Natural product inspired antibacterial tetramic acid libraries with dual enzyme inhibition†

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The application of natural product inspired synthesis has identified novel antibacterial tetramic acids which exhibit wide ranging antibacterial activity, and which provide potential lead structures for antibacterial drug discovery. Their phenotypic activity appears to correlate with action at two enzymes, UPPS and RNAP, which operate in independent metabolic pathways. SAR maps and identification of their relevant binding sites by molecular modelling has been achieved, and characterisation of the most active compounds suggests that these systems offer potential for topical antibiotics but that for oral and injectable use further optimisation is required.

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Introduction

Antibiotic discovery has become imperative as a result of the rapid emergence of virulent bacterial strains resistant to clinical drugs.¹ One approach is the identification of new classes of antibiotics with a novel mode of action (MOA), rather than further optimisation of existing antibiotics.^{2–5} Moreover, it has been suggested that antibiotics interacting at multiple targets rather than a single one might slow the development of target-based endogenous resistance.^{6,7} In this regard, two general avenues have been investigated:⁶ the first is to exploit interactions with multiple targets in a single metabolic pathway, and this has been widely applied in clinically effective mono-therapeutic antibiotics (e.g. β -lactams which mediate cell-wall biosynthesis and fluoroquinolones which mediate DNA replication). The second avenue is to exploit interactions with multiple targets operating in different metabolic pathways, and this concept underpins the successful widespread application of synergistic drug combinations for antibacterial therapy, including the couples gentamicin–vancomycin and sulphamethoxazole–trimethoprim.⁸ A related development, that of the hybridisation of existing antibiotics into a single chemical entity, has also been explored, since this was expected to lead to improved pharmacokinetic (PK) and ADMET properties.⁹

In this paper, we report an alternative paradigm for antibiotic development, based upon one chemical scaffold which possesses dual novel MOA at dual enzymes which mediate different metabolic functions. This approach exploits new

rather than existing MOAs, and might be expected to avoid the resistance mechanisms prevalent with current mono-therapeutic antibiotics along with the complications arising from combination therapies and hybridization strategies. We focused our attention on the tetramic acid (TA) scaffold, known to occur in range of antibacterially active natural products: for example, streptolydigin (3-acyl TAs), which are known to act as bacterial RNA polymerase (RNAP) inhibitors,^{10–12} but which are inactive (or only weakly active) against aerobic bacteria such as *Staphylococcus aureus* and *Escherichia coli*.¹³ However, of interest is that structurally closely related 3-carboxamide TAs act as undecaprenyl pyrophosphate synthase (UPPS) inhibitors with high levels of Gram-positive antibacterial activity.^{14–16} Noteworthy is that the binding sites in these enzymes have not yet been exploited in clinically effective drugs, although antibiotics targeting RNAP, such as rifampicin, have been launched.¹¹ Furthermore, reutericyclin and related 3-acyl TAs of quorum sensing products act by means of depolarization of the bacterial membrane, giving high levels of Gram-positive antibacterial activity, suggesting a further possible MOA of these systems.^{17–19} Other possible MOAs of natural 3-acyl TAs have recently been reported, such as kibelomycin²⁰ (bacterial type II topoisomerase inhibitor) and signermycin B²¹ (histidine kinase WalK inhibitor). With the inspiration of these reports and our successful efforts to develop efficient synthetic methodologies for the TA nucleus,^{22–26} we prepared an extensive library of substituted systems.²⁷ This permitted the identification of a significant number of TAs possessing dual novel MOAs as RNAP and UPPS inhibitors, with or without depolarization activity, accompanied by high levels of antibacterial activity, along with the establishment some structure–activity relationships (SARs). To the best of our knowledge, this is the first example of antibacterial agents with a single structural scaffold which acts at dual novel target enzymes operating in different metabolic

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pathways, and is quite distinct from the monotherapeutic, combination and hybridization strategies already reported.^{8,9}

Results and discussion

Molecular design and synthesis

162 analogues **1–7** and **9–14** (Fig. 1), sub-divided into mono-, bi- and tri-cyclic 3-carboxamide, bi-cyclic 3-acyl and bi-cyclic 3-enamine TAs, shown in detail in Fig. S1–S3 in the ESI,[†] were prepared. Templates **15A–J** and **16A–G** were prepared to give a range of diversity around the tetramic acid core (Fig. 1). It was

thought that bicyclic templates with variation at the 5–8 positions might give modifications of both RNAP and UPPS activity, an idea inspired from Novartis' SAR study for UPPS (e.g. **8A**)¹⁴ and the chemical structure of streptolydigin (**8B**). However, since streptolydigin and Novartis' library possess very different functionality types at the 3-position, it was decided to compare differing functionalities (3-carboxamide, 3-acyl and 3-enamine) at this position.

