Identification of an anti-MRSA dihydrofolate reductase inhibitor from a diversity-oriented synthesis†

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The screening of a diversity-oriented synthesis library followed by structure–activity relationship investigations have led to the discovery of an anti-MRSA agent which operates as an inhibitor of *Staphylococcus aureus* dihydrofolate reductase.

The discovery and development of antibacterial agents is widely regarded as one of the greatest successes of 20th century medicine.[1] However, bacteria have quickly become resistant to the most commonly prescribed antibiotics.[2,3] Combined with the lack of fundamental antibacterial research carried out by pharmaceutical companies over recent decades we are left with a legacy of few new drugs with an ever-decreasing efficacy.[4–7] Thus bacterial infection, particularly from multi-drug resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), remains a serious threat to human lives.[3,4,8–10] Consequently the identification and development of novel antibacterial agents is of paramount importance for human healthcare.[11]

Small molecules that exhibit antibacterial activity (so-called 'hits') can be identified through the phenotypic screening of structurally diverse small molecule collections.[12] However, a formidable challenge associated with the further development of these hits is the identification of the small molecule's biological target, which in turn provides information regarding the molecule's mode of action.[13] Herein, we report the results of screening experiments carried out on a small molecule library produced in a previous diversity-oriented synthesis (DOS) campaign, together with analogue synthesis, SAR analyses and target identification studies. This work has culminated in the discovery of a structurally novel antibacterial agent that displays activity against epidemic strains of MRSA (EMRSA) in cellular assays, and has been shown to act as a prokaryotic-selective uncompetitive reversible inhibitor of the EMRSA-16 dihydrofolate reductase enzyme DfrB ($DfrB_{EMRSA16}$) *in vitro*.

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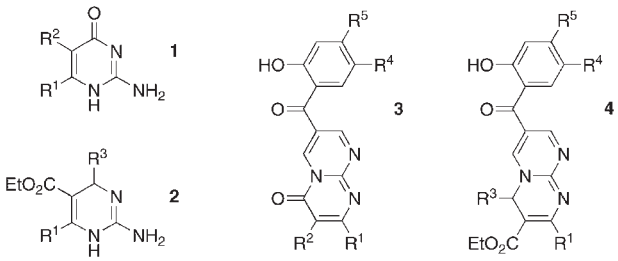
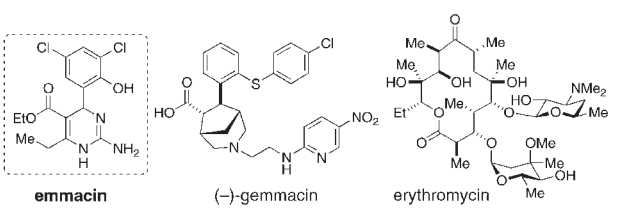


Fig. 1 Nitrogen-based molecular frameworks present in the majority of the DOS library compounds that exhibited anti-MRSA activity.

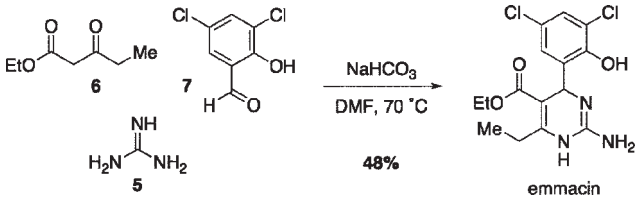
We have previously reported the synthesis of a structurally diverse small molecule library totalling 223 members *via* a DOS approach from a simple fluorous-tagged diazoacetate starting material.[14] Initial inhibition of proliferation phenotypic experiments identified 64 compounds that modulated the growth of EMRSA-15 and EMRSA-16 strains over a concentration range of 100 μM to 10 μM. Compounds based around four types of nitrogen-containing heterocyclic frameworks (**1–4**) were found to dominate as the active species (Fig. 1).

Substituents generally associated with increased levels of antibacterial activity were identified; for example, pyrimidine derivatives (**2**) were typically more active when R^3 = phenyl, thiophene or *iso*-butyl and R^1 = ethyl or aryl. In addition, it was found that heteroatom, in particular halogen, substitution on aryl ring substituents frequently produced compounds with

Table 1 Structures and activities (MIC_{50}) of emmacin, gemmacin, erythromycin and oxacillin, which display growth inhibitory activity against methicillin-susceptible and -resistant strains of *S. aureus*



	MIC_{50} (μg ml^{-1})		
	MSSA	EMRSA 15	EMRSA 16
emmacin	**2**	**9**	**9**
(–)-gemmacin	2	8	16
erythromycin	0.5	>64	>64
oxacillin	0.5	>32	>32



Scheme 1 One-pot synthesis of emmacin.

improved biological activity.[15] Thus various combinations of substituted β-keto ester, aldehyde and 3-formyl chromone 'building-blocks' were used to synthesize a focused collection of 35 compounds.[16]

Purified analogues were screened in inhibition of proliferation phenotypic assays against EMRSA-15 and EMRSA-16 strains.[17] The most potent compound identified by this study was named emmacin, which compared favourably with two clinical antibacterial agents and gemmacin, another anti-MRSA agent discovered recently (Table 1).[12a]

Emmacin was synthesised by a one-pot, three-component Biginelli-type reaction[18] of **5**, **6** and **7** (Scheme 1).[19]

