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Pyridine-3-carboxamide-6-yl-ureas as novel inhibitors of bacterial DNA gyrase: Structure based design, synthesis, SAR and antimicrobial activity.

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ABSTRACT

The development of antibacterial drugs based on novel chemotypes is essential to the future management of serious drug resistant infections. We herein report the design, synthesis and SAR of a novel series of *N*-ethylurea inhibitors based on a pyridine-3-carboxamide scaffold targeting the ATPase sub-unit of DNA gyrase. Consideration of structural aspects of the GyrB ATPase site has aided the development of this series resulting in derivatives that demonstrate excellent enzyme inhibitory activity coupled to potent Gram positive antibacterial efficacy.

Keywords: Antibiotics, bacterial resistance, DNA gyrase, pyridine-3-carboxamide, molecular modelling, *de novo* design.

Introduction

Due in part to disproportionate prescription of antibiotics during the latter half of the 20th century, the past 2 decades have seen the inevitable emergence of bacterial resistance to all currently available therapeutic agents.[1] Of particular concern are the so-called 'ESKAPE' pathogens (multi-drug resistant *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species), which cause infections associated with elevated rates of morbidity and mortality.[2, 3] The

need for antibiotics based upon novel chemotypes which act upon novel molecular targets has long been recognised as critical to the future management of the problem. A recent manifestation of this concept is the 'ten-by-twenty' initiative, a gauntlet thrown down by the Infectious Diseases Society of America (IDSA) to those involved in antimicrobial drug discovery, urging a more concerted effort with the goal of delivering 10 new antibacterial drugs by 2020.[4]

DNA gyrase and the structurally homologous enzyme topoisomerase IV (topo IV) are highly conserved ATP dependant bacterial type IIa topoisomerase enzymes which play an essential role within bacteria linked to the conservation of chromosomal integrity during DNA replication and transcription.[5] These enzymes have long been validated as therapeutic drug targets owing to the commercial and clinical success of members of the fluoroquinolone antibiotic class (e.g. ciprofloxacin, gemifloxacin) which target the catalytic (GyrA / ParC) sub-units in a dual-inhibitory fashion.[6] More recently, the development of small molecule ATPase inhibitors of gyrase (GyrB) and topo IV (ParE), aided by the elucidation of protein-ligand structures of GyrB, has gained attention as a means to inhibit these classical enzyme targets in the pursuit of novel antibacterial compounds.[7-10]

Encouraged by these reports, our interest in the discovery of novel ATPase inhibitors of GyrB / ParE began with the observation that a number of reports in the literature demonstrated that heterocyclic *N*-ethylurea derivatives to be conducive with potent dual-inhibitory ATPase activity coupled to antimicrobial activity.[7, 8, 10, 11]. Notably, benzimidazol-2-yl-ureas (**1**), benzathiazol-2-yl-ureas (**2**), imidazopyridin-2-yl-ureas (**3**), triazolopyridine-5-carboxamides (**4**), isoquinolin-3-yl-ureas (**5**) and pyridin-3-yl-ureas (**6**) reported by groups at Vertex[7], Prolysis[11], Pfizer[10], Evotec[8], Actelion[12] and AstraZeneca[13] respectively, exemplify the prevalence of this moiety amongst antibacterial GyrB / ParE inhibitors (Fig. 1).

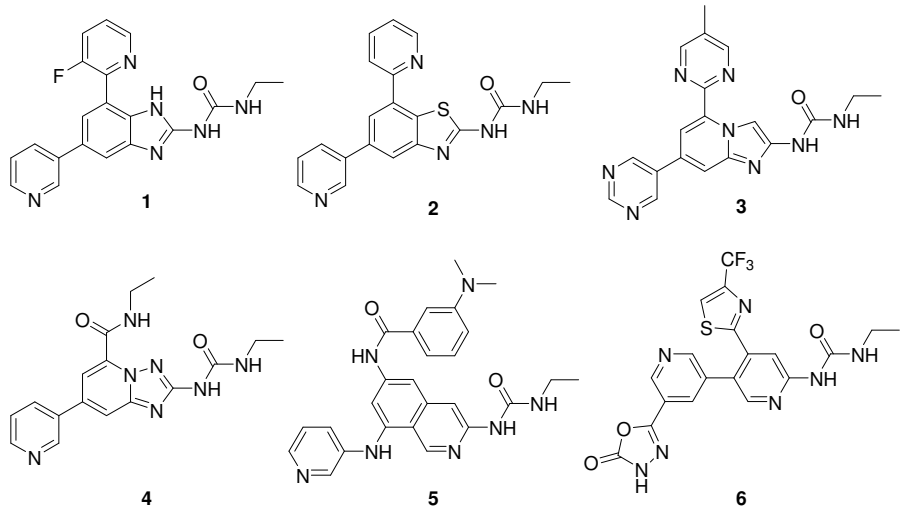


Figure 1. Reported GyrB / ParE inhibitors featuring the *N*-ethylurea moiety

Molecular design of inhibitors

Employing an in house crystal structure depicting the ATP binding domain of GyrB, we explored and modeled a number of alternative heterocyclic core scaffolds which, when coupled to the *N*-ethylurea side-chain, were predicted to have the potential for potent GyrB inhibitory activity whilst offering distinct advantages in terms of optimisation opportunities to compounds **1** – **6**. Specifically, the *de novo* design software SPROUT[14] aided the identification of putative inhibitors with features complementary to the ATP binding pocket.