In the library construction, efficient methodology based upon Dieckmann cyclization which has been reported by us^{22–24} was used for the synthesis of **15A**, **D**, **K** and **16A**, **B**, **H**, **I**, and **15B–G**

Fig. 1 Strategy, templates and representative analogues.

Scheme 1 Reaction conditions; (a) butyl chloroformate (1.2 eq.), DMAP (2.2 eq.), CH₂Cl₂, r.t.; (b) NHR₁R₂, toluene, reflux; (c) phenyl isocyanate (1.1 eq.), DMAP (1.3 eq.), CH₂Cl₂, r.t.; (d) RCO₂H (1.1 eq.), DCC (1.1 eq.), DMAP (1.2 eq.), CH₂Cl₂, r.t.; abbreviation; DCC; *N,N'*-dicyclohexylcarbodiimide, DMAP; 4-(dimethylamino)pyridine.

and **16C–G** were obtained either by using the same strategy or other known procedures^{26,28,29} (see Scheme S1 in ESI†). However, **15J**, possessing di-ester groups on both the 3- and 5-positions, could not be obtained *via* this Dieckmann cyclization approach, but we identified a new strategy in which **15J** was obtained efficiently from **16B** by treatment with butyl chloroformate (1.2 equivalent) in the presence of DMAP (2.2 equivalents, Scheme 1). Diastereoselectivities of relevant compounds were determined by NOE correlation (see Fig. S4 in ESI†). As shown in Scheme 1, the main route for the synthesis of 3-acyl TAs was by direct acylation of templates **16** with commercially available or conventionally prepared carboxylic acid (Scheme S2) by using DCC (1.1 equivalent) and DMAP (1.2 equivalent).²⁵ All 3-carboxamides, with the exception of **11O**, and 3-enamine TAs were prepared from 3-alkoxycarbonyls **15** and the corresponding 3-acetyl TAs, respectively, using a variety of commercially available amines, hydrazides and sulfinamides, providing good to excellent yields of the expected products (20–90%).^{14,30} Importantly, we found new reaction conditions which allowed the direct introduction of a carboxamide functionality at the

3-position of TA, using isocyanates in presence of DMAP (1.3 equivalents), and this approach was used to obtain 3-carboxamide **11O**. These approaches enable the introduction of a wide range of diversity into the library using one late-stage step at the end of the synthesis. Importantly, 3-acyl,²⁵ 3-carboxamide¹⁴ and 3-enamine³⁰ TAs may exist as four tautomeric forms in solution, and based on the literature as well as our own detailed NMR spectroscopic analysis, the dominant form of the 3-acyl and 3-enamine compounds is the *exo*-enol tautomer and of the 3-carboxamide is the *endo*-enol tautomer (see Fig. S6 in ESI†).

Enzyme activity

UPPS and RNAP enzyme activities of the library shown in Fig. S1† were determined and the data for selected analogues is summarized in Table 1 (see Table S1–S4 for full biological data in ESI†). Monocyclic 3-carboxamides **1A–E** derived from template **15K** and the Novartis analogue **8A** exhibited good UPPS activity as expected (IC₅₀ up to 0.1 μM), but were inactive against RNAP. Interestingly, bicyclic 3-carboxamides **2–4** inhibited not only UPPS but also RNAP, with activity which was dependent on the identity of the TA ring substituents. Thus, analogues with lipophilic aliphatic (*e.g.* (±)-**2A**, **E**) and bulky aromatic (*e.g.* (±)-**3F**) groups on a carboxamide pendant exhibited dual inhibition activity, whereas the IC₅₀ values dropped in the absence of the *tert*-butyl group at the 8-position (**4J** > **4M**) for both of the enzymes. These observations indicate that both of these bulky groups might play a major role in the establishment of favourable ligand–receptor interactions. In addition, bicyclic 3-acyl derivatives **5**, **6** also exhibited dual enzyme inhibitory activity when appropriately substituted. For example, 3-acyl **6C** along with 3-carboxamide (±)-**2A** and 3-acyl **6G**, all inspired by the structure of streptolydigin, exhibited dual enzyme activity. On the other hand, all of 3-enamines **7A–E**, structurally similar to the active 3-carboxamides **2–4** and the 3-acyls **5**, **6**, did not exhibit enzyme inhibitory activity, indicating that the oxygen atom of the 3-carboxamide/acyl groups might be a key element for UPPS and RNAP activity. However, enzyme inhibitory activities were only weakly affected by the substitution at the 5- and 6-position, the nature of the heteroatom at the 7-position, and the diastereomeric relationship of the bicyclic template (see

