

# Exploring the Chemical Space of Benzothiazole-Based DNA Gyrase B Inhibitors

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**ABSTRACT:** We designed and synthesized a series of inhibitors of the bacterial enzymes DNA gyrase and DNA topoisomerase IV, based on our recently published benzothiazole-based inhibitor bearing an oxalyl moiety. To improve the antibacterial activity and retain potent enzymatic activity, we systematically explored the chemical space. Several strategies of modification were followed: varying substituents on the pyrrole carboxamide moiety, alteration of the central scaffold, including variation of substitution position and, most importantly, modification of the oxalyl moiety. Compounds with acidic, basic and neutral properties were synthesized. To understand the mechanism of action and binding mode, we have obtained a crystal structure of compound **16a**, bearing a primary amino group, in complex with the N-terminal domain of *E. coli* gyrase B (24 kDa) (PDB: **6YD9**). Compound **15a**, with a low molecular weight of 383 Da, potent inhibitory activity on *E. coli* gyrase (IC<sub>50</sub> = 9.5 nM), potent antibacterial activity on *E. faecalis* (MIC = 3.13 μM) and efflux impaired *E. coli* strain (MIC = 0.78 μM), is an important contribution for the development of novel gyrase and topoisomerase IV inhibitors in Gram-negative bacteria.

**KEYWORDS:** DNA gyrase; topoisomerase IV; GyrB; ParE; antibacterial; benzothiazole;

The treatment of infections has advanced beyond recognition since the first discoveries of antimicrobial compounds. Widespread distribution, low price and high safety profile have established antimicrobial drugs as a cornerstone of modern medicine. However, due to rising resistance issues, the efficacy of existing infection treatments is rapidly decreasing. The dearth of new anti-infectives introduced to the market over recent years, and no discovery of novel classes, has deepened the problem.<sup>1-4</sup> Antimicrobial resistance is now a serious global health concern; infections that are currently readily treatable could become untreatable in the near future.<sup>5</sup>

DNA topoisomerases are enzymes that alter DNA topology, being involved in important biological processes in the cell (replication, transcription, chromosome condensation, etc.).<sup>6-11</sup> Bacterial DNA gyrase and DNA topoisomerase IV (topo IV) are attractive targets for antibacterial drug discovery due to their well-described structure and mechanism, as well as their absence from eukaryotes. Both enzymes are type II topoisomerases, sharing high functional and structural similarity. They change the topology of DNA by cleaving both strands of the double-helix. Gyrase has the unique function of introducing negative supercoiling ahead of the replication fork, while topo IV is involved in chromosome

decatenation. The enzymes consist of two pairs of subunits with different functions, forming heterotetrameric structures: A<sub>2</sub>B<sub>2</sub> (gyrase) or C<sub>2</sub>E<sub>2</sub> (topo IV). The main role of the GyrA and ParC subunits is to release torsional stress by breaking and rejoining strands of the DNA molecule. GyrB and ParE contain the ATP-binding site and provide the energy required for the enzyme function by ATP hydrolysis.<sup>12-13</sup>

DNA gyrase inhibitors can influence the enzyme action at different levels.<sup>14</sup> Inhibition of the GyrA subunit can cause stabilization of the DNA-enzyme complex, and thus broken strands of DNA cannot be re-joined. GyrA inhibitors, so-called gyrase poisons, are represented by fluoroquinolones, which are widely in clinical use, although struggling with side effects and growing resistance concerns.<sup>6</sup> These problems have encouraged further research on inhibitors with different mechanisms of action. GyrB inhibitors are not currently in clinical use; novobiocin, was withdrawn from the market in the 1960s due to significant side effects. Since then, no other GyrB inhibitor has passed clinical trials.<sup>7</sup> Low cross-resistance between potential GyrB inhibitors and fluoroquinolones is a promising and motivating factor for further research. Novel gyrase inhibitors based on various chemical structures were discovered in recent years.<sup>8</sup>

Our research group has recently published several articles focusing on ATP-competitive inhibitors of the DNA gyrase B protein (GyrB).<sup>15-17</sup> The compounds share a common pyrrole-

2-carboxamide moiety, originating from marine alkaloids such as oroidin.<sup>18</sup> Compounds are potent inhibitors of gyrase; however they lack potent antimicrobial activity. The mode of action for this series of compounds was confirmed by solving the crystal structure of complex of *E. coli* GyrB with a benzothiazole-type inhibitor **1** (PDB 5L3J).<sup>15</sup>

Our goal was to explore the chemical space of pyrrole-benzothiazole GyrB inhibitors by selecting those that retain potent enzyme inhibition whilst optimizing their physico-chemical properties. Several strategies of modification illustrated in Fig. 1, were followed: alteration of the pyrrole carboxamide moiety, alteration of the central scaffold, including variation of the substitution position, and most importantly modification of the oxalyl moiety.

