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Plants as a source of bacterial resistance modulators and anti-infective agents

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Abstract

The spread of multidrug-resistant (MDR) strains of bacteria necessitates the discovery of new classes of antibacterials and compounds that inhibit these resistance mechanisms. At present, there are no single chemical entity plant-derived antibacterials used clinically, and this chemically diverse group deserves consideration as a source for two major reasons. First, plants have exceptional ability to produce cytotoxic agents and second there is an ecological rationale that antimicrobial natural products should be present or synthesised *de novo* in plants following microbial attack to protect the producer from pathogenic microbes in its environment. We have been characterising plant-derived products that are either antibacterial in their own right, or modulators of resistance in bacterial strains possessing multidrug efflux mechanisms. These efflux transporters are responsible for resistance to certain antibiotics and antiseptics and occur in strains of methicillin-resistant *Staphylococcus aureus* (MRSA), a major clinical problem at present. We are also investigating plant sources for compounds with activity against mycobacteria with a view to discovering drug leads with potential activity toward tuberculosis (TB) producing species. This paper will briefly review the literature on plant derived bacterial resistance modifying agents and antibacterials. Examples in this area from our own work will be given. The activities of plant-derived antibacterials show that there are many potential new classes of antibacterial agents which should undergo further cytotoxicity, microbial specificity and preclinical studies.

Introduction

This paper gives a brief review of the literature on plant derived bacterial resistance modulators and antibacterial compounds and some examples are drawn from the authors own research. Most of the examples are drawn from the literature on anti-staphylococcal natural products as this is a most pressing area.

Bacterial resistance modulators are compounds which potentiate the activity of an antibiotic against a resistant strain. These compounds may target a resistance mechanism such as the inhibition of multidrug resistance (MDR), e.g. inhibition of the NorA efflux mechanism in *Staphylococcus aureus* (Gibbons et al., 2003a) or act in a synergistic fashion *via* an uncharacterised mechanism.

Staphylococcus aureus (SA) is commonly cited as being a major hospital-acquired pathogen (Perl, 1999). Strains of this species that are resistant to β-lactams, notably methicillin-resistant *Staphylococcus aureus* (MRSA) have been described from clinical sources for over 40 years (Jevons, 1961). The ability of this Gram-positive organism to acquire resistance to practically all

useful antibiotics is cause for great concern. In the UK there has been a significant increase in the number of death certificates which mention MRSA with 47 citations in 1993 rising to 398 in 1998 (Crowcroft and Catchpole, 2002). The threat of untreatable multidrug resistant bacteria has prompted a special report from the House of Lords 7th Report, 1998) and a report on hospital-acquired infections by the National Audit Office (Report by the Comptroller and Auditor General, 2000). The latter estimates that hospital-acquired infections and treatment of drug-resistant bacteria in the clinical setting cost the UK tax payer an estimated one billion pounds per annum.

The occurrence of a fully vancomycin resistant strain of MRSA in the US in 2002 indicates that the successful treatment of MRSA strains by the use of this glycopeptide antibiotic is not guaranteed. Linezolid (Zyvox®), a new member of the oxazolidinone group and the streptogramin quinupristin/dalfopristin mixture (Synercid®) are the newest anti-staphylococcal agents and have been heralded as a solution to MRSA infections. However, an isolated report of resistance to linezolid (Tsiodras et al., 2001) in a clinical isolate of *Staphylococcus aureus* demonstrates that there is a continual need for a pipeline of new agents to combat multidrug resistant bacteria.

Bacterial resistance modulators

The concept of using a compound that inhibits resistance in a bacterium which may be employed with a conventional antibiotic is well proven and Augmentin® is an important example. This product, uses a combination of amoxicillin (a beta lactam antibiotic) and clavulanic acid, a microbially derived inhibitor of beta lactamases. The inhibitor greatly increases the stability of amoxicillin to degradation by beta lactamases and the product is indicated for the treatment of patients with community-acquired pneumonia or sinusitis due to β-lactamase–producing pathogens.

Resistance modulators may also inhibit multidrug resistance (MDR) mechanisms. These are membrane proteins of varying substrate specificity that pump antibiotics from the bacterial cell resulting in a low intracellular ineffective concentration of the drug (Marshall and Piddock, 1997).

In combination with an antibiotic that is a substrate for these mechanisms, an inhibitor will increase the cellular concentration of the antibiotic therefore restoring its efficacy. It has also been shown that the use of such resistance modifying agents can reduce the emergence of antibiotic resistant variants (Markham et al., 1999). The following are examples of plant derived bacterial resistance modulators from the literature.

Much work on the ability of green tea and its constituents to inhibit methicillin-resistance in MRSA has been undertaken (Yam et al., 1998; Hamilton-Miller and Shah, 1999). A patent detailing the ability of 'compound P', which is also antibacterial (MIC = 280 μg/ml), to reverse methicillin-resistance has been described and the structure of this was revealed as epicatechin gallate (**1**) (Hamilton-Miller and Shah, 2000 (Figure 1).

This component acts by inhibiting the synthesis of penicillin binding protein 2′ (PBP2′) and is selective affecting only staphylococci that synthesise PBP2′. In the presence of a β-lactam antibiotic, epicatechin gallate renders MRSA strains sensitive, and an electron microscopy study has shown that **1** affects cell wall morphology of

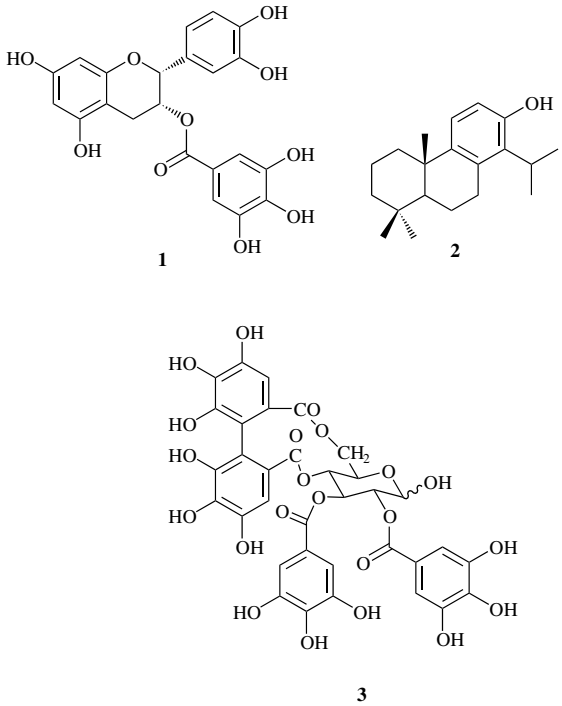


Figure 1.

resistant strains whereas sensitive strains are unaffected.