Table 1 Pharmacological properties of selected tetramic acids^{a,b}

	RNA (IC ₅₀ , μM)	UPP (IC ₅₀ , μM)	DEP (IC ₅₀ , μM)	RBC (IC ₅₀ , μM)	CAN (MIC, μg mL ⁻¹)	HEK (LD ₅₀ , μM)	PMB (LD ₅₀ , μM)	SOL (μM)	PPB (%)
(±)- 2A	17	0.38	10.8	>100	>32	30.3	30.3	75–150	— ^c
(±)- 2E	31	0.68	84.3	>100	>32	90.9	90.9	>300	96.6
(±)- 3F	7.3	0.78	>100	>100	>32	30.3	90.9	150–300	— ^c
4J	16	0.17	>100	>100	>32	90.9	90.9	>300	— ^c
4M	52	>10	— ^c	— ^c	>32	— ^c	— ^c	— ^c	— ^c
6C	4.0	3.2	>100	>100	>32	90.9	>90.9	>300	99.8
6 G	2.4	1.9	>100	>100	>32	10.1	>90.9	>300	— ^c

^a Abbreviation; RNA; *in vitro* activity against *E. Coli* RNAP, UPP; *in vitro* activity against *S. pneumonia* UPPS, DEP; *in vitro* activity in depolarization of *S. aureus* membrane, RBC; *in vitro* mammalian red blood cell membrane lysis activity, CAN; *in vitro* antifungal activity against *Candida albicans*, HEK; *in vitro* toxicities against human embryonic kidney 293 cells, PMB; *in vitro* toxicities against human peripheral blood cells, SOL; aqueous solubility at pH 7.4 (water with 2% DMSO), PPB; % ratio of plasma protein binding. ^b See ESI for the other analogues. ^c Not determined.

Fig. 2 Correlation between $-\log \text{MIC}$ values against MRSA and $-\log \text{IC}_{50}$ values against RNAP and UPPS taken (A) independently; and (B) jointly. 40 analogues for the RNAP correlation, 35 analogues for the UPPS correlation and 25 analogues for the joint RNAP and UPPS correlation were used after removing inactive analogues against each enzyme for Fig. 2A and any one of the enzymes for Fig. 2B where RNAP $\text{IC}_{50} > 100 \mu\text{M}$ and UPPS $\text{IC}_{50} > 10 \mu\text{M}$. The red line in Fig. 2B represents linear fit.

detailed discussion of SAR in ESI†). For UPPS activity, 3-carboxamides **2–4** (all with IC_{50} values up to $0.1 \mu\text{M}$) are more effective compared with 3-acyls **5, 6** (with IC_{50} values up to $1.9 \mu\text{M}$), with the values similar to those reported by Novartis for related analogues.¹⁴ On the other hand, 3-acyls **5, 6** (with IC_{50} values up to $1.6 \mu\text{M}$) compared with 3-carboxamides **2–4** (up to $4.3 \mu\text{M}$) are slightly more effective against RNAP and the activity is over 10-fold better than streptolydigin (IC_{50} found to be $38 \mu\text{M}$ in our assay but reported to be $7.5 \mu\text{M}$ in the literature³¹).