Emmacin was selected for preliminary mode of action studies and subjected to a battery of around 25 assays designed to identify antibacterial, fungicidal and herbicidal properties in addition to common cytotoxic effects. The assays were principally performed using insect or mammalian cell lines and only very moderate fungicidal or herbicidal properties were observed, providing an indication that emmacin is a selective antibacterial agent. Significantly, emmacin did not display activity in a range of cross-indication assays (100 μM emmacin) designed to investigate common cytotoxic modes of action, such as modulation of kinase activity, γ-aminobutyric acid receptors, protein synthesis, generation of reactive oxygen species and ATP synthesis uncoupling. Due to emmacin's structural features we were particularly intrigued by its lack of activity against bovine dihydrofolate reductase (DHFR), which is often associated with mammalian toxicity. DHFR is an enzyme present in all eukaryotic and prokaryotic cells which catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate using NADPH as a cofactor.[20,21] Tetrahydrofolate is involved in the biosynthesis of nucleotide bases of DNA; thus inhibition of the DHFR enzyme blocks DNA synthesis, thereby arresting cell growth.[22] Marked differences in the structures of mammalian and bacterial DHFR enzymes have been exploited in the development of several potent and selective bacterial DHFR inhibitors which have demonstrated antibacterial effects.[22,23] Emmacin contains a nitrogen-based

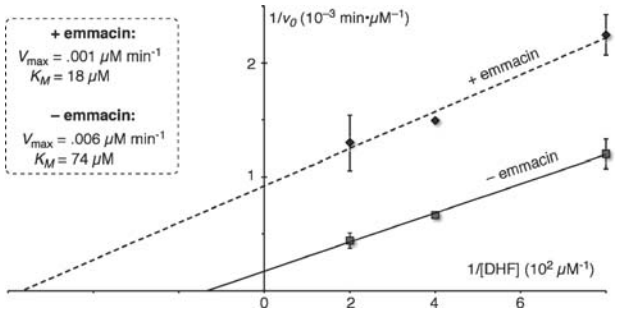


Fig. 3 Lineweaver–Burk plot of enzymatic reaction parameters for the conversion of dihydrofolate (DHF; 12.5–50 μM) to tetrahydrofolic acid by $DfrB_{EMRSA16}$ with constant NADPH concentration (60 μM). '+' and '–' emmacin indicates reaction in the presence (20 μM) or absence of emmacin; v_0 = steady-state reaction velocity (rate); K_M = substrate concentration required for the enzyme to reach half maximum velocity; V_{max} = maximum reaction velocity.

heterocyclic core which is reminiscent of known bacterial DHFR inhibitors (Fig. 2).

The assay results, together with structural similarities with existing bacterial DHFR inhibitors, raised the possibility that emmacin may inhibit *S. aureus* proliferation by acting as a prokaryote-selective DHFR inhibitor. Although there have been reports of anti-MRSA compounds that act as DHFR inhibitors,[23,24] the mechanism of the inhibition has not been well characterised. Therefore in this study we isolated the specific EMRSA-16 DHFR enzyme '$DfrB_{EMRSA16}$' by the expression of a cloned MRSA-16 DHFR gene (*dfrB*) in *E. coli* DH5α-cells. A DHFR inhibition assay was subsequently performed using the purified $DfrB_{EMRSA16}$ enzyme.[25] Emmacin was found to inhibit its enzymatic activity with an IC_{50} value of 5.4 μM (1.9 μg ml^{-1}). Analysis of the observed enzyme kinetics showed approximately parallel lines in the Lineweaver–Burk double reciprocal plot, where both K_M and V_{max} values decrease in the presence of emmacin (Fig. 3). Therefore, emmacin could be defined kinetically as an uncompetitive inhibitor with respect to dihydrofolate,[26] which has been observed for other DHFR inhibitors.[30] The inhibition appeared reversible as normal enzyme could be recovered.

Despite recent research into the discovery of new bacterial DHFR inhibitors,[22,23,27,28] the enzyme is still viewed as an underexploited target in the antibacterial field.[11] Interestingly, we could find no examples of substituted *dihydro*pyrimidine compounds, of the type exemplified in emmacin, having been applied to this therapeutic mode of action. The majority of disclosed agents are based around a hetero*aromatic* core with a 1,3-arrangement of amine groups,[29] which is believed to fit

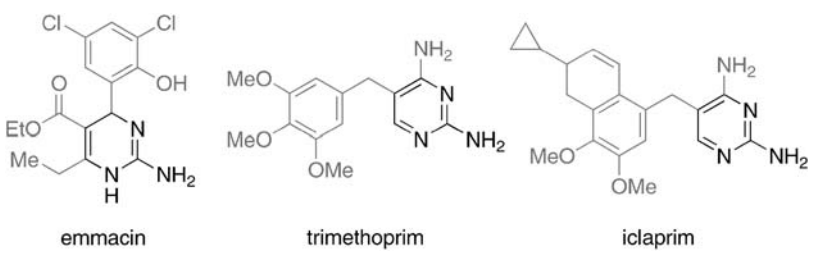


Fig. 2 Comparison of the structures of some known DHFR inhibitors (trimethoprim[11] and iclaprim[23]) with emmacin. The common elements of the nitrogen-based heterocyclic frameworks present in each structure are highlighted.

ideally into a narrow pocket in the active site of the enzyme.[11] Therefore, to the best of our knowledge, emmacin represents the first member of a new structural sub-class of bacterial-selective DHFR inhibitors.[31]

In conclusion, we have exploited a DOS small molecule collection in the discovery of a structurally novel antibacterial agent called emmacin. Emmacin inhibited the growth of two epidemic strains of MRSA *in vitro*, and crucially, was found to be inactive in a variety of mammalian cytotoxicity assays. It was shown to act as a prokaryote-selective, uncompetitive and reversible inhibitor of EMRSA-16 DHFR ($DfrB_{EMRSA16}$). Due to its *dihydro*pyrimidine core, emmacin represents a new structural sub-class of DHFR inhibitors, which could potentially be exploited in the development of critically-needed, new antibacterial agents.

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