Diterpenes such as totarol (**2**) also potentiate methicillin activity against MRSA *via* interference of PBP2′ expression (Nicholson et al., 1999). When incorporated into the medium at 1 μg/ml, compound **2** caused an eight-fold increase in methicillin activity against an MRSA strain and totarol was antibacterial (MIC = 2 μg/ml) and inhibited respiration in *S. aureus*.

Hydrolysable tannins also appear to have an effect as modulators and one of these tellimagrandin I (**3**), significantly lowers the minimum inhibitory concentrations (MIC) of tetracycline against some MRSA strains (Shiota et al., 2000).

The MDR inhibitor reserpine (**4**) caused a four-fold reduction in the MIC of tetracycline although erythromycin activity was unaffected. The effects of (**4**) have been studied against a variety of multidrug-resistant MRSA and MSSA strains (Schmitz et al., 1998; Gibbons and Udo, 2000) and this compound enhances the activity of tetracycline and norfloxacin against strains which possess efflux mechanisms such as the Tet(K) protein and the MDR transporter NorA, the major drug efflux pump in this pathogen (Hsieh et al., 1998). The NorA pump contributes significantly to decreased fluoroquinolone susceptibility. In one of these studies, reserpine reduced sparfloxacin, moxifloxacin and ciprofloxacin MICs up to four-fold in 11, 21 and 48 of 102 clinical isolates tested, respectively (Schmitz et al., 1998 (Figure 2).

From the highly productive research groups of Stermitz and Lewis, a number of inhibitors of

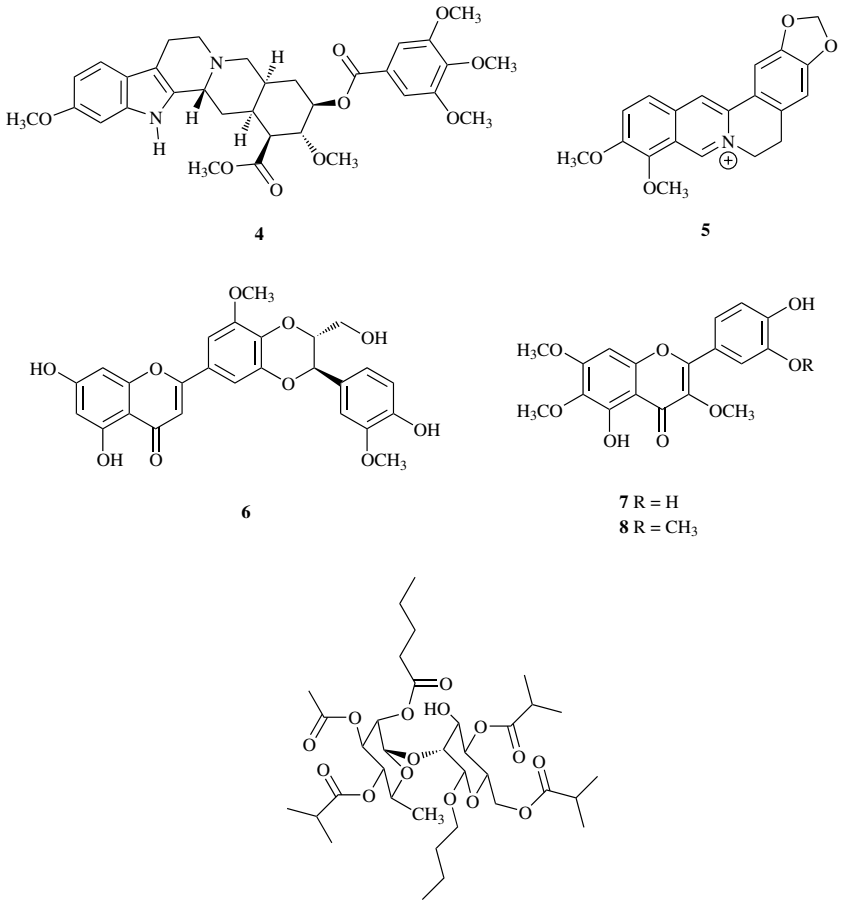


Figure 2.

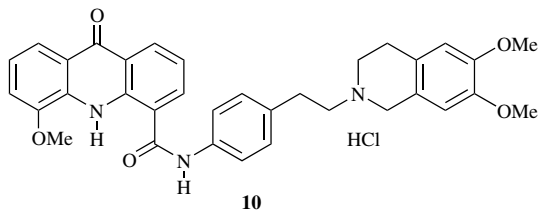
MDR in *S. aureus* have been described. These researchers studied the synergistic interaction between berberine (**5**), a plant antibacterial alkaloid from *Berberis fremontii* and another natural product, 5′-methoxyhydnocarpin (**6**) also present in this species (Stermitz et al., 2000; Guz and Stermitz, 2000). **6** potentiates the activity of berberine toward strains with the NorA MDR transporter, and MDR-dependent efflux of berberine was completely inhibited by this alkaloid. The authors postulate that the plant has evolved inhibitors against MDR pumps in plant pathogens, and that with the latent antibacterial products present in the plant (e.g. berberine), this offers an improved chemical defence. A key feature of these and many other MDR inhibitors is their large size and high degree of lipophilicity. These qualities are likely to be of importance for their solubility in the bacterial membrane and binding to the efflux transporters before inhibition can occur.

A number of methoxylated flavones (Stermitz et al., 2002) (**7**, **8**) and isoflavones (Morel et al., 2003) that potentiate the activities of berberine and the synthetic fluoroquinolone antibiotic norfloxacin have been described. The first two flavones from Wormwood, *Artemisia annua* (Asteraceae) were earlier reported to potentiate the activity of the antimalarial artemisinin against the causative agent *Plasmodium falciparum*, and it is likely that in *S. aureus* (and possibly *P. falciparum*), these compounds exert their effects by inhibition of MDR pumps.