Using SPROUT, key interactions within the GyrB ATP binding pocket, specifically involving Asn-55, Asp-82, Gly-86, Lys-145 and Arg-85 (Figure 2), were selected and an ethyl urea moiety was positioned so that it would form hydrogen bonding interactions to Asp-82 and Asn-55 (Figure 2). A variety of small structural fragments were also positioned in the other selected target sites (for Arg-85 aryl and heteroaryl fragments, for Lys-145 H-bond acceptors and carboxylates and for Gly-86 H-bond donors) and connected with linker fragments (consisting of aryl, amide and single carbon atoms) to give a selection of complete molecules. A number of such design runs were conducted within SPROUT involving variations in the specific fragments placed at each of the chosen residues and the order in which these were connected. Following the SPROUT runs the designed molecules were analysed based on their predicted binding scores and ease of synthesis.

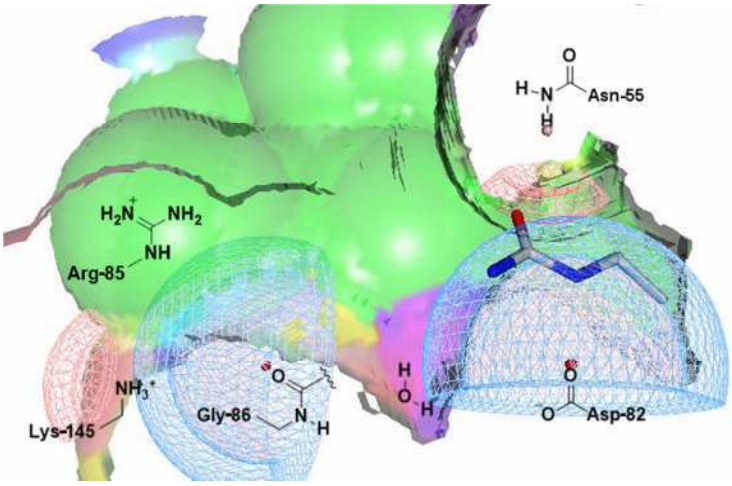
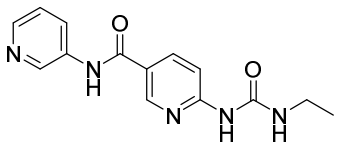


Figure 2. The first stages of *de novo* design using SPROUT. The ATP binding site of *Enterococcus faecalis* GyrB with several target sites selected and an ethyl urea fragment docked into the target sites for Asp-82 and Asn-55.

From a synthetic viewpoint, the most attractive structure to emerge from this process was based on a pyridine-3-carboxamide (**7**) core (Figure 3). Docking of this structure into the ATP binding site within the protein using AutoDock[15] acted as an independent check and confirmed that key binding interactions of known importance to GyrB / ParE inhibition would be maintained. These include hydrogen bonds to Asn-55 / Asp-82 and an aryl π-π stack interaction with Arg-85 (*Enterococcus faecalis* GyrB numbering, Figure 4)[16]. Although treated as neutral by AutoDock during the docking process, Arg-85 is likely to be positively charged under physiological conditions, and therefore interaction of this residue with the pyridine ring may be expected to have both π-π and π-cation character.



7

Figure 3. Simple pyridine-3-carboxamid-6-yl-urea **7** derived using *de novo* ligand design

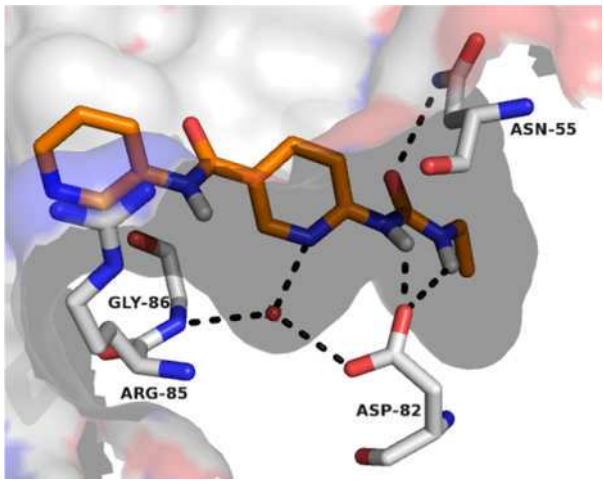
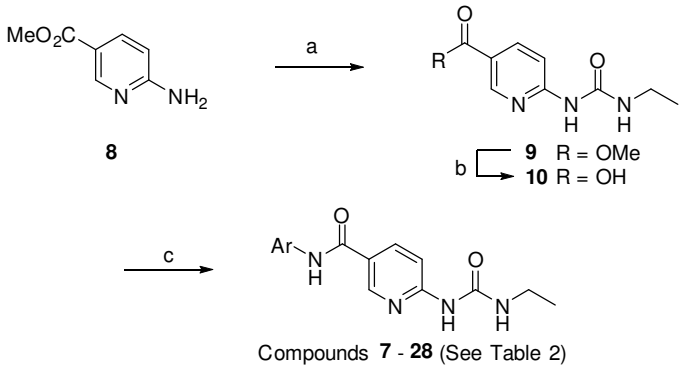


Figure 4. Compound **7** docked within the *E. faecalis* GyrB ATP binding site. Compound **7** is shown as orange sticks, key residues as grey sticks and polar interactions as dashed black lines.

Synthesis of putative inhibitors

To test the modelling hypothesis, compound **7** was prepared according to the procedure described in Scheme 1 from commercially available 6-aminonicotinate **8**.



Scheme 1. Reagents and conditions: (a) EtNCO, 1,4-dioxane, 100°C, 18 h, 63%; (b) 2M NaOH (aq), RT, 16 h, 95%; (c) $ArNH_2$, EDC.HCl, HOBT, DMF, RT-40°C, 16 h, 15-70%.

