A detailed study of antibacterial 3-acyltetramic acids and 3-acylpiperidine-2,4-diones

Dr Yong-Chul Jeong, Dr Zsolt Bikadi, Dr Eszter Hazai and Professor Mark G. Moloney*,

Addresses*

Abstract

Inspired by the core fragment of the antibacterial natural products, 3-acyltetramic acids and 3-acylpiperidine-2,4-diones have been synthesised from the core heterocycle by direct acylation with the substituted carboxylic acids using a strategy which permits ready access to a structurally diverse compound library. The antibacterial activity of these systems has been established against a panel of Gram positive and Gram negative bacteria, with activity mostly against the former, which in some cases is very potent. Data consistent with modes of action against undecaprenylpyrophosphate synthase (UPPS) and/or RNA polymerase (RNAP) for a small subset of the library has been obtained. The most active compounds have been shown to exhibit binding at known binding sites of streptolydigin and myxopyronin at UPPS and RNAP. These systems offer potential for their antibacterial activity, and further demonstrate the use of natural products as biologically validated start points for drug discovery.

Key words: antibacterial, drug discovery, tetramate, synthesis

Introduction

The emergence of persistant nosocomial and community acquired infections, caused particularly by bacterial strains resistant to current clinically effective drugs such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and *Enterococcus* (VRE) and multi-drug resistant *Streptococcus pneumoniae* (MDRSP), requires the urgent development of new antibacterial drugs.[1,2] However, a crisis era appears to have emerged in which the speed of new antibacterial development is slower than the emergence of resistance.[3] Approaches using new or hybridised forms of existing antibacterials, and combination therapies, have helped to redress the balance, but the discovery of new classes of

* Dr Yong-Chul Jeong[a], Dr Zsolt Bikadi[b], Dr Eszter Hazai[b] and Professor Mark G. Moloney[a]*,

[a] Chemistry Research Laboratory, University of Oxford, Mansfield Rd, University of Oxford, OX1 3TA, U.K.

Tel: +44 1865 275656; e-mail: mark.moloney@chem.ox.ac.uk

[b]Virtua Drug, 4C Csalogany Budapest, H-1015 Hungary

antibacterials is underexploited.[4-9] The identification of antibacterials with novel modes of action (MOAs), or which have new mechanisms at existing targets, is of particular interest, not least because it might be expected to slow the emergence of resistance development. Antibacterial drug development is now perceived to be expensive and of high risk, at least compared to other therapeutic areas, and few new antibacterial families have been launched since 1970, linezolid and the topical agent retapamulin being examples. However, small molecule natural products and their analogues derived from diverted synthesis provide major inspiration in drug discovery, and this is especially true for antibacterial agents.[10-12] Of interest are 3-acyltetramic acids (3ATs), found as the core structure in many natural products which possess intrinsic antibacterial activity, including for example magnesidin A (**1a**), reutericyclin (**1b**), quorum sensing product (**1c**), melophlin G (**1d**) and streptolydigin (**1e**) (Figure 1).[13-19] In a search for more effective compounds based on these natural products, the antibacterial activity of some unnatural 3ATs has been examined.[20-27] We have reported antibacterial bicyclic system 3AT **1f**, along with related 3-carboxamide tetramic acids, which exhibit bacterial RNA polymerase (RNAP) and undecaprenyl pyrophosphate synthase (UPPS) inhibitory activity.[28] The characteristic core in this family, the *N*-substituted 3AT system, has also been mimicked by the a ring expanded 3-carboxamide piperidine-2,4-dione **1g**, which shows antibacterial activity.[29] Since the SARs of such unnatural *N*-acyl 3ATs and 3-acylpiperidine-2,4-diones (3APs) have not been studied in great detail, we decided to systematically explore the antibacterial activity of *N*-unsubstituted, *N*-alkyl and *N*-acyl 3ATs and 3APs. We have established facile synthetic methodologies both for core tetramic acid templates, and for their acylation at the 3-position, routes which provide direct access to 3AT compound libraries bearing a large range of diversity.[30,31] In this paper, we report the antibacterial activity and SARs of 3ATs and 3APs, with three points of diversity, achieved by modified substitution at N(1), C(5) and the 3-acyl position.

Figure 1 near here

Results and discussion

Synthesis; In order to access a wide range of diversity at the N(1) and C(5) positions, 17 tetramate and piperidinone templates were prepared (Figure 2), seven of which (templates **2a**-**c**, **3b**-**d** and **5a**) have been reported previously; these were predominantly monocyclic ring systems, with the exception of bicyclic derivative **4**.[30] Tetramic acids **3a** and **5b** were accessed from **8a**, itself prepared from glycine ethyl ester hydrochloride, and commercially available *N*-benzoyl-DL-leucine **8b** (Scheme 1(i)), by coupling with Meldrum's acid followed by thermal rearrangement. Tetramic acid **4**, synthesized from (*S*)-**9** using the same method (Scheme 1(ii)), was found to be optically active ($[\alpha]_D^{22}$ = -15.7, c = 0.3 in MeOH), although the enantiomeric excess was not determined. Tetramic acids **6a**-**e** were prepared as shown in Scheme 1(iii); carboxylates **12b,d**,**e** (either made by *N*-acylation of L-methionine methyl ester hydrochloride **10** with the corresponding acid chlorides to give **11a-c**, followed by hydrolysis, or in the case of *N*-

acetyl-L-methionine (*S*)-**12a** and *N*-benzoyl-DL-methionine (±)-**12c** were commercially available) were coupled with Meldrum's acid and then cyclised to give tetramic acids **6a**-**e**. Noteworthy was that tetramic acids **5a**[30,32] and **6a**,**b**,**d**,**e**, synthesized from the optically active parent carboxylic acids, were optically inactive, indicating the high lability of the stereocentre on the C(5) position under these conditions. Application of this methodology to the synthesis of piperidine-2,4-diones **7a**,**b**[33] gave yields which were similar to those of the 5-membered tetramic acids (Scheme 1(iv)).

Scheme 1 and Figure 2 near here

For further derivatisation of the heterocyclic core of the tetramate or piperidine 2,4-dione systems **2-7**, 3-acylation with the desired carboxylic acid was found to be the most convenient (Scheme 2; see Supporting Information for full details), although acylation of 5-unsubstituted tetramic acids *via* *O*-acylation followed by acyl migration also provided indirect but effective access to modified tetramates.[31] Carboxylic acids for introduction as the C-3 side chain were chosen on the basis of systematic change of their structure, along with their hydrophobic/hydrophilic and hydrogen bond donor/acceptor properties, and were generally commercially available; acids **16a-c** and **17** were easily prepared as shown in Scheme 3. This gave a large range of tricarbonyl systems, typified by structures **18** and **19** (Figure 3). An immediate complication in these systems which was encountered is the existence of extensive tautomeric behaviour resulting from their tricarbonyl character, leading to a variety of possible keto-enol structures, and this is discussed in more detail below. Specifically, 3-acyl piperidinediones (3APs) **19a-f** (Figure 4), 3-acyl TAs (3ATs) **20–25** (Figure 5 and Table 1) and **26–31** (Figures 6 and 7), were accessed in this way, some of which have been reported previously.[31]