MDR pump inhibitors have even been described from popular horticultural taxa such as *Geranium* with some polyacylated neohesperidosides from *G. caespitosum* (Stermitz et al., 2003) for example **9**, showing potentiation activity of berberine by increasing berberine uptake by inhibition of MDR. **9** was also shown to be only weakly cytotoxic against three leukaemia cell lines.

Our own work in this area started with the evaluation of GG918 (**10**) a compound which is in clinical trials for the reversal of mammalian tumour resistance. GG918 is an inhibitor of *p*-glycoprotein, one of the major mechanisms associated with multidrug resistance in human tumours. Despite being synthetic, there are several features of this molecule that brought it to our attention and commend it to a natural product research group (Figure 3).

Firstly it contains acridone and tetrahydroisoquinoline moieties and these features are common amongst certain alkaloids belonging to the plant families Rutaceae, Ranunculaceae and Menispermaceae. Secondly other inhibitors of *p*-glycoprotein, for example reserpine, are also inhibitors of bacterial multidrug resistance and we reasoned that GG918 would have similar activity. This prompted us to test this compound against a panel of *S. aureus* strains possessing multidrug efflux mechanisms of resistance. We were able to



10

Figure 3.

Table 1. Susceptibilities of test strains[a].

Antimicrobial	RN4220 (MsrA)	CD-1281 (TetK)	SA-1199B (NorA)	ATCC25923
Norfloxacin	1	1	32	0.5
+ Reserpine[b]	0.25 (4)	0.25 (4)	8 (4)	0.125 (4)
+ 10[c]	0.25 (4)	0.25 (4)	4 (8)	0.25 (2)
Ciprofloxacin	0.25	0.25	8	0.125
+ Reserpine[b]	0.125 (2)	0.125 (2)	1 (8)	0.063 (2)
+ 10[c]	0.25 (nc)	0.125 (2)	1 (8)	0.125 (nc)

[a]Expressed in μg/ml.
[b]reserpine 20 μg/ml.
[c]G: GG918, 10 μg/ml.
Figures in parentheses are fold reductions in MICs; nc: no change.

demonstrate that **10** does in fact lower the minimum inhibitory concentrations of standard antibiotics against SA strains possessing MDR mechanisms (Table 1) (Gibbons et al., 2003a).

This potentiation of activity was possibly due to direct inhibition of the MDR mechanisms and in collaboration with Professor Glenn W. Kaatz of Wayne State University in Detroit, compound **10** was evaluated for its ability to inhibit ethidium bromide efflux from *Staphylococcus aureus* possessing the NorA MDR transporter. Ethidium bromide (EtBr) is a substrate for many MDR mechanisms and when inside bacterial cells it binds to DNA. This DNA-EtBr complex is fluorescent and therefore allows measurement of EtBr concentration (and therefore efflux) inside the bacterial cell. GG918 does in fact inhibit the NorA transporter and an uncharacterised efflux mechanism present in *S. aureus* strain SA-K2068 (Figure 4).

The IC_{50} of **10** is approximately 2 μM which compares favourably with the MDR inhibitor reserpine (IC_{50} = 10 μM). These results and the literature on plant derived natural product inhibitors of bacterial MDR indicated that further investigation of plant sources may prove fruitful.

We therefore investigated a series of European and Middle-Eastern plants for their ability to potentiate norfloxacin activity against a norfloxacin-resistant *Staphylococcus aureus* strain possessing the NorA MDR efflux transporter, with a view to characterising inhibitors of bacterial MDR processes. An extract of *Lycopus europaeus* L. (Lamiaceae) was screened, 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. *Lycopus 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 et al., 2000). Bioassay-guided

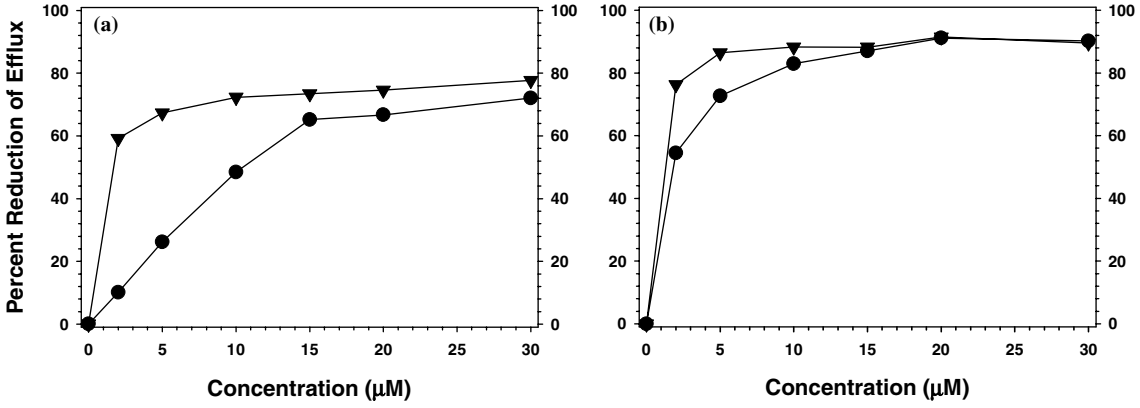
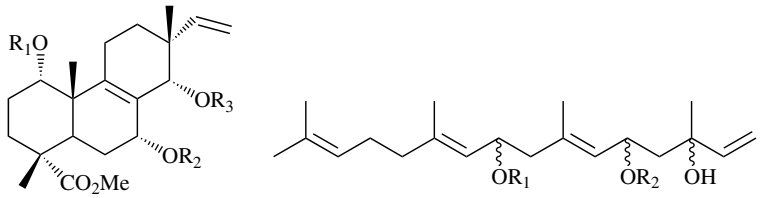


Figure 4. Effect of inhibitors on ethidium efflux; the data presented are means of duplicate experiments. A, SA-1199B; B, SA-K2068; ▼, GG918; ●, reserpine.

Table 2. MICs of test strains in the absence and presence of **11–16**[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 **11–16** are expressed in μg/ml.
[c]Figures in bold denote MICs in the presence of reserpine at 20 μg/ml.