Fig. 2A and B depict correlations between MIC values for methicillin-resistant *S. aureus* (MRSA) and their corresponding UPPS and RNAP activity. In order to minimise disruption of this correlation by other factors, any analogues which were inactive against either enzyme for Fig. 2A and B were removed, since although these were antibacterially active, their activity might result from other MOAs, such as any of those exhibited by reutericyclin, kibdelomycin or signermycin B. In addition, analogues which did not exhibit antibacterial activity were also removed, since although enzyme active, their phenotypic inactivity might be caused by cell impermeability or efflux pump transportation. Of particular interest is that the correlation between MIC and each enzyme activity individually is very weak (Fig. 2A), but much better when taken together (Fig. 2B). This feature suggests that the observed antibacterial activity from this compound class might result from a combination of dual targeting ability, although our library appears to be more effective against UPPS than RNAP (Fig. 2A).

In addition, the capacity to disrupt bacterial cell membranes was evaluated with 39 selected analogues (see Table S5 for full biological data in ESI†). 3-Carboxamides **1E**, ($\pm$)-**2A**, **E**, ($\pm$)-**3G**, **H** and **4C**, **D**, **L** were found cause membrane disruption, whilst amongst the 3-acyls, only **5D** exhibited such activity (up to $10.8 \mu\text{M}$ for ($\pm$)-**2A**), which is significantly more active than that of racemic reutericyclin ($88.0 \mu\text{M}$ in our assay). Surprisingly, none of the analogues disrupted mammalian red blood cells ($\text{IC}_{50} > 100 \mu\text{M}$), and were inactive against the fungus *Candida albicans* ($\text{MIC} > 32 \mu\text{g mL}^{-1}$), further supporting high selectivity. The toxicities against human embryonic kidney 293 cells (Hek293) and human peripheral blood cells (PMBC) were also evaluated for 39 of the analogues. Noteworthy was that monocyclic 3-carboxamides tended to be less toxic than bicyclic 3-carboxamides, and amongst the bicyclic 3-carboxamides, phenyl derivatives tended to be more toxic than alkyl derivatives. In addition, aqueous solubility was generally acceptable ($>150 \mu\text{M}$), with the exception only of ($\pm$)-**2A** and **5D**, **E**.

Overall, in terms of multiple targeting, dual novel MOA with major (UPPS) and minor (RNAP) targets (e.g. ($\pm$)-**3F**; 0.78 and $7.3 \mu\text{M}$ respectively), balanced activities (e.g. **6G**; 1.9 and $2.4 \mu\text{M}$ respectively) and major (UPPS) and minor (RNAP) targets along with the depolarization ability (e.g. ($\pm$)-**2A**; 0.38 , 17 and $10.8 \mu\text{M}$ respectively) were found. We believe that these IC_{50} values are appropriate for multiply targeting antibiotics; they may be compared with IC_{50} values of clinically exploited fluoroquinolones against *S. aureus* DNA gyrase and topoisomerase IV such as ciprofloxacin (61.7 and $3.0 \mu\text{M}$ respectively), gemifloxacin (5.6 and $0.4 \mu\text{M}$ respectively) and moxifloxacin (27.5 and $1.0 \mu\text{M}$ respectively).³²

Docking model

Docking calculations of 3-carboxamide **2A** and 3-acyl **6C**, considered as 4 possible tautomers, into one binding site of UPPS (PDB code: 1V7U)³³ and two binding sites of RNAP (PDB code: 1ZYR with streptolydigin¹⁰ and 3DXJ with myxopyronin³⁴) were carried out.³⁵ The calculated docking energies and the number of alternative conformations reflecting the specificity of the binding site (1 hit reflects the most specific binding according to the calculation, and means that all 100 independent docking runs of a given protein–ligand pair resulted the same docking geometry) are shown in Table S7 in the ESI†. All tautomers of **2A** and **6C** docked into these 3 sites yielded highly negative docking energies, consistent with all modes of docking being possible. In terms of binding site selectivity, docking to UPPS showed the lowest energies, reflecting higher binding affinity, unlike docking to RNAP (Fig. 2A). Although docking energies to the RNAPs were in the same range, the streptolydigin binding site (1ZYR) showed a lower number of possible conformations than the myxopyronin binding site (3DXJ), indicating the former site might be preferred over the latter. On the other hand, for tautomer selectivity, the differences of the binding energies ($<1 \text{ kcal mol}^{-1}$) and the number of conformations were small (≤ 1 , with the exception of **2A** to 1V7U), indicating tautomeric forms were not likely to affect binding affinities. Taking into account the docking results above, and