Scheme 2 and 3, Figures 3-7 and Table 1 near here

Tautomeric behavior; 3ATs **18** and 3APs **19** both exist as an equilibrium between four tautomeric forms A-D in solution (Figure 3).[31,34-36] For *N*-unsubstituted and *N*-alkyl 3ATs, the predominant forms were the CD external tautomeric pair (about 70-99 %), with internal tautomer D being the main contributor, and the 5-substituted *N*-alkyl 3ATs reported herein showed similar behaviour (see Figure S1 for HMBC NMR spectra of representative analogues and Electronic Supporting Information for the ratio of external tautomers of all analogues). By contrast, *N*-acyl 3ATs generally existed as a similar ratio of the two external tautomeric pairs (AB ↔ CD), in which A and B equally contributed to the external tautomer AB while D was the main contributor to external tautomer CD.[31] However, of greater interest was that the tautomeric behavior of 3APs was different, and in this case, the major external tautomer was assigned as the AB tautomer, with B being the major contributor, while for the minor external tautomer CD, D was

the main contributor (Figure S1).

This tautomeric behaviour is supported by calculation of ground state energies of simplified analogues **18** (n = 0, R_1 = $CH_3C(O)$, R_2 = H, R_3 = Me) and **19** (n = 1, R_1 = $CH_3C(O)$, R_2 = H, R_3 = Me) (Table 2). Unlike the tautomeric behavior of 3ATs,[31,34-38] this is the first study of such behavior for 3APs. Thus, for 5-membered **18**, the ground state energy of tautomers B and D were similar (Δ 0.17 kcal/mol), confirming the experimental ratio for external tautomers AB and CD. In addition, the energy difference between internal tautomers A and B (Δ1.42 kcal/mol) was smaller than for internal tautomers C and D (Δ 5.62 kcal/mol), supporting the experimental ratio in each external tautomer (similar contribution of tautomers A and B, and main contribution of tautomer D). Even though the energy difference was small (Δ 0.92 kcal/mol) in the calculation of the 6-membered **19**, the ground state energy of tautomers A and B, the major external tautomers as shown by NMR spectroscopy, were found to be more stable than tautomers C and D. In addition, tautomer B was more stable than tautomer A (Δ 0.51 kcal/mol), as observed by NMR spectroscopy.

Antibacterial activity; The activity of 104 different derivatives **17** and **20**-**31** against clinically relevant bacteria (8 strains of Gram-positive and 5 strains of Gram-negative bacteria) was screened, using linezolid and ciprofloxacin as standards (Table 1 and 3). Although it was found that antibacterial activity depended both on the structural diversity of the substrate as well as the bacterial strain, the ring size of the templates (5-membered tetramic acids versus 6-membered piperidine 2,4-diones) was not found to be a critical factor for the intrinsic antibacterial activity, as has been reported for 3-carboxamides.[29] Significantly, 3-acyl analogues were more effective against Gram-positive than Gram-negative bacteria. Amongst Gram-negative bacteria, no 3-acyl analogues exhibited activity (MIC ≥32 μg/mL) against either *P. aeruginosa* or efflux-positive and -negative *E. coli* strains, indicating that the inactivity is likely to be a result of cell impermeability. In addition, the activity against efflux-positive *H. influenzae* (H3) was usually weaker than that against the efflux-negative strain (H4), consistent with effective transport of 3-acyl analogues by membrane efflux pumps. However, the 3-acyl analogues not only showed excellent antibacterial activity against Gram-positive bacteria (MIC as low as ≤0.06 μg/mL), depending on their substituents, but also retained a good level of activity against all of the virulent Gram-positive strains, compared to non-resistant *S. aureus* (S4). By contrast, the activity of ciprofloxacin against MRSA and VRE decreased more than 50-fold for these organisms. The SAR of the 3-acyl analogues reported in this study are therefore similar to the bicyclic 3ATs described previously.[28]

Table 2 near here

The SAR of analogues possessing *n*-alkyl groups pendant on the 3-acyl moiety is shown in

Table 1. Amongst these analogues, *N*-Boc derivatives **20i-l** were inactive against all bacterial strains, while the activity of other analogues was found to critically depend on chain length.[28,17] However, the optimal chain length varied with the identity of the individual templates, and is probably related to lipophilicity as described below (see Table S1 in Supporting Information for the calculated physical properties). For example, amongst **20a-d** (derived from **2a)**, undecanoyl **20c** was the most active while hexyl **20a** was inactive, while amongst **20e-h** (derived from **2b)**, hexyl **20e** was the most active and undecanoyl **20g** was only mildly active. Amongst **21a-g**, each with a different chain length on the *N*-acyl group, undecanoyl **21b** and hexyl **21e** were the most active in each template system. Acetyl **22a** and **24h** were inactive while acetyl **23a**, the racemic form of reutericyclin,[28,32] was active.

The antibacterial activity of a further 56 analogues, with a large variety of 3-acyl groups, was also assessed (Figures 5-7 and Table 3, and see also Table S2 in the Supporting Information for calculated physical properties). Again, the activity was highly affected by lipophilicity, and varied with the templates: amongst the 3-alkylacyl analogues, **26c** ($ClogD_{7.4}$ = 0.40) compared with **20c** ($ClogD_{7.4}$ = 2.22), **27d**,**e**,**g**, (-2.65 ≤ $ClogD_{7.4}$ ≤ -1.75) compared with **21e** ($ClogD_{7.4}$ = 2.29), **27t**,**u** ($ClogD_{7.4}$; 2.88 and 2.43, respectively) compared with **21k** ($ClogD_{7.4}$ = 3.76), and **17b** ($ClogD_{7.4}$ = 1.40) compared with **25b** ($ClogD_{7.4}$ = 3.92) were all inactive; the $ClogD_{7.4}$ values of these are all significantly lower (more than 2-log) than the corresponding most active *n*-alkylacyl analogues in Table 1. By contrast, **26d**, **27h** and **31c** ($ClogD_{7.4}$: 1.81, 3.01 and 1.88, respectively) were all active; these possess similar lipophilicity to the corresponding active n-alkylacyl **20e**, **21e** and **24a** ($ClogD_{7.4}$; 2.55, 2.29 and 2.73, respectively). Similarly, analogues **28a**,**c**,**e-g**,**i**,**j**, which contained aromatic functional groups with various carbon bridges on the 3-acyl moiety, were inactive or only weakly active against most strains (-0.86 < $ClogD_{7.4}$ < 2.76), while **28b**,**d**,**h** were active (2.48 < $ClogD_{7.4}$ < 3.11). This tendency was also found for aromatic analogues of the other templates (**30a** ($ClogD_{7.4}$ = 4.71, active) versus **30b** ($ClogD_{7.4}$ = 3.02, inactive), and **17d**,**e** ($ClogD_{7.4}$: 2.19 and 2.34, respectively, both active) versus **17f** ($ClogD_{7.4}$ = 1.47, inactive). Interestingly, all of **27a**,**b**,**i-k**, **31d-g** and **17a** (1.25 < $ClogD_{7.4}$< 3.47), possessing non-polar cyclic-alkyl groups (cyclic hexyl, 2-norbonyl, and 1-adamantyl) with methylene bridges, as well as cyclohexyl **27c**,**o**,**s**, **31a** and **17c** (2.21 < $ClogD_{7.4}$ < 3.25) with longer chain bridges (ethylene, propylene and butylenes) showed good antibacterial activity. By contrast, **27f**,**l-n**,**p**,**q**, **30c** and **31b**,**h** (0.01 < $ClogD_{7.4}$ < 2.55), all containing a polar atom in the side chain, were inactive (especially against *S. aureus*) or less active against most strains, even though their lipophilicity is similar to the active n-alkyl analogues discussed above.