11 R_1 = Ac, R_2 = R_3= H
12 R_1 = R_3 = Ac, R_2 = H
13 R_1 = R_2 = R_3 = Ac
14 R_1 = R_2 = Ac, R_3 = H

15 R_1 = R_2 = H
16 R_1 = R_2 = Ac

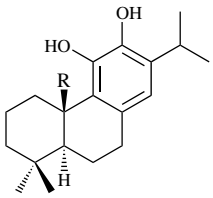
Figure 5.

fractionation of the hexanic extract led to the isolation of some isopimarane diterpenes and some straight-chain diterpenes e.g. **11–16**, which reduced the MICs of tetracycline and erythromycin by two-fold against strains possessing the Tet(K) and Msr(A) transporters which confer a high level of resistance to these antibiotics (Table 2) (Gibbons et al., 2003b (Figure 5).

Unfortunately no potentiation of norfloxacin activity was observed indicating that these compounds are unlikely to be inhibitors of the NorA pump.

There has been a literature report on the ability of extracts of *Rosmarinus officinalis* (Rosemary, Lamiaceae) to inhibit *p*-glycoprotein mediated MDR, a major efflux mechanism associated with human tumour resistance. This activity, coupled with the fact that this species is in the same plant family as Gypsywort encouraged us to make extracts and evaluate these for possible resistance modifying activity. Bioassay-guided isolation again led to the characterisation of diterpenoid natural products of the abietane class that are typical constituents of Rosemary chemistry e.g. **17** and **18** (Figure 6).

These compounds potentiate the activities of standard antibiotics against resistant strains possessing efflux mechanisms to these antibiotics (Table 3). These compounds (**17** and **18**) are



17 R = CO_2H
18 R = H

Figure 6.

carnosic acid and carnosol and both potentiate norfloxacin activity indicating that they are inhibitors of the NorA system.

We have recently conducted some experiments that indicate that one of these agents (carnosic acid) is an inhibitor of efflux (Oluwatuyi et al., 2004). Carnosic acid also showed an impressive eight-fold potentiation of erythromycin activity toward an erythromycin effluxing strain and no potentiation of erythromycin activity using the MDR inhibitor reserpine was seen.

A plant we collected in Kuwait and evaluated for resistance modifying activity was *Prosopis juliflora* (Mimosaceae). This is a small tree which also grows in the South Western part of the United States and is known as mesquite and is cultivated in Kuwait as a roadside tree due to its ability to

Table 3. MICs of *S. aureus* strains to antibiotics in presence and absence of (**17**) and (**18**) at 10 μg/ml.

Compound	XU212 (TetK) Tetracycline	RN4220 (MsrA) Erythromycin	SA199B (NorA) Norfloxacin
Carnosic acid (**17**)	16, 128	16, 256	16, 32
Carnosol (**18**)	32, 128	32, 256	64, 32

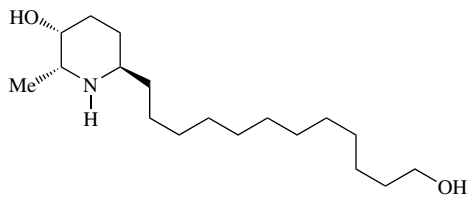
stand very high temperatures and survive in the arid desert of Kuwait. The species is known to produce piperidine alkaloids, e.g. julifloridine (**19**), juliflorine (**20**) and juliprosine (**21**) and some of these have been shown to have antibacterial properties (Ahmad et al., 1986) (Figure 7).

The methanol extract of the leaves of the tree potentiated norfloxacin activity against a norfloxacin resistant strain, but the active component required extensive bioassay-guided fractionation due to the way in which it behaved on various sorbents. Activity was lost on both silica and C_{18}, and alumina column chromatography was employed as an initial fractionation step followed by preparative thin-layer chromatography using a quaternary solvent system (acetone:methanol:THF: ammonia; 60:20:1.7:0.3) to afford the active potentiator.

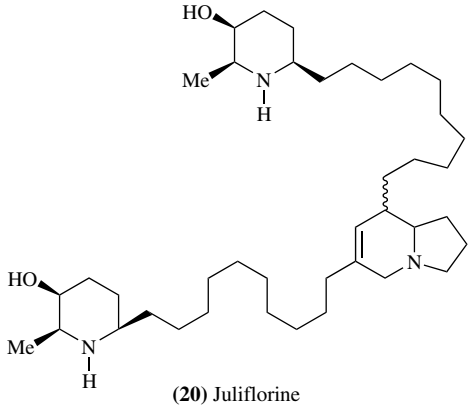
Accurate mass determination indicated a molecular formula of $C_{40}H_{72}N_3O_2$ and from the way in which this compound behaved in solvents and on sorbents, it was evident that it was both lipophilic and hydrophilic in nature. The ^{1}H NMR spectrum indicated the presence of many methylenes which were characteristic of a long alkyl chain and first inspection indicated that this compound might be a fatty acid. However, additional signals included aromatic resonances, deshielded methylenes, a methylene envelope and a doublet methyl resonance which integrated for six protons indicating two secondary methyl groups. COSY spectra elucidated spin systems for a piperidine ring and the integration in the ^{1}H NMR spectrum indicated that there were two of these moieties which is characteristic for *Prosopis* natural products and is also found in juliflorine and juliprosine (Figure 8).

HMBC spectra were highly informative for the aromatic resonances (Figure 9) and suggested that the active potentiator had a pyridine ring system attached to a four-membered ring which should be highly strained and unstable.

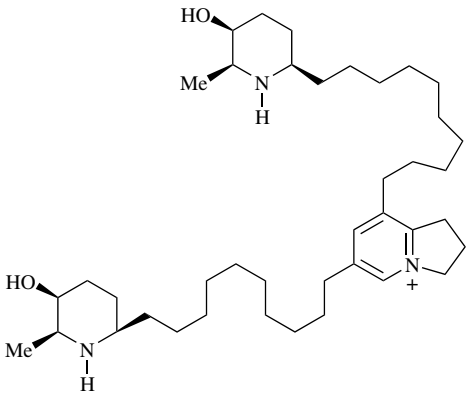
The ^{13}C and ^{1}H resonances for this system were consistent for its structure and furthermore the ^{1}H–^{13}C correlations supported the suggestion of a 6:4 heterocyclic ring system with a quaternary nitrogen atom between the 6 and 4 membered rings (Figure 9). Extensive mass spectrometric studies were undertaken and key fragmentation suggested that the compound possessed a piperidine ring with thirteen methylenes attached to it



(19) Julifloridine



(20) Juliflorine



(21) Juliprosine

Figure 7.