Compound **7** was screened *in vitro* for GyrB inhibitory activity (ATPase, *E. coli*) and minimum inhibitory concentrations (MICs) against *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *Streptococcus pyogenes* (ATCC 51339) and *Haemophilus influenzae* (ATCC 49247) (Table 1).

Table 1.
Comparison of compound **5** with reported GyrB inhibitors

Compound	GyrB IC_{50} (μM)	MIC (μg / mL)			
		SA	*EF*	*SP*	*HI*
1[a]	0.004	0.03	nd	nd	1
2[b]	< 0.75	nd	<0.25	nd	nd
3[c]	0.053	0.5	nd	0.5	nd
4[d]	0.041	8	4	8	nd
7	6.0	>256	256	32	>256

E. coli GyrB; *SA*, *S. aureus* ATCC 29213; *EF*, *E. faecalis* ATCC 29212; *SP*, *S. pyogenes* ATCC 51339; *HI*, *H. influenzae* ATCC 49247; nd, not determined; [a] data (Ki) from Charifson et al.[7]; [b] data from Haydon et al.[11]; [c] data from Starr et al.[10]; [d] data from East et al.[8]

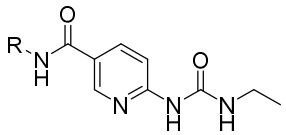
As indicated from inspection of the data in Table 1, although compound **7** synthesised as part of the present work was 1 – 2 orders of magnitude less potent against GyrB than those previously reported, this was encouraging given the lower molecular weight / complexity of this inhibitor. Furthermore, the antimicrobial activity found for this compound, albeit weak, indicated the potential for optimisation. We therefore elected that our continuing investigation would focus on further developing these pyridine-3-carboxamides.

C3 position SAR

Our initial SAR investigation concerned variation of the aryl moiety at the pyridyl C3 position of structure **7** and the target molecules were accessed conveniently through coupling of nicotinic acid **10** with commercially available aromatic amines (Scheme 1, Table 2). We reasoned that variation here could increase the importance of the π-π stacking with Arg-85. Biological data is given in Table 2. In general, substituted phenyl derivatives (entries **11** – **21**) led to improvements in enzyme potency and some MICs were also improved relative to compound **7**. In particular the *ortho*-chloro compound **12** (GyrB IC_{50} 1.6 μM, MIC 16 μg / mL *SA*, *EF*, *SP*) demonstrated a good balance between enzyme and cellular potency. Potent GyrB inhibitors were also identified when the C3 substituent was a 5-membered heterocycle. Thiazole compounds **25** and **26** have IC_{50}s of 420 nM and 960 nM respectively, though these compounds did not demonstrate measurable antibacterial activity in the strains tested (Table 2). Compounds **12**, **15**, **16** and **20**, which possessed antibacterial activity against the wild type strains, were further tested for MICs against a *S. aureus* GyrB mutant (T173N) and a *S. pyogenes* ParE mutant (A53S) respectively. The compounds showed diminished antibacterial activity relative to that observed for the wild-type, consistent with a target based, dual-inhibitory mode of action. None of the compounds tested demonstrated antibacterial activity

against the Gram negative organism *H. influenzae*, highlighting the challenges involved in targeting Gram negative organisms.

Table 2.



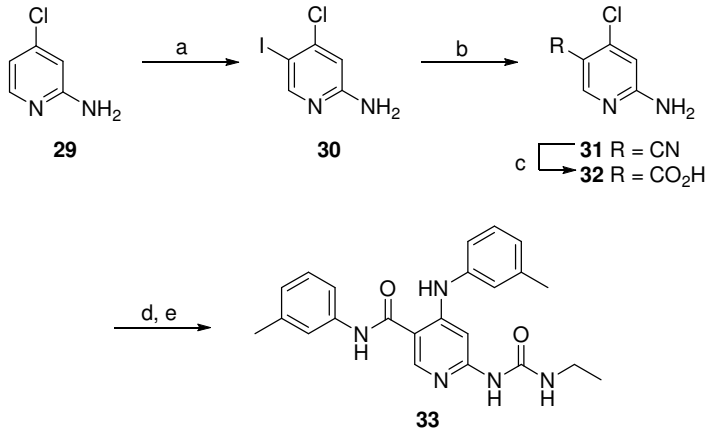
GyrB inhibitory activity and MICs for pyridine-3-carboxamides

Cmpd.	R	GyrB IC_{50}	MIC (μg / mL)					
		(μM)	*SA*	*SA* (T173N)	*EF*	*SP*	*SP* (A53S)	*HI*
7	pyridin-3-yl	6.0	>256	nd	256	32	nd	>256
11	phenyl	7.7	>64	nd	>16	>16	nd	>16
12	3-chlorophenyl	1.6	16	>64	16	16	>64	>64
13	2-chlorophenyl	2.1	>64	nd	>64	>64	nd	>64
14	4-chlorophenyl	3.3	>64	nd	>64	>64	nd	>64
15	3-flourophenyl	3.0	32	>64	32	32	>64	>64
16	3-methylphenyl	3.4	32	>64	32	32	>64	>64
17	3-cyanophenyl	3.8	>64	nd	>64	32	nd	>64
18	3-carbamoylphenyl	24.5	>256	nd	>256	>256	nd	>256
19	3-ethylphenyl	40.4	>256	nd	>256	>256	nd	>256
20	3,4-bismethoxyphenyl	6.2	>64	>64	32	32	>64	>64
21	5-chloro, 2-methylphenyl	7.5	>64	nd	>64	>64	nd	>64
22	pyridin-4-yl	3.5	>64	nd	64	64	nd	>64
23	2-methyl pyridin-4-yl	5.3	>64	nd	>64	>64	nd	>64
24	4-CO_2Me pyridin-2-yl	14.0	>64	nd	>64	>64	nd	>64
25	thiazol-2-yl	0.42	>64	nd	>64	>64	nd	>64
26	5-CO_2H thiazol-2-yl	0.96	>64	nd	>64	>64	nd	>64
27	cyclohexyl	23.1	>64	nd	>64	>64	nd	>64
28	4-methylcoumarin-7-yl	8.4	>64	nd	>64	>64	nd	>64