Table 3 near here

In order to understand lipophilicity-activity relationships, plots of $ClogD_{7.4}$ against molecular surface area (MSA) of the 3-acyl analogues from this study (104 examples), along with the 3-acyl and the 3-carboxamide bicyclic TAs from our previous study (152 examples) were made

(Figure S2A-C).[9,28,39,40] In the SAR of MRSA and efflux-negative *H. influenzae* (H4) (Figure S2A,C), $ClogD_{7.4}$ of the active analogues is approximately proportional to MSA, with active 3-acyls (red-filled circles) from the current study tending to be smaller than the previously reported active 3-acyls (blue-filled circles) and more lipophilic than previously reported 3-carboxamides (green-filled circles). As noted above, these analogues were readily transported by the efflux pump of *H. influenzae* (Figure S2B,C). Of particular interest is that the efflux pump efficiency is affected not only by lipophilicity (ClogP > 4.0) but also molecular volume (MV > 620 $Å^3$).[28]

There is a similar trend in the plots of ClogP against MSA as shown in Figure S3, but no strong tendency is found in the plot of polar surface area (PSA) against MSA (Figure S4). In the plot of relative-PSA (rel-PSA) against MSA in Figure S5, however, the active analogues are seen to occur at a narrow range of rel-PSA (9 % < rel-PSA < 15 % for MRSA and 11 % < rel-PSA < 15 % for H3). These analogues possess a range of physicochemical properties tending to higher PSA and rel-PSA compared with previously reported unnatural 3AT libraries, as depicted in Figure S2D-S5D.[28] This tendency results from the fact that our library has di- or tri-substitution on *N*(1), C(5) and at the 3-acyl groups, whereas previous libraries usually have only one or two points of diversity and are generally without polar atoms. However, these analogues possess a narrower range of MSA and PSA compared with natural 3ATs, and the library has a higher hit ratio (ratio of active analogues/ total analogues) than these previous libraries, probably as a result of their greater diversity.

In addition, the antifungal activity against *Candida albicans* of the 3-acyl analogues (fluconazole as a standard) was also evaluated, but none were active (minimum inhibitory concentration (MIC) ≥32 μg/mL, data not shown). This is evidence for phenotypic selectivity against bacteria.

Mode of action; Since the MOA of reutericyclin (**1b**)[15] and quorum sensing product **1c**[17] has been reported to be depolarization of the bacterial cell membrane, the depolarization ability at *S. aureus* membrane (DEP) was evaluated with selected 3-acyl analogues (Table 4).[41] Of particular interest is that this disruption ability was found to be sensitive both to the identity of the templates, and the functionalities on N(1) and C(5). In the assay, racemic reutericyclin **23a** exhibited disruption activity (IC_{50}; 88.0 μM), and the more lipophilic substrates **23b**,**c,** with longer side chains, were even more active (IC_{50}; 49.5 and 19.6 μM respectively). In addition, 6-membered **25a**,**b**,**d** (69.0 ≤ IC_{50} ≤ 94.5 μM), with the exception of **25e** (IC_{50}; >100 μM), were active, but 5-unsubstituted (**20e**,**g**,**h**, **21e**-**g**,**i**, **26d** and **27a**,**c**,**i**,**k**), 5-substituted (**24c**,**e**,**i**,**j**,**m**, **30a** and **31f**,**g**,**j**) and bicyclic (**22b**) 3ATs did not exhibit such membrane disruption capacity, with the exception of 5-substituted **24k** and **31e** (IC_{50}; 68.8 and 91.4 μM respectively). In order to determine the selectivity between bacterial and mammalian membranes, red blood cell lysis (RBCL) activity was also evaluated at a concentration of 100 μM. For 3ATs, reutericyclin derivatives **23a**-**c** did not show RBCL activity, whereas *N*-branched alkylacyl **24k** and **31e**

disrupted both membranes. Amongst *N*-acyl derivatives, RBCL ability of the analogues derived from *N*-acyl leucines (**23a-c** and **30a**; inactive) tended to be weaker than that of the analogues derived from *N*-acyl methionines (**24e** and **31f**, inactive; **24c**,**i-k**,**m** and **31e**,**g**,**j**, active). However, 5-unsubstituted **20e**,**g**,**h**, **21f**,**g**, **26d** and **27a** did not show RBCL activity even though **21i** and **27i**,**k** showed such activity. In the assay of 3APs, **25a**,**b** selectively disrupted bacterial membranes, while **25d** disrupted both membranes.

Table 4 near here

Another possible MOA of 3ATs, namely inhibition of bacterial RNAP,[19] was also examined for several selected analogues. 5-Substituted systems **24a**,**b**, **31b** and **24i**,**j** all showed RNAP inhibition activity in the range of $16 \leq IC_{50} \leq 34$ μM and these values are similar to the value of the known natural product inhibitor, streptolydigin (IC_{50}; 38 μM in our assay[28] and 7.5 μM in a previous report[42]). By contrast, 5-unsubstituted **27c**,**h** and bicyclic **22b**, bearing less-hindered groups around N(1) and C(5), and 6-membered **25a**,**b**, were inactive or only weakly active. In keeping with the observation that bicyclic **1f** has been reported to be RNAP active,[28] 5-unsubstituted **27i**,**k** (IC_{50}; 3.1 and 6.7 μM respectively) showed better activity than 5-substituted **31d**,**e**,**f** (IC_{50}; >100, 11 and 40 μM respectively). However, dioxolanyl **30c**, norbornyl **31g** (similar steric effect to the adamantyl group) and the less sterically hindered bicyclic **29a** were inactive or only weakly active. In addition, phenyl **28h** also showed RNAP inhibition activity (IC_{50}; 33 μM) while other phenyl derivatives **28c**,**g** and **31j** were inactive or weakly active.

An alternative possible MOA, inhibition of undecaprenyl pyrophosphate synthase (UPPS), was also evaluated for 3 analogues. Similarly to 3-carboxamide **1g** reported in a Novartis' study (IC_{50}; 0.04 μM),[29] 6-membered 3-acyl **25b** (only weakly active against RNAP (IC_{50}; 86 μM)) was found to be strongly UPPS active (0.6 μM). Notably, monocyclic **27i** showed dual MOA ability as an RNAP (IC_{50}; 3.1 μM) and UPPS (IC_{50}; 0.4 μM) inhibitor, similar to bicyclic **1f**.[28] However, 3AT **28g**, which showed mild antibacterial activity, did not show either RNAP (IC_{50}; >100 μM) or UPPS (IC_{50}; >10 μM) activities.