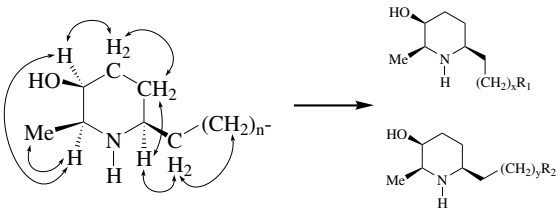


Figure 8.

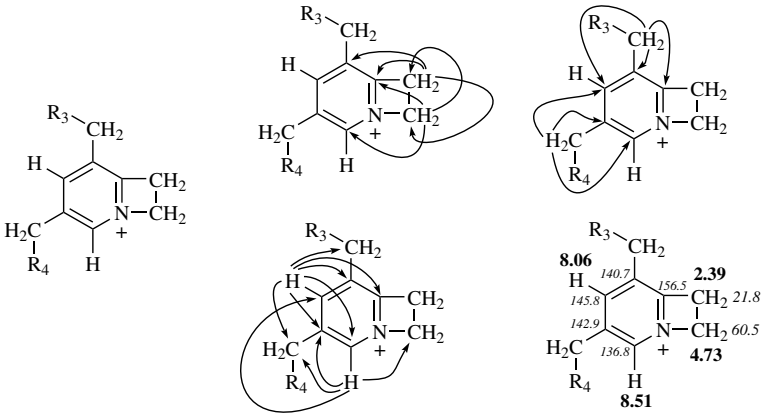


Figure 9.

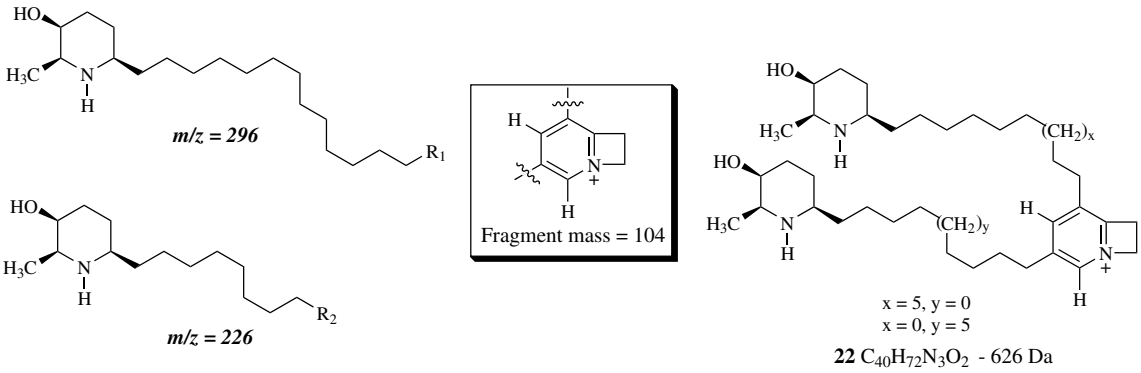


Figure 10.

and a piperidine ring with eight methylenes attached to it (Figure 10). Together with the heterocyclic system this suggested the correct molecular formula. Unfortunately there is ambiguity concerning the position of attachment of the two piperidine/methylene systems, i.e. it is not possible to ascertain at which of the two remaining aromatic positions these moieties are attached. Neither NMR spectroscopy nor mass spectrometry can resolve this ambiguity and only single crystal x-ray structural analysis will resolve this dilemma and at present, the hydrophilic and lipophilic nature of the potentiator (**22**) (Figure 10) has so far prohibited the growth of a crystal of suitable quality for diffraction studies. Four-membered rings possessing a quaternary nitrogen are very rare both in nature and as synthetic compounds and those described from the synthetic literature are brominated on the four-atom ring system (Anderson et al., 1976).

22 was able to inhibit EtBr efflux from *S. aureus* cells with the NorA transporter with an IC_{50} of 7 μM which is marginally better than reserpine (10 μM) but poorer than GG918. Additionally, **22** is antibacterial with MIC values of 4 μg/ml against a panel of MDR MRSA strains and this would compare favourably with one of the newest antibacterial agents daptomycin (cidecid) which had similar levels of *in vitro* potency (2–4 μg/ml).

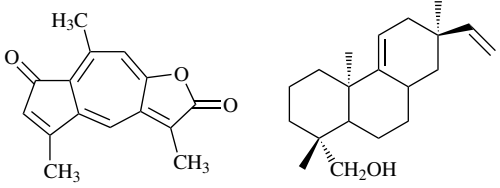
Anti-infective natural products

We are also interested in characterising compounds which have antibacterial activity as bacteriostatic and bacteriocidal agents and there is much potential to exploit plants as a source of novel antibacterial lead molecules. The area has recently been reviewed (Gibbons, 2004) and there is much opportunity to utilise this resource

providing that adequate preliminary evaluation of compounds is undertaken, for example screening against clinical isolates, resistant strains and evaluation of mammalian cell cytotoxicity to ensure that the margin between bacterial and mammalian cytotoxicity can be exploited.

Some of the examples from the literature (see below) are exceptional. The guaianolide sesquiterpene **23**, from *Artemisia gilvescens* (Kawazoe et al., 2003) showed excellent potential against a clinical strain of MRSA (MIC, 1.95 μg/ml) and only minor cytotoxicity toward a human colon carcinoma cell line (IC_{50} = 16 μM (Figure 11)).

An excellent study has been conducted by Timmermann and co-workers (Woldemichael et al., 2003) on the isopimarane diterpene **24**, using SA and MRSA strains and activity was also shown against *Bacillus subtilis* (MIC; 2, 2 and 4 μg/ml respectively). These authors showed that an oxymethylene group at C-19 is important for activity and its replacement with a carboxylic acid reduced activity. **24** non-specifically inhibited uptake and incorporation of radio-labelled thymidine, uridine and amino acids at MIC levels. It was also suggested that **24** functions by a membrane damaging effect. However, using human red blood cells, no haemolytic effect was observed until a concentration of 32 μg/ml was used. The compound was evaluated *in vivo* for its ability, at a subcutaneous dose, to afford protection against SA infection but no protection was seen in a murine model.