E. coli GyrB; *SA*, *S. aureus* ATCC 29213; *EF*, *E. faecalis* ATCC 29212; *SP*, *S. pyogenes* ATCC 51339; *HI*, *H. influenzae* ATCC 49247; *SA* T173N, *S. aureus* GyrB mutant; *SP* A53S, *S. pyogenes* ParE mutant; nd, not determined.

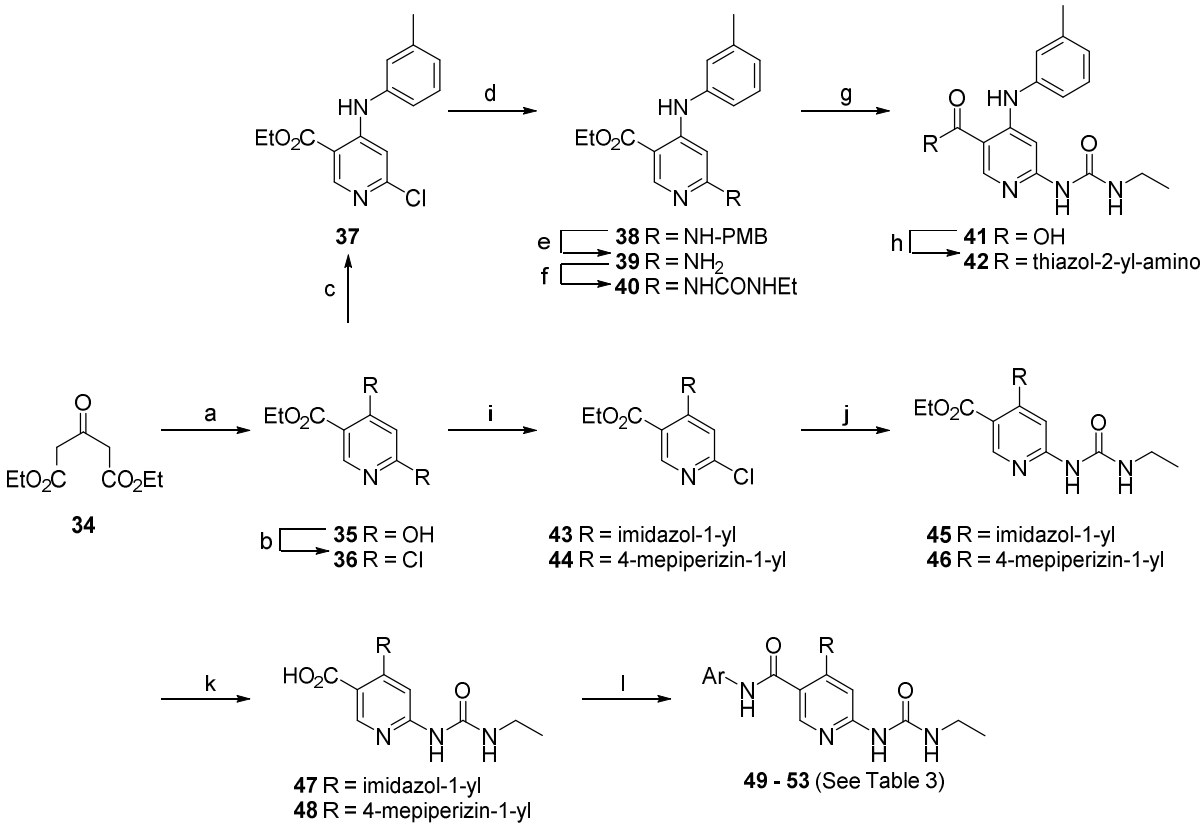
Variation of the C4 position: Synthesis and SAR

According to our earlier modelling (Fig. 3), a sizeable sub-pocket was predicted such that substitution at the pyridyl C4 position in structure **7** was highlighted as an option for structural optimisation. Synthetic routes to these compounds are summarised in Schemes 2 - 5. Our initial route proceeded *via* 5-iodopyridine **30**, which was prepared following regioselective iodination of 2-amino-4-chloropyridine. A modified Rosenmund-von Braun reaction[17] installed the carbonitrile functionality as in **31**, which was hydrolysed under aqueous acidic conditions to give tri-substituted nicotinic acid **32**. Coupling under standard conditions with an appropriate aniline led, unexpectedly, to the 6-amino-3,4-bisanilino intermediate which, when treated with ethylisocyanate, gave pyridin-6-yl-urea **33** (Scheme 2).