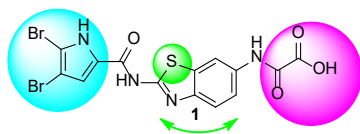


Fig. 1. Structure of previously-discovered compound **1** (PDB 5L3J) with highlighted positions selected for structure modifications.

The 4,5-dibromo-1*H*-pyrrole-2-carboxamide interacting with the protein through hydrogen bonding interactions of the pyrrole nitrogen and adjacent carbonyl with Asp73, either directly or via a network of hydrogen bonds involving conserved water molecule, respectively does not permit substantial changes. Modification to 3,4-dichloro-5-methyl-1*H*-pyrrole-2-carboxamide has several potential beneficial properties. The key role of the pyrrole nitrogen and non-substituted carboxamide moiety remains unchanged. Smaller chlorine atoms (compared to bromine) are likely to fit better into the *E. coli* topo IV hydrophobic pocket and the *Staphylococcus aureus* gyrase hydrophobic pocket, which has a smaller volume than the *E. coli* gyrase hydrophobic pocket.<sup>19</sup> The 3,4-dichloro-5-methyl-1*H*-pyrrole-carboxamide moiety is present in the natural antibiotics kibelomycin<sup>20-22</sup> and amycolamicin<sup>23,24</sup> and was used in the Astra Zeneca pyrrolamide series of gyrase B inhibitors.<sup>25-27</sup>

To explore the influence of the central scaffold we interchanged the substituents on positions 2 and 6 of benzothiazole, resulting in interchanging the position of the benzene ring and thiazole ring of the benzothiazole moiety<sup>28</sup> in the binding site and its  $\pi$ -cation interactions with Arg76, as well as influencing the acidity of pyrrole amide proton. An obvious bioisosteric approach was the replacement of the ring sulphur (benzothiazole) with nitrogen to give a benzimidazole central scaffold. Indeed, benzimidazole-ureas were presented as potent dual inhibitors of bacterial topoisomerases in a recent study by Vertex Pharmaceuticals.<sup>29</sup> In this manner, crucial cation- $\pi$  interactions between the core benzene ring and Arg76 and the Glu50-Arg76 salt bridge interactions were preserved. Additional interactions might have been introduced by enlarging the central scaffold, but we did not plan to increase the scaffold MW.

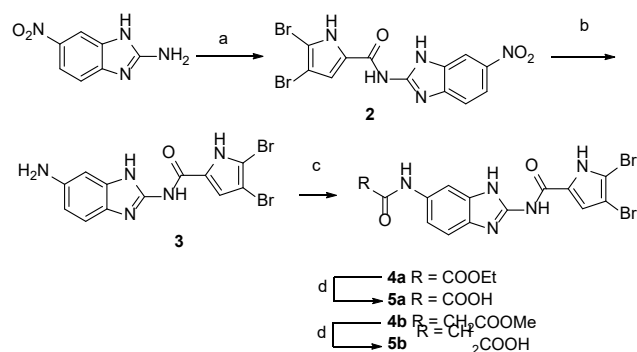
The focus of the optimisation strategy was the replacement of the oxalyl moiety, which is likely too acidic and might impair permeation into bacteria and thus diminish antimicrobial activity. As confirmed by the crystal structure of **1**, the oxalyl moiety extending out of the binding pocket and making only a single direct hydrogen bond to the protein, via the carbonyl group to Arg136, can be replaced without loss of

inhibitory activity. We systematically changed the acidic oxalyl moiety to i) a neutral small aliphatic acyl moiety (acetamide group) ii) a neutral aromatic moiety (isonicotinamide moiety) iii) a neutral polar group with H-bond donor/acceptor potential (urea derivative) iv) and an aliphatic primary amino group (derivatives of glycine and beta alanine).

Synthesis of benzimidazole compounds, illustrated in Scheme 1, starts with coupling of 6-nitro-1*H*-benzo[d]imidazol-2-amine with 2,2,2-trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethan-1-one and a weak base in DMF. The nitro group of intermediate **2** is reduced using tin(II) chloride dihydrate to give amino-derivative **3**, further acylated with respective acyl chloride to obtain esters **4a-b**. Corresponding carboxylic acids **5a-b** were obtained by alkaline hydrolysis.