the ligand stability in which tautomer C was found to be less stable both by NMR and computational studies,²⁵ ligand–protein interactions of **2A** as tautomer A and **6C** as tautomer B to 1V7U (Fig. 3A) and **2A** and **6C** as tautomer B to 1ZYR (Fig. 3B) were investigated in detail (see also Fig. S7–S13 for full docking models of **2A** and **6C** as each tautomer into 1V7U, 1ZYR and 3DXJ in the ESI†). Both **2A** and **6C** were found to bind to 1V7U with hydrophobic and hydrophilic interactions. The adamantyl group in **6C** is located near to a hydrophobic helix composed of residues GLY46, ALA47 and VAL50 and the *tert*-butyl group forms hydrophobic interactions with ILE24, MET25 and TYR68. Strong hydrogen bonds are formed between the oxygen on the 3-acyl moiety with ASN28 and HIS43, and the carbonyl oxygen on the pendant ester with ASN74. However, the conformation of **2A** compared with **6C** is flipped at the binding site, where hydrogen bonds between the oxygen with TRP221 and the nitrogen with ALA69 in the 3-carboxamide moiety are formed, while there are hydrophobic interactions between the adamantyl group and ILE24, MET25 and TYR68 and the *tert*-butyl group with LEU85, LEU88, PHE89 and ALA92. This binding model supports the experimental observation that the oxygen with a lipophilic pendant on the 3-acyl/carboxamide moiety (compared with carbon on the 3-enamine) and *tert*-butyl

groups are essential. In the binding model to 1ZYR (Fig. 3B and S7†), **2A** and **6C** bind in a similar fashion to the StI-pocket and the helix where the streptolol moiety of streptolydigin interacts, but not to the trigger loop region where the TA moiety of streptolydigin interacts.¹⁰ However, the adamantyl moiety is buried into the hydrophobic region formed by ALA447, ALA1082, ALA1085, ILE449 and LEU1086. The positively charged ARG428 forms strong hydrogen bonds with the oxygens on C-2 and the 3-acyl moiety in **6C** and the 3-carboxamide moiety in **2A**, which explains the inactivity of 3-enamines **7** against RNAP. Negatively charged GLU421 additionally forms hydrogen bonds with oxygen on C(2) in **6C**. The well-separated hydrophobic adamantyl and hydrophilic TA moieties are ideal for binding to this site.

Antibacterial activity

Whole cell antibacterial activity was assessed against clinically relevant bacteria, and representative analogues which exhibited both antibacterial and enzyme activity, are shown in Table 2 (see Table S1–S4† for full data). In the assay, skeletons comprising tetramic acids **16** and 3-alkoxycarbonyl derivatives **15** as well as enzyme inactive 3-enamines **7** did not show antibiotic activity against any Gram-positive and -negative strains. However, side chain modified 3-carboxamides and 3-acyls which were enzyme active also showed broad-spectrum activity against various Gram-positive strains and Gram-negative *Haemophilus influenzae*, depending on their physicochemical properties (MIC up to $\leq 0.06 \mu\text{g mL}^{-1}$), but did not show activity against Gram-negative *E. coli*, including an efflux-negative strain, and *Pseudomonas aeruginosa*, probably caused by impermeability to the outer cell membrane. As shown in Table S6,† **1D** and **5C** exhibited mild activity against a TolC negative strain of *E. coli* (MIC; $8 \mu\text{g mL}^{-1}$) and *Klebsiella pneumonia* (MIC; $>64, 16 \mu\text{g mL}^{-1}$, respectively), but were inactive against both of the wild type strains as well as the AcrB negative strain of *K. pneumonia* (MIC; $>64 \mu\text{g mL}^{-1}$), indicating that RND pumps (especially TolC protein) play a major role in impermeability. Of particular interest is that the phenotypic activities were maintained against virulent Gram-positive strains which cause significant public health infections, such as MRSA, vancomycin-susceptible *S. aureus* and *Enterococcus faecalis*, vancomycin resistant *E. faecium* (VRE), erythromycin susceptible *S. pneumonia* and multi-drug resistant *S. pneumonia*.³⁶ By comparison, the MICs of ciprofloxacin against MRSA and VRE and the Novartis analogue **8A** against MRSA were more than 60-fold weaker than against the non-resistant strain, *S. aureus* 4. However, the activity against efflux-positive *H. influenzae* (HI3) tended to be weaker and that against efflux-negative *H. influenzae* (HI4) tended to be stronger than Gram-positive strains.