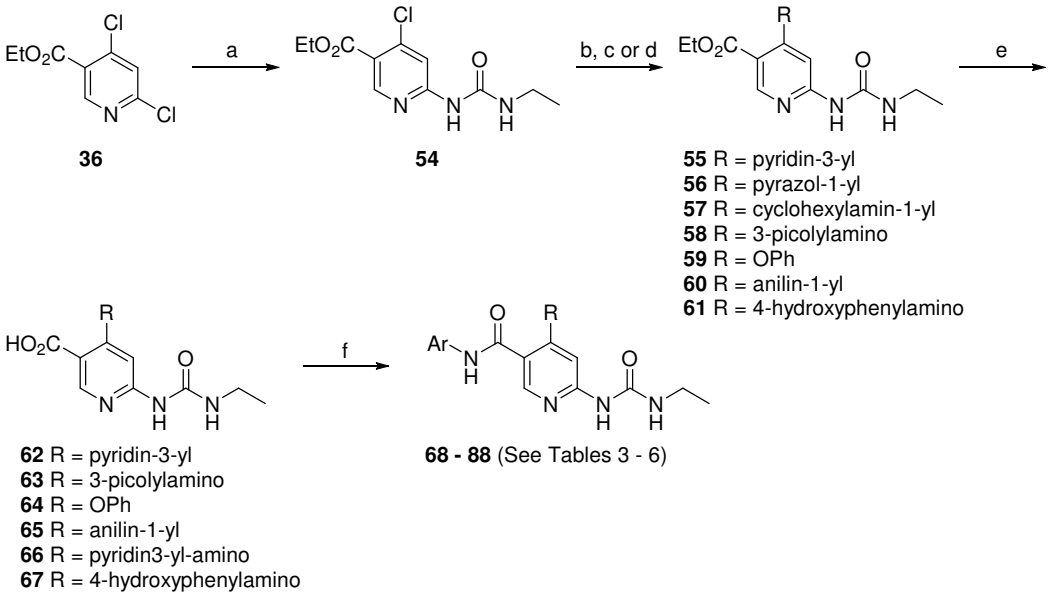


Scheme 2. Reagents and conditions: (a) NIS, DMF, rt, 18h, 60%; (b) $Zn(CN)_2$, $Pd(PPh_3)_4$, NMP, 135°C, 2h, 60%; (c) 1:2 c.H_2SO_4-H_2O, 100°C, 18h, 81%; (d) *m*-toluidine, HOBT, EDC.MeI, DMF, rt, 5h; (e) EtNCO, 1,4-dioxane, 80°C, 16h, 17% (over 2 steps).

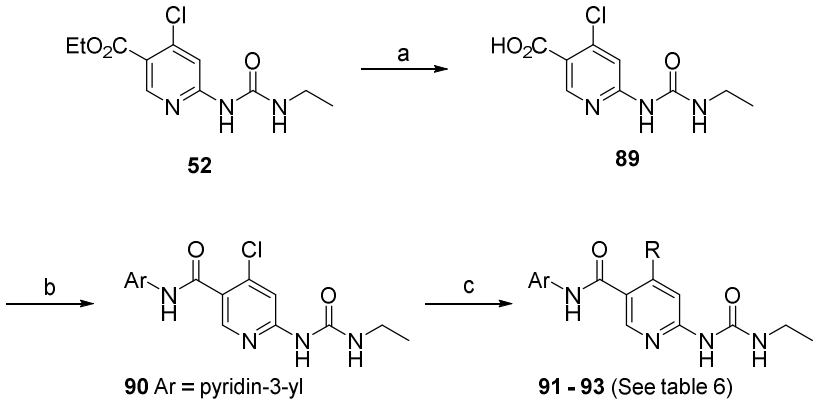
Alternatively, C4 substituted compounds were prepared *via* dichloronicotinate **36**[18] using a range of synthetic methodologies, as indicated in Schemes 3, 4 and 5. Sequential S_NAr at the C4 and C6 positions of pyridine **36**, followed by facile benzyl deprotection using TFA and triethylsilane, gave 6-amino-4-anilinonicotinate **39**. This intermediate was treated with ethyl isocyanate to give the *N*-ethylurea **40** as per Scheme 3. Ester hydrolysis under more forcing conditions than those used previously, preceded synthesis of the target molecules under standard amide coupling conditions. In an improvement to this synthetic sequence, we discovered that the *N*-ethylurea side-chain could be installed directly to C4 functionalised-6-chloro-nicotinates (i.e. compounds **43** and **44**) *via* a Buchwald-Hartwig type coupling[19]. The target nicotinamides were thus accessed following ester hydrolysis (Scheme 3). A key improvement was realised upon direct reaction of dichloro compound **36** with *N*-ethylurea under the same conditions to give the 4-chloro-pyridin-6-yl-urea intermediate **54** with C6 regioselectivity. Variation at the C4 position was then readily achieved *via* S_NAr, Buchwald-Hartwig coupling or Suzuki-Miyaura coupling accordingly either following or prior to nicotinamide synthesis using the previously described conditions (Scheme 4). Further C4-substituted derivatives were synthesised using a variant synthetic route. Ester hydrolysis of the 4-chloro-pyridin-6-yl urea intermediate **52** followed by amide coupling using T3P gave 4-chloro-pyridine carboxamide **90** which was converted to C4 substituted compounds **91** - **93** via S_NAr reactions (Scheme 5).



Scheme 3. Reagents and conditions: (a) $HC(OEt)_3$, Ac_2O, NH_3(aq), 120°C, 2h, 55%; (b) $POCl_3$, 110°C, 2.5h, 79%; (c) *m*-toluidine, HCl, EtOH, 80°C, 3h, 51%; (d) *p*-methoxybenzylamine, PhMe, reflux, 72h, 62%; (e) TFA, Et_3SiH, DCM, rt, 4h, 98%; (f) EtNCO, 1,4-dioxane, 100°C, 48h, 43%; (g) 2M NaOH(aq), 75°C, 48h, 90%; (h) EDC.HCl, HOBT, $ArNH_2$, DMF, 40°C, 18h, 35%; (i) ImH, NaH, DMF, 0°C – RT, 34% or *N*-methylpiperazine, TEA, EtOH, 0°C, 3h, 80%; (j) $Pd(OAc)_2$, Xantphos, KO*t*Bu, *N*-ethylurea, 1,4-dioxane, H_2O, 100°C, 16h, 79-92%; (k) 2M NaOH (aq), rt or 40°C, 2h, 56-97%; (l) $ArNH_2$, EDC.HCl, HOBT, DMF, rt-40°C, 5-51%.