23 **24**

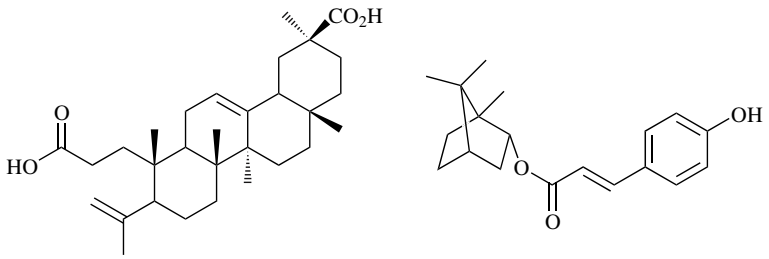
Figure 11.

Triterpenes are well represented in the literature (Muhammad et al., 2000) with a report of a *seco*-A-ring oleanene, koetjapic acid (**25**), from *Maytenus undata* (Celastraceae) (MIC; 50 and 12.5 μg/ml; SA). The *seco* compound is probably related to other oleananes present in the plant *via* a Baeyer-Villiger type oxidation (Figure 12).

Compounds of mixed biosynthesis such as **26**, bornyl coumarate, possessing both phenylpropanoid and monoterpene moieties, had excellent activity against a standard SA strain (0.6 μg/ml) (Setzer et al., 1999). Presumably, the lipophilic monoterpene portion of the molecule allows membrane permeability of this compound and the phenolic coumarate may act as an ionophoric moiety.

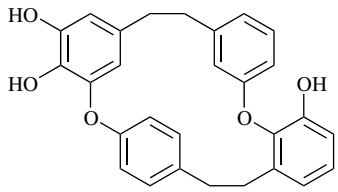
Marchantin A (**27**) from a Hungarian liverwort *Marchantia polymorpha* has exceptional activity against a panel of both Gram-positive and Gram-negative bacteria (SA; MIC = 6.8 nM (!)) (Kamory et al., 1995) and as this compound is cyclic and has lipophilic and hydrophilic domains, it is possible that it functions by forming pores in cell membranes resulting in cell lysis (Figure 13).

Piper gibbilimbum (Piperaceae) is a scrambling shrub used in Papua New Guinea as an antiseptic to heal abscesses, ulceration of the skin and also to treat fevers. Fractionation of the petroleum extract of the leaves of this plant afforded several alkenylphenols (e.g. gibbilimbol B; **28**, MIC = 2 μg/ml, SE) which were evaluated using Brine Shrimp



25 **26**

Figure 12.



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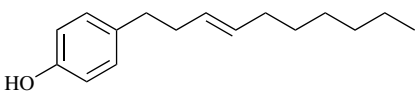

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Figure 13.

and KB nasopharyngeal carcinoma cells (ED_{50} (**28**) 3.9 μg/ml) (Orjala et al., 1998).

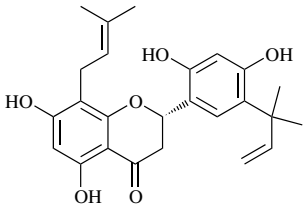
The flavonoids are exceptionally well represented in the literature but specifically it is the flavanones which are the most widely reported anti-staphylococcal compounds of this class. Many reported here possess either prenyl (sometimes more than one) or geranyl groups that presumably contribute to the lipophilicity and membrane solubility of these compounds. Of particular note within this group is **29** with excellent potency toward standard and MRSA strains (MIC = 1.56 μg/ml against both resistant and sensitive strains; Nanayakkara et al., 2002) (Figure 14).

There is a very good rationale that plant alkaloids should possess antibacterial activity, particularly given the number of cytotoxic drugs and templates from this source such as the vinca alkaloids (vincristine and vinblastine) and camptothecin and its synthetic derivatives (topotecan and irinotecan). From *Clausena heptaphylla* (Rutaceae), **30** has a broad spectrum of activity with MIC values of 3, 6 and 20 μg/ml against SA, *Escherichia coli* and *Pseudomonas aeruginosa* (Chakraborty et al., 1995). This activity toward the Gram-negative species, which are generally harder to find hits against, and the simple nature of this carbazole alkaloid are intriguing.

Xanthones are metabolites commonly found in the Clusiaceae (Guttiferae) family and frequently occur as prenylated, geranylated or farnesylated products. From the guttiferous plant, *Garcinia dioica*, rubraxanthone (**31**) has even better *in vitro* potency than vancomycin and is one of the most potent anti-MRSA agents from plants having MIC values ranging from 0.313-1.25 μg/ml toward MRSA and MSSA strains (Iinuma et al., 1998) (Figure 15).

The lipophilic nature of this compound is probably responsible for good bacterial uptake and the authors anticipate that these xanthones will have wide pharmaceutical uses.

Oils from the Garlic genus for example garlic itself (*Allium sativum*, Alliaceae) are rich in sulphur containing natural products (e.g. allicin) and are know to be strongly antimicrobial (Barnes et al., 2002). A series of diallyl sulphides, including diallyl tetrasulphide (**32**) have been evaluated (Tsao and Yin 2001) using SA and MRSA strains with MIC values of 0.5 and 2.0 μg/ml, respectively. The pure compounds and the parent oils



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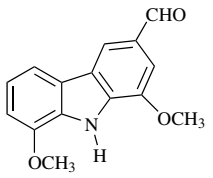

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Figure 14.

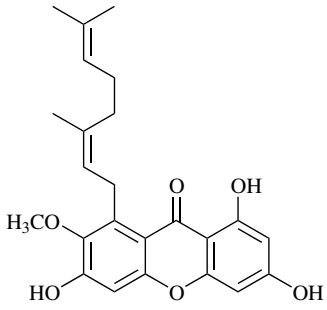

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Figure 15.

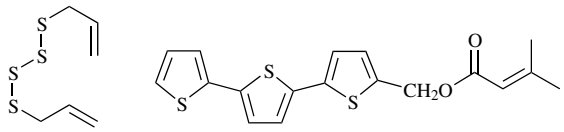

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Figure 16.

from garlic and Chinese leek (*Allium odorum*) were also active against *Candida* and *Aspergillus* species and are probably produced by *Allium* as latent antimicrobial substances (Figure 16).