Binding model; In order to gain insight to the possible modes of substrate binding to RNAP and UPPS, docking calculations with 3AP **25b** and 3AT **27i** were carried out in which one binding site of UPPS (PDB code: 1V7U)[43] and two binding sites of RNAP (PDB code: 1ZYR with streptolydigin[19] and 3DXJ with myxopyronin[44]) were considered, based on the structural similarity of the compound libraries with these ligands (Table 5).[28,45] In order to determine whether there are specifically favoured tautomeric forms for binding, calculation using each tautomeric form was carried out. Although binding energies of 3AP **25b** to both of the RNAP sites yielded substantial negative docking energies, a non-specific geometric conformation was observed for all tautomers, suggesting that 3AP **25b** is unlikely to specifically bind to the RNAP sites. In the docking model for the UPPS site, however, tautomers B and C of 3AP **25b** yielded

not only significant negative docking energies but also a specific docking geometry, while the tautomers A and D had no specific geometric preference. Based on the docking results, as well as from the analysis of the tautomeric behaviour as described above in which the tautomer C is unlikely to be a significant contributor, tautomer B of 3AP **25b** is favoured to bind at the UPPS site. These docking results are in agreement with the experimental data where 3AP **25b** is strongly active at UPPS (IC_{50}; 0.6 μM) but only weakly active at RNAP (IC_{50}; 86 μM). On the other hand, all the docking models of 3AT **27i** yielded significant negative docking energies in which the binding energy at 1V7U was lower than at 1ZYR and 3DXJ. Furthermore, the low number of specific geometric conformations, with the exception of tautomer C to 1ZYR and tautomer A to 3DXJ, support the experimental observation that 3AT **27i** is strongly active at RNAP and UPPS (IC_{50}; 3.1 μM and 0.4 μM, respectively). These results are of interest and suggest that 3AT **27i** might be able to bind to the 3 active sites (multi-targeting ability); if true, this may have significance for the prevention of rapid target-based endogenous resistance,[46] as discussed further below.

Table 5 near here

Figures 9A,B present the docking model of tautomer B of 3-acyl derivatives **25b** and **27i** at the UPPS site. In these models, the phenyl group in 3AP **25b** and the adamantyl group in 3AT **27i** form hydrophobic interactions with the amino acids LEU85, LEU88 and PHE89. The flexible alkyl chains in 3-acyls **25b** and **27i** also form hydrophobic interactions with ILE24, MET25 and ALA69. In addition, the oxygen on the 3-acyl group in 3AP **25b** interacts with ASN28. Analyzing the geometry of tautomer B of **27i** bound at the streptolydigin binding site (1ZYR) of RNAP reveals that the molecule is intercalated between two helices (Figure S6C). The adamantyl group is in hydrophobic contact with PHE1241 and the hexyl chain forms hydrophobic interactions with LEU1094, LEU1256 and ILE1260, even though these amino acid residues do not interact with streptolydigin (Figure S7A). The oxygen of the 3-acyl moiety in 3AT **27i** also accepts a hydrogen bond from ARG1087. Moreover, THR1237 and ASP1090 are in polar interaction with the oxygen of the tetramic acid ring. Of particular interest is that the binding geometry of monocyclic 3AT **27i** is different to bicyclic 3AT **1f**, in which the adamantyl group in monocyclic **27i** is positioned near the binding pocket of the sugar-pendant on the tetramic acid scaffold of streptolydigin, while that of bicyclic **1f** is positioned near the binding pocket of the bicyclic pendant in the streptolol moiety of streptolydigin.[19,28] On the other hand, analysis of the geometry of tautomer B of **27i**-RNAP complex at the myxopyronin binding site (3DXJ) reveals two hydrogen bonds as the driving force of the complexation. Thus, the hydroxyl group of the 3-acyl moiety accepts a hydrogen bond from the backbone amide of GLY620, while the carbonyl oxygen on C(4)-position accepts a hydrogen bond from the backbone amide of GLU1034. In addition, the adamantyl group is in hydrophobic interaction with LEU619 and ILE1467. However, in this case, the hexyl chain does not form favourable hydrophobic interactions, while the alkyl chain, which contains a carbamate, forms a polar interaction (Figure S7B). This result supports

the observation that the shorter chain analogue **27k** is active at RNAP (IC_{50}; 6.7 μM).

Toxicity; The toxicities against human embryonic kidney 293 cells (HEK293) and human peripheral blood cells (PMBC) (Table 4) depended on both the identity of the templates, as well as the functionalities on N(1), C(5)) and the 3-acyl group, although a clear SAR pattern could not be discerned. 3ATs **24e** and **31f,** both derived from **6b**, were less toxic than counterparts **24b**,**c** and **31e,** derived from the less lipophilic **6a**. *N*-Hexyl **20e**,**g**,**h** and **26d** all exhibited lower toxicities (LD_{50}s; ≥90.9 μM) against both cell lines, with the exception of **20g** (HEK293 LD_{50}; 10.1 μM) whereas LD_{50}s of 3ATs **24i-k** and **31g**,**j** derived from **6d** against HEK293 and PMBC were 10.1 and 30.3 μM, respectively. One exception was **24k** against PMBC (LD_{50}; 90.9 μM). However, 3ATs **27c**,**h**,**i**,**k** and **28h** derived from **3a**,**b** (except **27a**) tended to be more toxic against HEK293 than PMBC. Of particular interest is that LD_{50}s of 6-membered **25a**,**b**,**d**,**e** and **17d** against both cell lines were ≥90.9 μM. In addition, reutericyclins **23a**,**b** showed low toxicities (LD_{50}; 90.9 μM) against both cell lines, while the longer chain **23c** (as compared to **23a**,**b**) led to increased toxicity (LD_{50}; 30.3 μM). This dependence of toxicity on chain length was also observed in the other analogues: lengthening of the n-alkyl chain on N(1) and the 3-acyl group increased PMBC toxicity (**21e** → **21f** → **21g** and **24b** → **24c**; **21b** → **21f**) although again no clear relation between the chain length and HEK293 toxicity could be found. In order to judge the balance between antibacterial activity and toxicity, a selectivity index (SI) was calculated in which the ratio of MIC value against *S. aureus* 2 (MRSA strain) against the lower LD_{50} value of HEK293 and PMBC was taken. 3ATs **24e** and **31f** derived from **6b** and reutericyclins **23b**,**c** (with the exception of **23a** (SI; 4.0)) exhibited high selectivities (13.7 < SI <75). Other analogues **20e**, **21e**, **22b**, **27a** and **30a** possessing low toxicity and good antibacterial activity (LD_{50}s; 90.9 μM, MICs; 2-4 μg/mL) also provided high SI values (6.7-19). However, SI values over 10 for **21b**, **24b** and **27c**,**h** possessing mild toxicity and good antibacterial activity were also found. Even though **24i-k**, **27i** and **31g**,**j** were found to be toxic (LD_{50}s; 10.1 μM), their SI values are favorable (8.3 < SI < 16) because of their excellent antibacterial properties (MICs; 0.5 μg/mL), and these SI values are similar to 3APs **25a,d**,**e** and **19d** (8.4 < SI < 9.8) (with the exception of **25b** (SI; 73)). By contrast, highly toxic **20g** and **21g** also possessing weak antibacterial activity had low SI values (SI; 0.23 and 0.06, respectively).