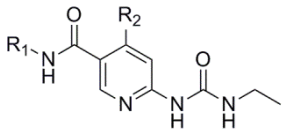
Scheme 4. Reagents and conditions: (a) $Pd(OAc)_2$, Xantphos, KO*t*-Bu, *N*-ethylurea, 1,4-dioxane, H_2O, 100°C, 16h, 40%; (b) NaH, NuH, DMF, 0°C - 70°C, 48h, 21-32%, or RNH_2, EtOH, reflux, 68-71%, or $ArNH_2$, HCl, EtOH, 60°C, 16h, 93%; (c) $Pd(PPh_3)_4$, $ArB(OH)_2$, 2M Na_2CO_3(aq), 1,4-dioxane or THF, reflux, 2-16h, 63%; (d) $Pd(OAc)_2$, Xantphos, KO*t*-Bu, $ArNH_2$, 1,4-dioxane, H_2O, 100°C, 3h; (e) 2M NaOH(aq), rt-70°C, 47%-quantitative or LiOH (aq), THF, EtOH, 60°C, 3h, 95%; (f) $ArNH_2$, EDC.HCl, HOBT, DMF, rt-40°C, 22-55%.



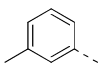
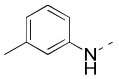
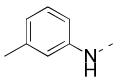
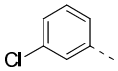
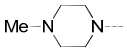
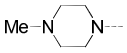
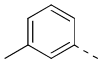
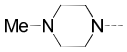
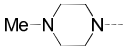
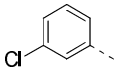
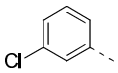
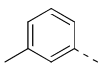
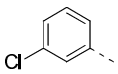
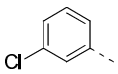
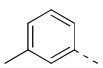
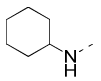
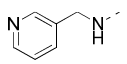
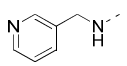
Scheme 5. Reagents and conditions: (a) 2M NaOH(aq), reflux, 80 °C, 18h, 83%; (b) $ArNH_2$, T3P, EtOAc, rt, 18h, 34-57%; (c) $ArNH_2$, HCl, EtOH, 60°C, 16h, 46-83%.

Biological data for the C4 substituted pyridine-3-carboxamides is given in Tables 3 and 4. Additionally, in order to probe the suitability of these compounds for broad spectrum therapeutic use, selected compounds were further tested for MICs against an extended panel of Gram positive (*S. epidermidis* ATCC 12228, *S. pneumonia* ATCC 49616), Gram negative (*E. coli* ATCC 25922, *E. coli* N43 efflux knock-out) and mutant (*S. aureus* T173N GyrB, *S. pyogenes* A53S ParE, *S. aureus* T173N / A53S GyrB-ParE dual mutant) bacterial species. These additional data are presented in Table 5.

Table 3.



GyrB inhibitory activity and MICs for C4 functionalised pyridine-3-carboxamides

Cmpd.	R_1	R_2	GyrB IC_{50} (μM)	MIC (μg / mL)			
				SA	*EF*	*SP*	*HI*
33			0.099	1	0.5	32	>64
42			0.098	>64	>64	>64	>64
49			5.14	>64	>64	>64	>64
50			5.66	>64	>64	>64	>64
51			18.0	>64	>64	>64	>64
52			33.2	>64	>64	>64	>64
53			0.55	>64	32	64	>64
68			0.56	>64	64	64	>64
69			1.13	>64	64	64	>64
70			3.02	>64	64	64	>64
71			7.27	>64	64	>64	>64
72			0.81	>64	4	>64	>64
73			2.96	8	1	4	>64
74			2.12	>64	>64	>64	>64
75			0.66	>64	8	8	>64
76			4.81	>64	2	8	>64

E. coli GyrB; *SA*, *S. aureus* ATCC 29213; *EF*, *E. faecalis* ATCC 29212; *SP*, *S. pyogenes* ATCC 51339; *HI*, *H. influenza* ATCC 49247.

As indicated from inspection of the data in Table 3, significant improvements in enzyme potency and antimicrobial activity were realised upon the addition of a substituted aniline to the pyridyl C4 position. In particular, compound **33** has an IC_{50} of 99 nM and MIC of 0.5 µg / mL against *E. faecalis*. Thiazol-2-yl nicotinamide **42**, being structurally analogous, demonstrated comparable enzyme inhibition (GyrB IC_{50} 98 nM) but was not antibacterial within the concentration ranges tested, illustrating the importance of structural modification on bacterial membrane penetration. Structural departure from an amino aryl substituent at C4 (compounds **49** – **76**) led to reduced enzyme potency and antibacterial activity, with the exception of pyrazol-1-yl compound **73** which, despite having moderate enzyme potency (GyrB IC_{50} 2.96 µM), retained good activity against Gram positive bacterial species. The 4-methylpiperazine moiety at C4 (compounds **49** – **52**) is conducive with both a reduction in enzyme inhibitory activity and a loss of antibacterial activity, whilst directly linked aromatic groups at this position (compounds **53** – **73**) resulted in inhibitors with broadly similar activity profiles to the parent unsubstituted compounds (Table 2). It was evident that a single atom-linked aromatic ring at the C4 position of the pyridine core was important to inhibitor efficacy. The reduced potency demonstrated by C4-cyclohexylamino derivative **74**, further confirmed this apparently strict requirement. In order to aid our understanding, compound **33** was modelled within the *E. faecalis* ATP binding domain (Fig. 5). Again, important polar contacts were predicted to be maintained and it appeared plausible that ligand 'pre-organisation' through an internal hydrogen bond involving the carbonyl at C3 and the NH at C4 may explain the improvement in potency.