Structure–activity relationship

In order to understand SARs, detailed cheminformatic and bioinformatic analysis was conducted.^{5,37} Fig. 4 presents a plot of ClogP against molecular surface area (MSA). In addition, plots of ClogD_{7.4}, polar surface area (PSA) and relative PSA (rel-PSA (=PSA/MSA $\times 100\%$)) against MSA are also presented in

Fig. 3 Docking model of **2A** (yellow) and **6C** (light blue) to (A) 1V7U and (B) 1ZYR.

Table 2 *In vitro* antibiotic activity (MIC, $\mu\text{g mL}^{-1}$) of tetramic acids^{a,b,c}

	S1	S26	S4	S2	E1	E2	P1	P9	P9B	HI3	HI4
(±)-2A	1	2	1	1	1	1	1	1	1	2	1
(±)-2E	4	4	4	2	2	1	2	1	1	4	0.12
(±)-2I	0.25	0.5	0.25	0.25	0.25	0.25	0.12	0.25	4	>64	4
(±)-3F	1	4	1	0.5	0.5	0.5	0.5	0.5	2	1	0.5
4D	0.25	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.5	2	≤0.06
4G	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	1	1	≤0.06
4H	0.25	0.5	0.25	0.12	0.12	0.12	0.5	0.5	1	1	≤0.06
4J	0.5	1	1	1	0.5	1	0.5	0.5	8	>64	1
5E	0.5	0.5	0.5	0.5	0.12	0.12	0.25	0.12	1	>64	2
6C	2	2	2	1	2	1	0.5	0.5	1	64	0.25
6G	0.5	1	1	0.5	1	0.5	2	1	4	>64	2
8A	0.12	0.12	1	64	≤0.06	≤0.06	2	2	2	>64	0.5
Cip	0.12	0.5	0.12	16	1	32	1	1	1	0.5	≤0.06

^a Abbreviation; Cip; ciprofloxacin as a positive control, S1; *S. aureus* 1, ATCC13709 *in vivo* (methicillin sensitive), S26; *S. aureus* 26, ATCC25923 (vancomycin susceptible), S4; *S. aureus* 4, Oxford, S2; *S. aureus* 2, (MRSA *in vivo*), E1; *E. faecalis* 1, ATCC29212 VanS (vancomycin susceptible), E2; *E. faecium* 1, VanA (vancomycin resistant), P1; *S. pneumonia* 1, ATCC49619 (erythromycin susceptible), P9; *S. pneumonia* 9, (multi-drug resistant), P9B; *S. pneumonia* 9 in presence of 2.5% horse blood, HI3; *H. influenzae* 3, ATCC31517 MMSA, HI4; *H. influenzae* 4, LS2 Efflux knock over. ^b All analogues are inactive (MIC > 64 $\mu\text{g mL}^{-1}$) against *E. coli* 1, ATCC25922 (non pathogenic strain), *E. coli* 50, Ec49 No Efflux and *P. aeruginosa* 1, ATCC27853. ^c See ESI for the other analogues.

Fig. 4 Plot of ClogP against MSA of bicyclic 3-acyl (66 examples) and 3-carboxamide (86 examples) TAs against (A) *S. aureus*, (B) *H. influenzae* 3 and (C) efflux-negative *H. influenzae* 4. For comparison, (D) gives MICs against *S. aureus* without consideration of the species of strains of natural (34 analogues) and unnatural (66 analogues) 3-acyl TAs and Novartis' 3-carboxamide TAs (10 analogues) reported in the literature. Active, mild and inactive correspond to MIC ≤ 4 $\mu\text{g mL}^{-1}$, 4 $\mu\text{g mL}^{-1}$ < MIC ≤ 32 $\mu\text{g mL}^{-1}$ and MIC > 32 $\mu\text{g mL}^{-1}$, respectively. In order to compare with Fig. 4A–C in increased resolution, Fig. 4D has been cut off at 250 < MSA < 1100. See Fig. S17† for the full version.