When induced by UVA irradiation, terthiophenes have antibiotic activity towards viruses, bacteria, fungi nematodes and eggs and larvae of insects. In a study of nine terthiophenes, typified by **33**, Ciofalo et al. (1996) have shown that these compounds are highly active when UV irradiated (MIC = 0.022 μg/ml(!), SA, amikacin = 5 μg/ml) and are inactive at 10 μg/ml to *Pseudomonas aeruginosa*. This natural product exhibits an astounding level of potency toward *S. aureus* and whilst this activity must be initiated by UV light there may be opportunities to use this class as topical antibacterial agents.

The acylphloroglucinols are natural products based on an aromatic ring that in many cases has been reduced or has a keto–enol form. The majority of these products are prenylated and/or farnesylated and possess simple acyl groups such as 2-methylpropanoyl which is found in hyperforin (**34**). This metabolite occurs in *Hypericum perforatum* (St John's Wort) and is commonly used as a herbal antidepressant product. Much work has been done on the antibacterial evaluation of hyperforin and *in vitro* activity is exceptional with MIC values ranging from 0.1 to 1 μg/ml against penicillin-resistant SA (PRSA) and MRSA strains (Schempp et al. 1999; Reichling et al., 2001). These results substantiate the use of St John's Wort in several countries as a treatment for superficial burns and wounds that heal poorly. A recent article describes that exposure of SA to hyperforin leads to a reduced sensitivity to this agent, although it is suggested that resistance cannot be acquired at the doses of which St John's Wort is given for antidepressant activity (Hübner, 2003). The potential use of this agent as an antibacterial is supported by the observation that no resistance occurred at low concentrations of hyperforin and that even in strains with reduced susceptibility, no cross resistance with clinically used antibiotics could be detected. These findings highlight the opportunity to exploit the acylphloroglucinol class as anti-staphylococcal drug-leads and although this compound is known to be unstable (Trifunovic et al., 1998) even the degradation products display moderate activity (50 μg/ml, SA) (Vajs et al., 2003).

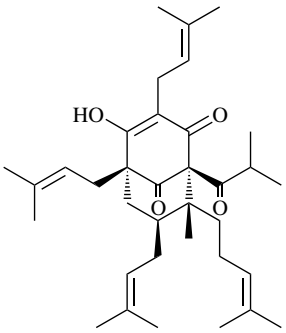
In a collaboration with Professor Giovanni Appendino, we have been evaluating acylphloroglucinol natural products from myrtle, *Myrtus communis* (Myrtaceae) which is used in some Mediterranean countries as a topical antiseptic agent (Figure 17).

The major components of this species include myrtucommulone A (**35**) and semimyrtucommulone (**36**) and these metabolites, particularly **35**, exhibit excellent anti-staphylococcal activity (Table 4), (Appendino et al., 2002).

We have also been investigating the plant family Apiaceae for antibacterial agents and this group have enormous importance as a food source and as medicinal herbal products, particularly in the traditional Chinese Medicine (TCM) system. One of these TCM products is *Angelica dahurica*. The plant is a perennial herb growing to 2.5 m with a hollow stem, large 3-branched leaves and umbels bearing many white flower heads. It grows wild in thickets in China, Japan, Korea and Russia and the cultivated herb is mainly from central and eastern regions of China. The roots are known as Bai Zhi in traditional Chinese medicine, where they are classified as a sweat-inducing drug able to counter harmful external influences on the skin, such as cold, heat, dampness and dryness (Chevalier, 2001). Bai Zhi is also claimed to be effective in the treatment of acne, erythema, headache, toothache, sinusitis, colds and flu (Wagner, 1999). The use of this plant to treat acne indicated to us that perhaps there is an anti-staphylococcal component present and that further investigation was warranted.

Bioassay-guided fractionation of an hexane extract prepared from the roots led to the isolation of the polyacetylenic natural product falcarindiol (**37**), unfortunately a well-known antibacterial substance (Figure 18).

The absolute stereochemistry of this compound was confirmed by careful ^{1}H NMR analysis of its (*R*)- and (*S*)-Mosher ester derivatives as the 3(*R*),



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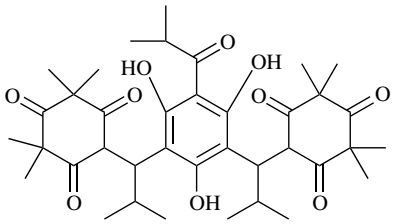

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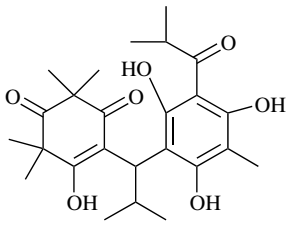

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Figure 17.

8(*S*) isomer. Activity was tracked using a *Mycobacterium fortuitum* screening assay and the purified product was evaluated against multidrug-resistant and methicillin-resistant strains of *Staphylococcus aureus* (MRSA). The minimum inhibitory concentrations (MIC) of this metabolite ranged from 8 to 32 μg/ml highlighting the potential of the acetylene natural product class as antibiotic-lead compounds (Lechner et al., 2004).

We are also interested in evaluating compounds with activity against the mycobacteria. The World Health Organisation has estimated that tuberculosis kills over two million people each year and represents a global epidemic that is worsening due to the occurrence and proliferation of strains of multidrug-resistant (MDR) *Mycobacterium tuberculosis*. New agents are urgently needed to meet the threat of multiple drug-resistant tuberculosis and to manage infection with the naturally resistant non-tuberculosis fast-growing mycobacteria (Gillespie et al., 2001). The spread of TB is cause for considerable concern and there have been outbreaks in major cities such as New York and more recently in a School in Leicester in the UK. With the dearth of agents that can be used against sensitive and resistant strains, new chemistry is needed to address this threat.

Table 4. MICs of *S. aureus* strains to myrtucommulone A (**35**) and semimyrtucommulone (**36**) (μg/ml).