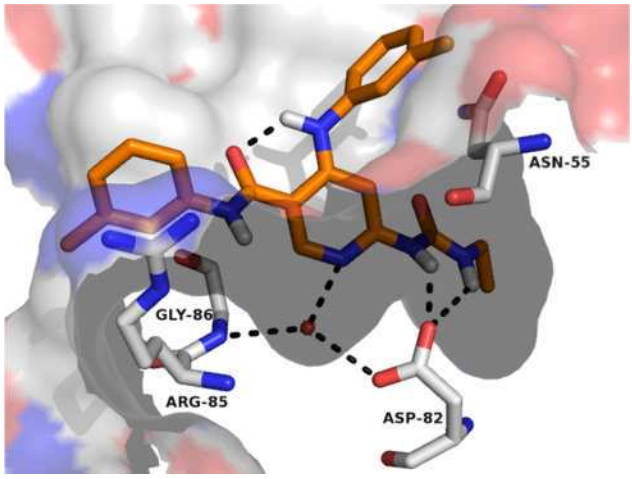
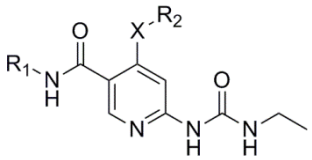


Figure 5. Compound **33** docked within the *E. faecalis* GyrB ATP binding site. **33** is shown as orange sticks, key residues (*EF* numbering) as grey sticks and predicted polar contacts as dashed lines.

A number of compounds were synthesised to further explore this phenomenon through preservation of the C4 amino-aryl motif (Table 4). In this instance, simple, unsubstituted phenyl / pyridine-3-yl moieties were chosen and found to be adequate replacements for the *m*-toluidyl group present in compound **33**. Compounds featuring the amino-aryl appendage were predominantly found to have potent GyrB inhibitory activity (IC_{50}s < 500 nM) and Gram positive antibacterial activity (MICs 0.125 – 2 µg / mL). Tellingly, C4 *O*-aryl compound **85**, which lacks the ability to form an internal hydrogen bond, is comparatively inactive and had no antibacterial activity (GyrB IC_{50} 4.9 µM). Para substitution at the C3 aromatic group was found to be well tolerated, with compounds **81** – **83** designed to probe the solvent exposed area of the binding pocket using morpholine, acetamide and triazole side-chains respectively. In particular, compound **82** is a highly potent GyrB inhibitor (GyrB IC_{50} 39 nM), presumably forming additional contacts with Lys-145 (Arg-136 *E. coli* GyrB) through the acetamide carbonyl oxygen. Incorporating a 3-chlorophenylamine moiety at C3 identified compound **79** as the most potent anti Staphylococcal agent tested here (MIC *S. aureus* 0.5 µg / mL). Where MICs against *S. aureus* were determined in the presence of plasma proteins (horse serum, compounds **80** – **82**, **86**), MIC shifts of 4 to 8-fold were observed, indicating that compounds of this class are not greatly impeded by high plasma-protein binding (Table 4).

Table 4



GyrB inhibitory activity and MICs for C4 amino-aryl pyridine-3-carboxamides.

Cmpd	R_1	R_2	GyrB IC_{50} (µM)	MIC (µg / mL)				
				SA	*SA* + HS	*EF*	*SP*	*HI*
77	phenyl	phenyl	0.040	1	nd	0.25	2	>64
78	3-methylphenyl	phenyl	0.140	1	nd	0.125	2	>64
79	3-chlorophenyl	phenyl	0.150	0.5	nd	0.125	4	>64
80	pyridin-3-yl	phenyl	0.170	1	4	0.125	1	>64
81	6-(morpholin-1-yl)pyridin-3-yl	phenyl	0.110	2	16	0.25	1	>64
82	6-(acetamid-1-yl)pyridin-3-yl	phenyl	0.039	2	8	0.125	0.5	>64
83	6-(1,2,4-triazol-1-yl)pyridin-3-yl	phenyl	0.150	>16	nd	0.25	>16	>64
84	3-picolylamin-1-yl	phenyl	2.0	>16	nd	0.5	1	>64
85[a]	phenyl	phenyl	4.9	>16	nd	>16	>16	>64
86	phenyl	pyridin-3-yl	0.43	1	2	0.25	1	>64
87	pyridin-3-yl	pyridin-3-yl	0.093	8	nd	0.5	1	>64

E. coli GyrB; *SA*, *S. aureus* ATCC 29213; *SA* + HS, *S. aureus* + 50% horse serum; *EF*, *E. faecalis* ATCC 29212; *SP*, *S. pyogenes* ATCC 51339; *HI*, *H. influenzae* ATCC 49247. Unless stated, X = NH; [a] X = O; nd, not determined.

