1362.

Yatagai, M., Shirato, T., Hayashi, Y., Fukuhara, N., Takahashi, T., 1978. Pisiferic acid, a new phenolic diterpene carboxylic acid from *Chamaecyparis pisifera* S. et Z. Mokuzai Gakkaishi 24, 267–269.