Fig. S14–S17.† Although molecular weight has been generally used to parallel steric effects in the literature, we selected MSA as a better measure. The SARs against both MRSA and efflux-negative HI4 bacteria show similar trends, in which ClogP, ClogD_{7.4} and PSA of active analogues changes approximately in proportion to MSA (Fig. 4A and C, S14A and C and S15A and C†), while rel-PSA changes in inverse proportion (Fig. S16A and C†). Remarkably, trends resulting from transportation by the efflux pump in *H. influenzae* were also found (Fig. 4B and C); thus, analogues possessing bigger (MSA > 620), more lipophilic (ClogP > 3, ClogD_{7.4} > 1 and rel-PSA < 12%) and higher PSA (PSA > 90) values tend to be more easily transported.^{38–40} In addition, the SAR of the 3-carboxamides along with natural and unnatural 3-acyls against *S. aureus* was compared (Fig. 4A and D). In fact, previously reported unnatural TAs, usually possessing simple functionality without polar atoms on the 1-, 3- and 5-positions, tend to have more limited diversity and to be more lipophilic than natural 3-acyl TAs, while our analogues are more diverse, with the 3-carboxamides in particular being smaller and less lipophilic. In terms of future drug design, antibacterial active 3-carboxamides ($\pm$)-2A, E and 4A, C, G–I, with multiple enzyme targeting ability, possess similar physicochemical properties (530 < MSA < 600, 65 < PSA < 80, 11 < rel-PSA < 14, 1.2 < ClogP < 2.5, $-1.1 < \text{ClogD}_{7.4} < 0$) to clinical fluoroquinolones (380 < MSA < 550, 60 < PSA < 120, 13 < rel-PSA < 24, 0.6 < ClogP < 3.7, $-2.7 < \text{ClogD}_{7.4} < 0.5$) and they also possess favourable rotatable bond counts and hydrogen donor/acceptor counts (see Table S8–S15 for physical properties ESI†). On this basis, these would be of interest for selection for further optimization.

Unfortunately, the activity against *S. pneumonia* 9 in the presence of 2.5% blood was generally shifted to higher MICs to an extent depending on the identity of the template substituents, and the MICs of *S. aureus* Sa26 in the presence of 10% serum were >64 $\mu\text{g mL}^{-1}$ with the exception of 1E (32 $\mu\text{g mL}^{-1}$), 1D (64 $\mu\text{g mL}^{-1}$) and 5E (64 $\mu\text{g mL}^{-1}$). This finding, supported by experimental measurements indicating high plasma protein binding (PPB, 96.6–100%) indicates that further optimisation of these series is needed for their development as oral and injectable actives. As a confirmation of the consequences of high protein binding resulting in low therapeutic concentrations, 1E, ($\pm$)-2E and 5E were tested *in vivo* in a mouse model of thigh muscle infection with *S. aureus*. Even at high doses, the compounds were completely ineffective (data not shown). For further optimisation, SARs for PPB with the physicochemical properties were analyzed (Fig. S18†), and it was found that PPB correlated with lipophilicity (ClogP and rel-PSA) as described in the literature,^{41,42} but not with MSA and PSA. As indicated by the green line in Fig. S19,† analogues defined by ClogP > 2 and rel-PSA < 16%, are likely to show high PPB.

Conclusion

Natural product inspired bicyclic TAs which target novel active sites in two enzymes and which exhibit broad-spectrum activity against Gram-positive bacteria, including virulent strains, have been found. As a result of their dual targeting ability, good antibiotic activity, high selectivity, low toxicity and acceptable

solubility, ($\pm$)-2E, 4G, I and 6C offer promise for application as topical antibiotics for skin, ear and respiratory tract infections, indications which are mainly caused by the pathogens *S. aureus* and *S. pneumoniae*.^{36,43} Furthermore, ($\pm$)-2E and 4G, I with appropriate lipophilicity and drug-likeness are viable candidates for optimization to overcome PPB effects for oral and injectable use. Our results suggest that a strategy developing small molecule agents which target dual novel active sites in dual enzymes might similarly be useful for the identification of new antibacterials, especially since favourable activity, PK and ADMET profiles within the one TA scaffold can exist. Moreover, this work demonstrates that a strategy based upon the use of natural products as start points for drug discovery, promoted by Waldmann amongst others,⁴⁴ and one which has been previously proposed^{45,46} as a way out of the drug discovery deficit,⁴⁷ will provide access to novel antibacterials.⁴⁸ Other recent successes deploying a similar approach have been reported,⁴⁹ and this naturally inspired approach offers significant opportunity for novel antibacterial drug discovery.

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