Table 5 details additional biological profiling of the most promising C4 substituted pyridine-3-carboxamides. Firstly, excellent antibacterial activity was demonstrated against *S. epidermidis* and *S. pneumonia* (MICs 0.125 – 1 µg / mL), confirming the compounds possess a broad spectrum of Gram positive activity. Furthermore, though none of the compounds tested showed activity versus *E. coli*, it is significant that certain compounds (**73**, **77** and **80**) were active against the efflux knock-out mutant indicating that active efflux plays a role in the lack of Gram negative activity of this compound class. The MICs for these three compounds were higher against the efflux-knock out *E. coli* than against wild type *S. aureus* suggesting that they are not able to permeate Gram negative cells as effectively as Gram positive cells.

Table 5
MICs for selected compounds against GyrB / ParE mutants, additional Gram positive species and *E. coli*

Cmpd.	MIC (µg / mL)								
	SA	*SA* T173N (GyrB)	*SA* T173N/ T167N (GyrB / ParE)	*SP*	*SP* A53S (ParE)	*SE*	*SPn*	*EC*	*EC* (N43)
73	8	32	>64	4	16	2	1	>64	32
77	1	2	>64	2	16	0.25	1	>64	8
78	1	>64	>64	2	>64	0.5	0.5	>64	>64
79	0.5	1	>64	4	32	0.5	1	>64	>64
80	1	4	>64	1	2	0.25	0.25	>64	8
81	2	>64	>64	1	4	1	0.5	>64	>64
82	2	>64	>64	0.5	2	0.25	0.125	>64	>64

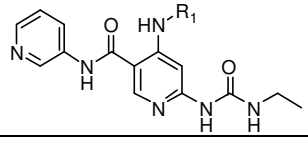
SA, *S. aureus* ATCC 29213; *SA* T173N, *S. aureus* GyrB mutant; *SA* T173N / T167N, *S. aureus* dual GyrB / ParE mutant; *SP*, *S. pyogenes* ATCC 51339; *SP* A53S, *S. pyogenes* ParE mutant; *SE*, *S. epidermidis* ATCC 12228; *SPn*, *S. pneumonia* ATCC 49616; *EC*, *E. coli* ATCC 25922; *EC* (N43), *E. coli* efflux pump mutant.

Finally, mutant data provided useful insights into the mode of action, dual-inhibitory nature and resistance vulnerability of these compounds. In all cases, MIC shifts were observed versus GyrB / ParE mutants suggesting bacterial cell death to be target specific rather than due to promiscuous cell damaging effects. Compounds **33** and **78**, whilst potent against wild-type species, were ineffective against *S. aureus* and *S. pyogenes* single-step GyrB and ParE mutants respectively. Compounds **81** and **82** lost activity against the *S. aureus* GyrB mutant but maintained activity against the *S. pyogenes* ParE mutant. This would suggest GyrB to be the primary target for these compounds. Pleasingly, compounds **73**, **77**, **79** and **80** were active against both GyrB and ParE single-step mutants but lost all antibacterial activity versus the *S.*

aureus dual GyrB / ParE mutant. The indication is therefore that these compounds are potent dual inhibitors of GyrB / ParE, are not vulnerable to single-step resistance and would require a statistically unlikely spontaneous double mutation for resistance to be conferred.

Subsequently a small library of compounds was synthesised with the goal of investigating alternative substitution at the C4 position (Schemes 4 and 5). Hydroxy- and methoxy-phenyl derivatives were chosen, as docking indicated that the C4 group would be oriented in a solvent exposed area, and more polar substituents would be predicted to experience stabilisation in the water-exposed region. Enzyme inhibition was measured against both GyrB and ParE enzymes from *E. coli* and *S. aureus* and MICs were measured against both *S. aureus* and *E. coli* (Table 6). In keeping with our previous observations, none of the compounds synthesised showed meaningful activity against the Gram negative species suggesting that simple variation at the C4 position is insufficient to provide compounds with Gram negative activity. All compounds showed good inhibitory activity against GyrB and reasonable activity against ParE, although they were less active against ParE from *E. coli* which may offer an additional barrier to activity of these compounds in Gram negative bacteria.

Table 6
GyrB and ParE inhibitory activity and MICs for C4 amino-aryl pyridine-3-carboxamides



Compound	R_1	IC_{50}s (μM)				MIC (μg / mL)	
		GyrB		ParE			
		SA	*EC*	*SA*	*EC*	*SA*	*EC*
88	4-hydroxyphenyl	0.024	0.028	0.086	0.94	8	>64
91	3-hydroxyphenyl	nd	0.036	nd	0.78	nd	>64
92	2,4-bismethoxyphenyl	0.041	0.046	0.80	42	1	>64

S. aureus and *E. coli* GyrB; *S. aureus* and *E. coli* ParE; *SA*, *S. aureus* ATCC 29213; *EC, E. coli* ATCC 25922; nd, not determined.

Conclusion

In summary we have reported the discovery, synthesis and an initial SAR study of a novel series of antibacterial GyrB inhibitors. Through structural optimisation we have developed the pyridine-3-carboxamide-6-yl-urea core to offer derivatives with excellent GyrB inhibitory activity and Gram positive antibacterial efficacy. A key activity breakthrough *via* the introduction of amino-aryl i.e. NH-aryl functionality at the pyridyl C4 position is reported.

Encouragingly, inhibitors were identified (e.g. compound **80**) which demonstrate broad spectrum Gram positive antibacterial activity, activity against a Gram negative efflux pump mutant, and activity versus single-step GyrB / ParE mutants. These observations may prove important in the future therapeutic utility of this compound class. Compounds reported in this paper have been patented[20]. Further optimisation of compounds in this series is ongoing, with a focus on modification of physicochemical properties with the hopes of broadening their spectrum of activity to include Gram negative organisms.

Acknowledgements

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Supporting information

Associated content including full experimental details, HPLC method, purity analysis and example NMR spectra for tested compounds is available in the supporting information document.

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