Other biological activities; In order to profile general pharmacological properties, selected analogues were subjected to ADME assays. The 3-acyl analogues were generally soluble in water at pH 7.4 (>150 μM), with the exception of **22b** and **25e** (<100 μM). Unfortunately, high affinity towards plasma protein binding (91.9-100 %) of many substrates interrupted their antibacterial activity, and almost all MICs against *S. pneumonia* 9 (P9B) were shifted to higher values in the presence of 2.5 % blood. MICs were also shifted in the presence of 10 % serum against *S. aureus* Sa26, and only **24b**, **25a**,**b** and **27i** showed some weak activity (16 ≤ MIC ≤

64, data not shown). For the same reason, **24b** and **27i** were inactive in a mouse thigh infected by septicaemia *in vivo* assay (data not shown). Figure S8 presents the relationships of the PPB effect against various physicochemical properties. The PPB effect correlates with ClogP and rel-PSA in which only those analogues possessing lower than a value of ClogP of 1.8 and more than 15 % of rel-PSA are likely to show low PPB affinity. Analogues showing a large MIC difference (over 15 times) are bunched in regions of high ClogP (>3.2) and low rel-PSA (<13.0 %).[47,48]

Conclusion

A series of 3ATs and 3APs bearing a large range of substituents on the N(1), C(5) and 3-acyl groups has been prepared, based on several monocyclic core tetramate templates. The direct 3-acylation promoted by 1.3 equivalent of DMAP offers the easiest way to incorporate the required 3-acyl groups at a late synthetic stage and thereby provides ready access to diversely substituted libraries. Their tautomeric behavior is mainly affected by ring size, with tetramic acids and piperidine-2,4-diones exhibiting very different behaviour, in which the identity of *N*-substitution (rather than C(5)- or 3-acyl substitution) dominates. Thus, *N*-unsubstituted and *N*-alkyl 3ATs favor tautomer D, *N*-acyl 3ATs favor tautomers AB and D, and *N*-acyl 3APs favor tautomer B.

3ATs and 3APs exhibited good antibacterial activity against various Gram-positive strains including MRSA, VRE and MDRSP, along with activity against Gram-negative *H. influenzae*. A strong relationship between antibacterial activity and lipophilicity ($ClogD_{7.4}$ and ClogP) rather than polar surface area (PSA) was found. This SAR study provides a guide for the design of new antibacterial 3ATs. Limited MOA information at two targets (RNAP, UPPS) has been developed, and 5-substitution of 3ATs appears to be necessary for bacterial membrane disrupting ability (e.g. **24a**-**c**,**k** and **31e**; IC_{50}; up to 19.6 μM) while the adamantyl pendant on the 3-acyl position with appropriate functionality on N(1) and C(5) provides for RNAP inhibition activity (e.g. **27i**,**k** and **31e**,**f**; IC_{50}; up to 3.1 μM). This indicates that MOAs should be capable of modulation by incorporation of appropriate functionality. Noteworthy is that **27i** and **27k**, which exhibited low micromolar inhibition of RNAP, and **24b, 24i** and **24j** only moderate RNAP inhibition, were strongly active against the panel of bacteria (Table 2) while **27c, 27h, 31g, 31j, 31f** (Table 3) which were essentially inactive at RNAP nonetheless also showed potent phenotypic antibacterial activity. This RNAP activity is perhaps unsurprising, given the known activity of the natural product parent, streptolydigin, at RNAP for example.[49-51] Moreover, none of these compounds exhibited depolarisation activity (Table 4). This shows that, although a subset of the library may exhibit RNAP activity, this activity is neither necessary nor sufficient for antibacterial activity, and this outcome points to other as yet unidentified modes of action. This could include UPPS activity, since both **25b** and **27i** showed potent activity at this enzyme, but is clear that extensive investigation of these compounds is required in order to fully delineate their MOA. Toxicity against HEK293 and PMBC weakly depended on the

functionalities of the 3-acyl analogues rather than their lipophilicity. Unfortunately, this activity was generally lost in the present of serum, consistent with strong plasma protein binding of these systems.

Of all the systems examined, those of interest appear to be 3ATs **23b**, **27h**,**l** and **31g** which may be promising for topical use (Figure 8), since these were uniformly active against *S. aureus* and *S. pneumonia* (MICs ≤0.5 μg/mL), organisms associated with skin, ear and respiratory tract infections.[52-54] Moreover, such topical applications may not be so affected by plasma protein binding affinity. Also, the possibility of multiple targeting abilities of 3AT **27i** at RNAP (possessing two active sites) and UPPS, and with IC_{50} values amenable for practical application (3.1 and 0.4 μM, respectively) is of interest.[28,55] Importantly, these targets are related to different metabolic functions in bacteria, namely cell wall (UPPS) and RNA synthesis (RNAP), which have not been exploited by current antibacterial agents. By contrast, previously reported multiple targeting antibacterials with novel MOA have been related only to single metabolic functions (e.g. platencin for fatty acid biosynthesis (FabF and FabH)[56] and thiazolidin-4-one-based inhibitor for peptidoglycan biosynthesis (MurD and MurE ligases[57]). Moreover, the depolarization selectivity for bacterial membranes is important, especially since this activity is known to be targeted by some natural products (e.g telavancin[58] and ceragenin[59]) and has been highlighted as being underdeveloped[58] and may provide opportunities for further development, especially in the treatment of slow growing organisms.[60,61] Furthermore, recent work has suggested that appropriate chemical modification of the tetramate/piperidinedione ring system may ameliorate serum binding activity, and this offers significant opportunity for future development.[62]

Figure 8 near here

The tetramates and piperidinediones reported herein provide synthetically accessible but unusual systems worthy of further investigation, for which suitable optimisation might provide novel approach for the treatment of bacterial infections.[63] This results further demonstrates the use of natural products as lead structures with biologically validated start points for drug discovery, as has been promoted by Newman[64] Waldemann[65-67] and Quinn.[68,69]

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Supplementary Information
Supporting Information Available: Experimental detail, spectroscopic data, NMR spectra and Tables of Data.

Experimental

Tautomeric behavior; 3ATs **18** and 3APs **19** both exist as an equilibrium between four tautomeric forms A-D in solution (Figure 3).[31,34-36] Each set of external tautomers (AB and CD) are usually easy to distinguish, since their interconversion rate is slow on the nuclear magnetic resonance (NMR) time scale. By contrast, the internal tautomers of each set (A ↔ B and C ↔ D) are observed as broadened peaks because of their rapid interconversion. After assigning each external tautomer by analysis of HMBC NMR spectra, their ratio could be readily determined from the intensity of separate peaks (usually the C(5) proton) in the ^{1}H NMR spectra. The ratio of internal tautomers was roughly estimated from the chemical shifts of the carbonyl carbons in the ^{13}C NMR spectra. In the ^{1}H NMR and ^{13}C NMR spectra of 3AP **19a**, two sets of signals were observed in a ratio of about 20:80. In order to assign these signals, the HMBC spectrum was acquired and the correlations for the main ring established as shown in Figure 6, in which the major external tautomer was assigned as the AB tautomer (in the HMBC correlation, resonances for the free carbonyl on C(2) in the external tautomer AB (about 165 ppm) and the free carbonyl on C(4) in external tautomer CD (about 190 ppm), which are not affected by the ratio of each internal tautomer, were indicative). The hydrogen-bonded carbonyl on C(4) in the external tautomer AB (about 195 ppm) and the hydrogen-bonded carbonyl on C(2) in the external tautomer CD (about 175 ppm) indicated that tautomers B and D were the main contributors to each external tautomer.