Bacterial Strain (Efflux mechanism)	**35**	**36**	Tetracycline
RN4220 (Msr(A))	0.5	32	0.5
XU212 (Tet(K))	1	32	256
SA1199-B (Nor(A))	1	32	32
ATCC 25923	2	64	0.5

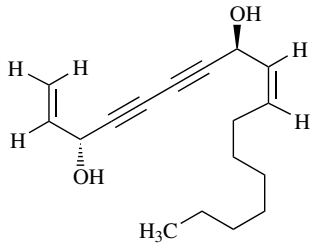

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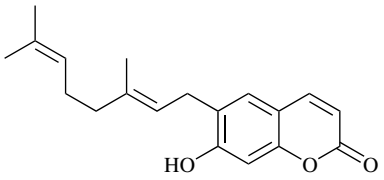
Figure 18.



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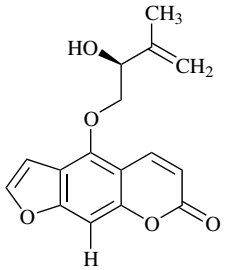

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Figure 19.

We have been using a *Mycobacterium fortuitum* model assay to evaluate plant extracts and purified compounds (Gillespie et al., 2001). This can be performed within 72 h and the assay is conducted in a class II cabinet and there is good correlation with anti-TB drugs. This is an important factor as 'in house' access to TB producing strains is not available to us but through collaboration, so it was vital to use a species of *Mycobacterium* which is a good detector for anti-TB activity.

We have focused on bioassay-guided fractionation of extracts of roots of plants belonging to the Apiaceae with dereplication of known antibacterials components such as the polyacetylenes which display many bioactivities. Our rationale for studying the roots is that mycobacteria are common soil components and it is likely that the roots of plants contain antimicrobial substances as a defence against microbes in their environment. It makes perfect sense to us that these compounds have evolved to afford protection against bacteria and fungi which may be invasive and pathogenic.

In collaboration with Dr Franz Bucar of Karl-Franzens University in Graz, Austria, we have been evaluating species of Apiaceae for their antimycobacterial components. One of Dr Bucar's students, Andreas Schinkovitz visited us and undertook bioassay-guided fractionation of the roots of Masterwort, *Peucedanum ostruthium* (Apiaceae). The most active natural product from this species was ostruthin (**38**) which had MIC values of between 3.4 and 6.7 μM (against four strains of fast-growing mycobacteria (*M. aurum*, *M. phlei*, *M. smegmatis* and *M. fortuitum*) (Schinkovitz et al., 2003) (Figure 19).

This compound highlights the potential of the coumarin class as antimycobacterial leads and ostruthin is currently being evaluated against *Mycobacterium tuberculosis*. We have also been evaluating desert species of Apiaceae, notably *Ducrosia anethifolia* which is a herb that grows along the Arabian Gulf coast of Kuwait. Bioassay-guided fractionation led to the isolation and characterisation of another coumarin natural product pangelin (**39**) which was less active than ostruthin against the same four strains (64 – 128 μg/ml) but again suggest that further exploitation of the Apiaceae and the coumarin group is worthwhile (Stavri et al., 2003). Microbially produced coumarins, for example novobiocin and coumermycin are represented therapeutically and it has been suggested that coumarin antibacterials exert their effects by inhibition of DNA gyrase (Chatterji et al., 2001).

Conclusion

There is a great deal of research pertaining to antibacterial plant natural products and the literature on plant derived anti-staphylococcal natural products has recently been reviewed by the author (Gibbons, 2004). It is however unusual that no single entity plant derived antibacterials are used clinically, especially given the ability of plants to produce cytotoxic agents, many of which are in clinical use e.g. the vinca alkaloids and taxanes. It is also surprising given that there are herbal medicinal products which are in use, for example in the treatment of urinary tract infections by consumption of cranberry juice (Stapleton, 2003).

It is hard to understand why plant antibacterials have not been exploited, but foremost among these reasons is probably that pharmaceutical companies prefer to pursue microbially derived products, of which there are many first class drug examples that can be readily fermented with few

re-supply issues. Companies have in the majority of cases neglected natural products and opted to utilise combinatorial chemistry libraries as a source of chemistry. Unfortunately these libraries lack the true chemical diversity that natural products display (extensive functional group chemistry and chirality) and are poor for discovery purposes but highly useful in lead optimisation. A series of important reviews (Rouhi, 2003) have focused on this deficit and highlight the value of natural products as a screening resource. It is likely that pharmaceutical companies will once again turn their attention to plants, microbes and marine organisms.

Plant sources of antibacterials should not be overlooked as the resistance modifying activity and antibacterial activities are in many cases appreciable. Several of the examples cited here are exceptional with great potential and access to large quantities of these products could be facilitated by large-scale cultivation, failing an economically viable synthesis.

It is most important that these leads undergo further profiling against other Gram-positive and Gram-negative bacteria, particularly against resistant clinically relevant species. Of paramount importance is the evaluation of mammalian cell cytotoxicity to see if the margin between bacterial and mammalian cell toxicity can be exploited and ideally small *in vivo* experiments should be conducted to gauge efficacy.

The burden of multidrug-resistant (MDR) strains will increase pressure to find novel antibacterials with new modes of action and I believe this will drive exploitation of plant sources as antimicrobials. This is highly logical given the ecological rationale that plants produce natural products as a chemical defence against microbes in their environment.

There is also opportunity to discover and characterise bacterial resistance modifying agents. These are analogous to human tumour resistance modifying agents and could readily mirror their successes, for example inhibitors of *p*-glycoprotein, one of the major MDR mechanisms. There is an ecological rationale for the production of natural products that modify microbial resistance. It has been speculated that plants have evolved compounds which evade MDR mechanisms and that plant antimicrobials might be developed into broad spectrum antibiotics in combination with inhibitors of MDR (Tegos et al., 2002). More and more of these MDR proteins are being described and it is likely that their purpose in nature is to remove foreign toxic substances from the bacterial cell. These do however confer resistance to many clinically relevant antibiotics and therefore give a selectional advantage to the organism. The development of broad spectrum resistance modifying agents will be of importance as it is likely that many new efflux resistance mechanisms will be characterised in the future and perhaps the case of polypharmacy can be made where a series of efflux inhibitors are given in combination. This mirrors the use of herbal medicinal products where the plant has produced a series of closely related analogues that may circumvent a variety of efflux mechanisms.

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