Calculation of physicochemical properties; The energies in equilibrium geometry at ground state were carried out with Density Functional B3LYP (6-31G*) in Spartan 02 in each tautomeric isomer. In the calculation of physicochemical properties, tautomer D was selected. MSA, PSA, ClogP, $ClogD_{7.4,}$ hydrogen bond donor count, hydrogen bond acceptor count and rotatable bond count were obtained from MarvinSketch version 5.3.8. (www.chemaxon.org), in which ClogP and $ClogD_{7.4}$ by using VG method and MSA by using van der Waals method were performed and sulfur atoms were excluded in the calculation of PSA. rel-PSA were calculated from (PSA/MSA) x 100.

Docking study; Docking calculations were performed by using the DockingServer methodology as described in our previous paper.[28] In the course of docking calculations, 100 independent docking runs were carried out and the results were clustered according to docking geometry. A conformation was considered to be relevant if the cluster had more than 10 members, and the lowest docking energy of the given conformation was within 1 kcal/mol of the lowest energy hit. Low numbers of alternative conformations implies potentially specific binding, whereas if the frequency of each conformation is below 10%, there is no significantly favoured complex geometry at the binding site, suggesting no specific inhibition at the binding site.

Biological testing; RNAP and UPPS enzyme assay, MIC determination for bacteria and *Candida albicans* strains, cytotoxicity test in in HEK293 and PBMC cell cultures, solubility assay, plasma protein binding assay, bacterial cell membrane depolarization and red blood cell lysis

were performed as described previously.[28]

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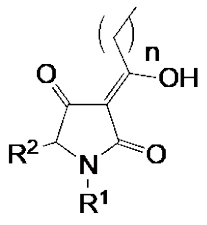
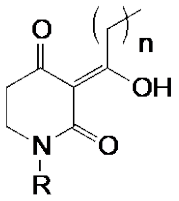
Table 1. *In vitro* antibiotic activity (MIC, μg/mL) of 3-acyl analogues **20**-**25** (for identity of templates, see Figure 2).[a-d]



3ATs **20-24**



3APs **25**

Comp-ound	Tem-plate	*n*+1	Antibacterial strains (MIC, μg/mL)										
			S1	**S26**	**S4**	**S2**	**E1**	**E2**	**P1**	**P9**	**P9B**	**H3**	**H4**
20a	**2a**	6	>64	>64[d]	-[c]	>64	>64	>64	>64	-[c]	-[c]	>64	-[c]
20b		9	8	16[d]	-[c]	16	16	16	64	-[c]	-[c]	>64	-[c]
20c		11	1	1[d]	-[c]	1	0.5	1	64	-[c]	-[c]	>64	-[c]
20d		13	8	1[d]	-[c]	4	4	0.5	64	-[c]	-[c]	>64	-[c]
20e	**2b**	6	4	4[d]	-[c]	4	2	2	8	-[c]	-[c]	4	-[c]
20f		9	4	-[c,d]	-[c]	4	0.5	0.12	8	-[c]	-[c]	>64	-[c]
20g		11	32	32	-[c]	16	16	0.12	8	-[c]	-[c]	>64	-[c]
20h		13	>64	>64	-[c]	>64	>64	>64	16	-[c]	-[c]	>64	-[c]
20i	**2c**	1	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
20j		9	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
20k		11	>64	>64[d]	-[c]	>64	>64	>64	>64	-[c]	-[c]	>64	-[c]
20l		13	>64	>64[d]	-[c]	>64	>64	>64	>64	-[c]	-[c]	>64	-[c]
21a	**3a**	9	2	8	8	4	1	2	1	1	4	8	2
21b		11	0.25	2	1	1	0.25	0.5	<0.06	0.12	2	4	2
21c		13	0.25	16	4	1	2	<0.06	0.12	<0.06	1	>64	4
21d	**3b**	4	32	32	32	32	16	16	8	4	8	4	1
21e		6	1	8	8	4	2	2	1	1	4	8	2
21f		11	8	8[d]	-[c]	8	8	0.12	2	-[c]	-[c]	>64	-[c]
21g		13	64	64[d]	-[c]	64	8	1	2	-[c]	-[c]	>64	-[c]
21h	**3c**	9	0.5	2	2	2	1	1	0.5	0.25	4	>64	16
21i		11	2	8	4	4	4	0.5	1	1	8	>64	>64
21j	**3d**	8	32	32	32	32	32	16	4	4	8	32	8
21k		10	2	8	8	4	2	2	1	0.5	2	32	4
22a	**4**	1	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
22b		13	4	16	4	4	16	4	16	2	64	>64	>64
23a	**5a**	1	8	16	8	8	8	4	2	1	4	64	4
23b		3	<0.06	0.5	0.25	0.25	0.25	<0.06	0.25	0.12	1	>64	2
23c		5	0.25	0.5	0.5	0.5	0.25	0.12	<0.06	<0.06	2	>64	-[c]
23d	**5b**	5	16	32	16	32	16	16	16	16	16	32	8
23e		7	4	16	4	4	4	8	4	2	8	>64	16
24a	**6a**	10	0.5	8	4	2	0.5	0.5	0.5	0.25	2	8	4
24b		11	0.25	2	1	1	<0.06	<0.06	<0.06	<0.06	0.5	2	2

24c		12	0.5	4	2	2	0.12	0.25	<0.06	<0.06	2	>64	8
24d	**6b**	5	2	4	2	4	2	2	2	2	4	32	4
24e		7	0.5	1	2	0.5	0.25	0.5	0.12	0.12	1	16	2
24f	**6c**	8	8	8	8	8	8	4	1	1	4	8	2
24g		10	1	2	2	2	2	0.5	0.25	0.25	2	>64	2
24h	**6d**	1	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
24i		5	0.25	1	0.5	0.5	0.5	0.25	0.25	0.25	2	8	2
24j		7	0.5	1	0.5	0.5	0.25	0.25	0.12	0.12	4	>64	2
24k		9	0.5	1	0.5	0.5	0.5	0.25	0.25	0.12	2	>64	64
24l	**6e**	7	4	8	8	8	8	2	1	1	4	8	2
24m		9	2	4	2	2	2	0.5	0.5	0.25	4	16	4
25a	**7a**	9	2	4	2	4	2	4	0.5	0.5	4	16	8
25b		11	0.5	1	0.5	0.5	0.25	0.5	0.25	0.25	1	8	4
25c		13	2	4	1	2	2	1	1	1	8	>64	32
25d	**7b**	9	8	8	4	4	4	4	2	2	8	64	8
25e		11	4	4	1	4	1	1	0.5	0.5	8	>64	8
linezolid			2	2	2	2	2	2	1	0.5	0.5	16	4
ciprofloxacin			0.12	0.5	0.12	16	1	32	1	1	1	0.5	≤0.06

[a]; Abbreviation; **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* (methicillin resistant), **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, PenR (penicillin and erythromycin resistant), **P9B**; *S. pneumonia* 9 in presence of 2.5 % horse blood, **H3**; *H. influenzae* 3, ATCC31517 MMSA, **H4**; *H. influenzae* 4, LS2 Efflux knock-out, [b]; All analogues are inactive against *E. coli* 1, ATCC25922 (non Pathogenic strain), *E. coli* 50, Ec49 No Efflux and *P. aeruginosa* 1, ATCC27853 (MIC > 32 μg/ml), [c]; Not determined. [d]; Not determined against *S. aureus* 26 in presence of 10 % serum, *E. coli* 50, and *P. aeruginosa* 1.

Table 2. Calculated energy of the ground state of 3AT **18** and 3AP **19**[a]

Compound	Calcd relative energy (kcal/mol)[b,c]			
	Form A	Form B	Form C	Form D
3AT **18**	+1.59	+0.17	+5.62	0
3AP **19**	-0.40	-0.91	+0.01	0

[a]; 3AT **18**; n = 0, R_1 = acetyl, R_2 = H, R_3 = Me, 3AP **19**; n = 1, R_1 = acetyl, R_2 = H, R_3 = Me in Figure 3. [b]; The energy difference between the each tautomer related to tautomer D, [c]; Calculated by using DFT B3LYP (6-31G*) in Spartan 02.

Table 3. *In vitro* antibiotic activity (MIC, μg/mL) of 3-acyl analogues **19** and **26-31** (for identity of compounds, see Figure 5).[a-f]

	S1	S26	S4	S2	E1	E2	P1	P9	P9B	H3	H4
26a	>64	>64[d]	-[c]	>64	>64	>64	>64	-[c]	-[c]	>64	-[c]
26b	>64	>64[d]	-[c]	>64	>64	>64	>64	-[c]	-[c]	>64	-[c]
26c	>64	>64[d]	-[c]	>64	>64	>64	>64	-[c]	-[c]	>64	-[c]
26d	8	8[d]	-[c]	8	16	16	16	-[c]	-[c]	4	-[c]
27a	1	4	2	2	1	2	1	0.5	4	4	1
27b	2	4	4	4	2	2	1	0.5	2	4	0.5
27c	0.25	1	0.5	1	0.5	0.12	0.12	<0.06	2	2	1
27h	0.25	0.5	0.5	0.5	0.25	0.25	<0.06	<0.06	1	2	2
27i	0.25	0.5	0.25	0.25	0.12	0.25	0.12	0.12	1	2	1
27j	16	16	16	16	8	8	4	4	4	16	4
27k	2	2	2	2	0.5	1	1	0.5	1	8	4
27l	>64	>64	>64	>64	16	32	8	16	16	64	8
27m	32	>64	>64	64	16	32	16	8	8	>64	16
27n	>64	>64	>64	>64	8	2	0.5	0.5	8	>64	2
27o	4	8	16	4	4	2	0.5	0.5	4	8	2
27r	>64	>64	>64	>64	64	32	16	32	64	>64	16
27s	16	16	16	16	8	8	2	2	16	32	4
28b	2	4	2	2	2	1	0.5	0.25	2	8	2
28c	64	>64	>64	>64	64	64	64	32	32	64	32
28d	4	32	16	8	1	2	0.5	0.5	4	4	1
28e	64	>64	>64	>64	16	32	16	16	16	32	8
28f	>64	>64	>64	>64	64	64	32	32	32	32	16
28g	16	>64	>64	64	4	8	4	4	8	16	8
28h	1	4	4	2	0.5	0.5	0.5	0.5	4	8	4
28i	64	>64	>64	64	64	32	32	32	64	64	2
30a	2	4	2	2	2	2	1	1	4	>64	8
31a	16	64	32	16	8	8	8	8	16	>64	16
31b	>64	>64	>64	>64	>64	>64	>64	32	32	>64	>64
31c	64	64	64	64	64	32	32	16	16	>64	4
31d	16	32	16	32	16	16	8	8	8	32	4
31e	2	1	4	4	4	4	2	2	4	32	4
31f	0.5	1	0.5	2	0.5	1	1	0.5	2	32	2
31g	0.12	0.5	0.5	0.5	0.25	0.25	0.5	0.25	2	32	2
31i	8	>64	16	8	2	1	2	1	4	>64	8
31j	0.5	1	1	0.5	1	0.25	0.5	0.25	8	>64	8
19a	8	16	16	16	16	16	8	8	8	32	8
19c	1	2	2	2	2	2	1	1	4	16	8

19d	2	4	4	4	4	4	2	1	4	16	8
19e	4	4	4	4	2	2	2	2	8	>64	16

[a]; Abbreviation; see foot note in Table 1, [b]; All analogues are inactive against *E. coli* 1, ATCC25922 (non Pathogenic strain), *E. coli* 50, Ec49 No Efflux and *P. aeruginosa* 1, ATCC27853 (MIC > 32 µg/ml), [c]; Not determined. [d]; Not determined against *S. aureus* 26 in presence of 10 % serum, *E. coli* 50, and *P. aeruginosa* 1. [e]; **27d**,**e**,**g**,**p**,**t**,**u**, **28j**, **29b**, **30b**,**c** and **31h** were inactive against all strains (MIC > 32 µg/ml). [f]; **27f**,**q** (MIC; 32 µg/ml), **28a**, **29a** and **17b**,**f** (MIC; 16 µg/ml) were active against H4 but inactive against the other strains (MIC; > 32 µg/ml).

Table 4. Pharmacological properties of selected tetramic acids[a-f]

	RNAP (IC_{50}, µM)	**DEP** (IC_{50}, µM)	**RBCL**[d] (IC_{50})	**HEK293** (LD_{50}, µM)	**PMBC** (LD_{50}, µM)	**SI**	**SOL** (µM)	**PPB** (%)
20e	-[c]	>100	Inactive	>90.9	90.9	6.7	-[c]	-[c]
20g	-[c]	>100	Inactive	10.1	90.9	<0.23	-[c]	-[c]
20h	-[c]	>100	Inactive	>90.9	90.9	-[c]	-[c]	-[c]
21b	-[c]	-[c]	-[c]	30.3	90.9	11	>300	99.9
21e	-[c]	>100	-[c]	90.9	90.9	7.3	-[c]	-[c]
21f	-[c]	>100	Inactive	30.3	30.3	1.5	-[c]	-[c]
21g	-[c]	>100	Inactive	>90.9	10.1	<0.06	-[c]	-[c]
21i	-[c]	>100	Active	30.3	90.9	2.9	-[c]	-[c]
22b	>100	>100	Active	90.9	>90.9	8.6	38-75	-[c]
23a	-[c]	88.0[e]	Inactive	90.9	90.9	4.0	-[c]	-[c]
23b	-[c]	49.5	Inactive	90.9	90.9	137	-[c]	-[c]
23c	-[c]	19.6	Inactive	30.3	30.3	25	-[c]	-[c]
24b	25	-[c]	-[c]	30.3	90.9	12	>300	93.6
24c	-[c]	>100	Active	30.3	30.3	6.2	>300	99.9
24e	-[c]	>100	Inactive	>90.9	>90.9	>75	>300	-[c]
24i	33	>100	Active	10.1	30.3	<8.3	>300	100
24j	19	>100	Active	10.1	30.3	<8.9	>300	100
24k	-[c]	68.8	Active	10.1	90.9	<9.5	>300	-[c]
24m	-[c]	>100	Active	30.3	90.9	6.9	>300	-[c]
25a	>100	83.6	Inactive	>90.9	>90.9	>8.4	>300	98.9
25b	86 (0.6)[b]	94.4	Inactive	>90.9	>90.9	>73	>300	-[c]
25d	-[c]	69.0	Active	90.9	90.9	9.1	150-300	-[c]
25e	-[c]	>100	Active	90.9	90.9	9.8	19-38	-[c]
26d	-[c]	>100	Inactive	>90.9	90.9	3.3	-[c]	-[c]
27a	-[c]	>100	Inactive	90.9	90.9	16	-[c]	-[c]
27c	>100	>100	-[c]	30.3	90.9	11	>200	100
27h	>100	-[c]	-[c]	30.3	90.9	21	150-300	91.9
27i	3.1(0.4)[b]	>100	Active	10.1	30.3	<16	67-200	99.9

27k	6.7	>100	Active	30.3	90.9	5.7	>300	-[c]
28h	33	-[c]	-[c]	30.3	90.9	6.4	>300	100
30a	-[c]	>100	Inactive	>90.9	90.9	19	>300	-[c]
31e	11	91.4	Active	30.3	90.9	3.2	>300	-[c]
31f	40	>100	Inactive	90.9	>90.9	21	>300	-[c]
31g	>100	>100	Active	10.1	30.3	<9.1	>300	-[c]
31j	91	>100	Active	10.1	30.3	<11	>300	-[c]
19d	-[c]	-[c]	-[c]	90.9	90.9	9.7	>300	-[c]

[a]; Abbreviation; **RNAP**; *In vitro* activity against *E. Coli* RNAP, **DEP**; *In vitro* activity in depolarization of *S. aureus* membrane*,* **RBCL**; *In vitro* mammalian red blood cell membrane lysis activity, **HEK293;** *in vitro* toxicities against human embryonic kidney 293 cells, **PMBC**; *in vitro* toxicities human peripheral blood cells, **SI**; Selectivity index; LD_{50} of one of lower value between HEK293 and PMBC divided by MIC against *S. aureus* 2, MRSA *in vivo* after converting the unit of LD_{50} from μM to μg/mL, **SOL**; aqueous solubility at pH 7.4 (water with 2% DMSO), **PPB**; ratio of plasma protein binding, [b]; *In vitro* activity against *S. pneumonia* UPPS (IC_{50}), [c]; Not determined, [d]; Active; IC_{50} <100 μM or inactive; IC_{50} >100 μM, [e]; reported in our previous paper.[28] [f]; UPPS inhibition activity of **28g** (>10 μM) and RNAP inhibition activity of **24a** (16 μM), **30c** (85 μM), **31b** (34 μM) and **28c**,**g**, **29a**, **31d** (>100 μM), were also determined.

Table 5. Docking results of 3AP **25b** and 3AT **27i**

		UPPS (1V7U)		RNAP (1ZYR[a])		RNAP (3DXJ[b])	
Comp	Tautomer	docking energy (kcal/mol)	number of conformations	docking energy (kcal/mol)	number of conformations	docking energy (kcal/mol)	number of conformations
3AP	A	-8.05	Non specific	-6.08	Non specific	-7.96	Non specific
25b	B	-9.27	1	-7.03	Non specific	-7.32	Non specific
	C	-8.09	1	-6.44	Non specific	-8.27	Non specific
	D	-8.96	Non specific	-7.28	Non specific	-7.11	Non specific
3AT	A	-9.18	2	-7.49	1	-8.13	Non specific
27i	B	-9.69	3	-8.41	1	-8.20	3
	C	-9.43	1	-6.73	Non specific	-7.65	1
	D	-9.14	4	-7.84	2	-8.01	1

[a]; active site of streptolydigin, [b]; active site of myxopyronin.

Scheme 1. Synthesis of tetramic acids and piperidine-2,4-diones. *Reaction conditions*; (a) Meldrum's acid (1.1 eq), DCC (1.1 eq), DMAP (1.1 eq), CH_2Cl_2, r.t. (b) ethyl acetate, reflux; (c)

acid chloride (1.1 eq), triethylamine (2.2 eq), CH_2Cl_2, 0 ℃-r.t.; (d) LiOH (3.0 eq), H_2O: CH_3OH: THF (2:1:1), 0 ^oC-r.t.; Abbreviation; DCC; *N*,*N*′-dicyclohexylcarbodiimide, DMAP; 4-(dimethylamino)pyridine.

Scheme 2. Synthesis of 3-acyltetramic acids and 3-acylpiperidine-2,4-diones*. Reaction conditions*; (a) R_3CO_2H (1.1 eq), DCC (1.1 eq), DMAP (0.1 eq), CH_2Cl_2, r.t.; (b) R_3COCl (1.1 eq), triethylamine (1.2 eq), CH_2Cl_2, r.t.; (c) $(CH_3)_2C(OH)CN$ (0.5 eq), triethylamine (2.0 eq), CH_3CN, r.t.; (d) DMAP (1.3 eq), CH_2Cl_2, r.t.; (e) R_3CO_2H (1.1 eq), DCC (1.1 eq), DMAP (1.3 eq), CH_2Cl_2, r.t.; (f) trifluoroacetic acid, CH_2Cl_2, r.t.

Scheme 3. Synthesis of carboxylic acid derivatives*. Reaction conditions*; (a) (a) RC(O)Cl (1.1 eq), triethylamine (2.2 eq), CH_2Cl_2, 0 ^oC-r.t.; (b) LiOH (3.0 eq), H_2O: CH_3OH: THF (2:1:1), 0 ^oC-r.t.; (c) 2-amino-4,5-dimethylthiazole hydrochloride (1.1 eq), triethylamine (2.2 eq), CH_2Cl_2, 0 ^oC-r.t.

Figure 1. Some naturally occurring and some synthetic 3-acyltetramic acids

Figure 2. 2,4-Dione templates

Figure 3. Tautomerisation in 3-acyltetramic acids and 3-acylpiperidine-2,4-diones

Figure 4. 3-Acyl analogues (see Table 1 and 3)

Figure 5. 3-Acyl analogues (see Table 1 and 3)

Figure 6. 3-Acyl analogues (see Table 1 and 3)

Figure 7. 3-Acyl analogues (see Table 1 and 3)

Figure 8. Chemical systems with optimal properties