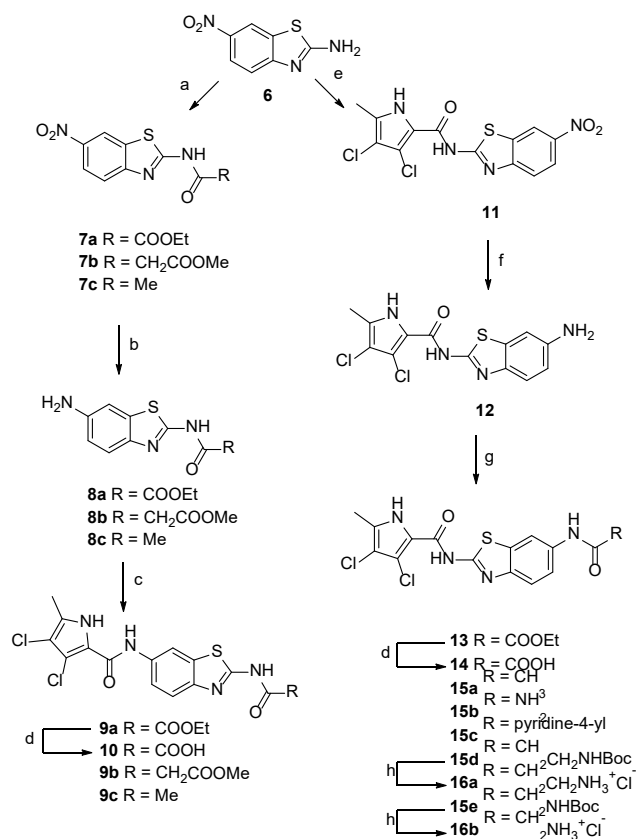
Synthesis of benzothiazole-based compounds is illustrated in Scheme 2. Acylation of 6-nitro-benzo[d]thiazol-2-amine (**6**) with ethyl oxalyl chloride / methyl malonyl chloride / acetyl chloride gives intermediates **7a**, **7b** and **7c** respectively.

### Scheme 1. Reagents and conditions



(a) 2,2,2-trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethan-1-one, Na<sub>2</sub>CO<sub>3</sub>, DMF, 80 °C, 16h; (b) SnCl<sub>2</sub>·2H<sub>2</sub>O, EtOH, reflux, 12h; (c) ethyl oxalyl chloride (for **4a**) or methyl malonyl chloride (for **4b**), Et<sub>3</sub>N, 1,4-dioxane, rt, 12h; (d) 1M NaOH, 1,4-dioxane, rt, 16 h.

### Scheme 2. Reagents and conditions



(a) corresponding acyl chloride, Et<sub>3</sub>N, 1,4-dioxane, rt, 4 h; (b) H<sub>2</sub>, 10% Pd/C, EtOH (for **8a** and **8c**) or MeOH (for **8b**), rt, 24 h; (c) 3,4-dichloro-5-methyl-1H-pyrrole-2-carbonyl chloride, pyridine, DCM, (d) 1 M NaOH, MeOH, rt, 24 h, (e) 3,4-dichloro-5-methyl-1H-pyrrole-2-carbonyl chloride, toluene, reflux, 16h; (f) SnCl<sub>2</sub>·2H<sub>2</sub>O, EtOH, reflux, 12h; (g) ethyl oxalyl chloride, Et<sub>3</sub>N, 1,4-dioxane, rt, 8 h (for the synthesis of **13**); (h) Ac<sub>2</sub>O, Et<sub>3</sub>N, DCM, rt, 2h (for the synthesis of **15a**); or CDI, DMF, rt, 3h; NH<sub>3</sub>, rt, 16h (for the synthesis of **15b**); or nicotinic acid, EDC, NMM, HOBT, DMF, rt, 12h (for the synthesis of **15c**); or corresponding Boc-amino acid, EDC, NMM, HOBT, DMF, rt, 12h (for the synthesis of **15d-e**); (h) 4M HCl, 1,4-dioxane, rt, 5 h

**Table 1. Inhibitory activity of series I of compounds with benzimidazole central scaffold.**

Cmpd	n	R	IC <sub>50</sub> (μM) or RA (%) <sup>a</sup>			
			<i>E. coli</i> gyrase	<i>E. coli</i> topo IV	<i>S. aureus</i> gyrase	<i>S. aureus</i> topo IV
<b>4a</b>	0	Et	4.0 ± 1.6 μM	100 %	100 %	100 %
<b>4b</b>	1	Me	7.0 ± 3.4 μM	100 %	100 %	100 %
<b>5a</b>	0	H	0.60 ± 0.32 μM	12 ± 2 μM	80 ± 23 μM	31 ± 1 μM
<b>5b</b>	1	H	1.5 ± 0.2 μM	100 %	100 %	100 %
<b>1</b>			0.058 μM	13 μM	>100 μM	10 μM
<b>novobiocin</b>			0.17 μM	11 μM	0.041 μM	27 μM

<sup>a</sup> Residual activity of the enzyme at 10 μM concentration of the compound.

The nitro group in position 6 of the benzothiazole was then reduced using hydrogen and palladium catalyst to obtain compounds **8a-c**. Coupling with 3,4-dichloro-5-methyl-1H-pyrrole-2-carboxylic acid (activated in situ with oxalyl chlo-

ride) in DCM in the presence of pyridine as base, gave compounds **9a-c**. The ethyl oxalyl derivative **9a** was further hydrolysed to carboxylic acid **10**. Synthesis of the reversed isomers with the pyrrole moiety at position 2 is more demand-

ing. The coupling of benzothiazole **6** with the pyrrole-2-carbonyl chloride moiety was done in refluxing toluene without any base, to obtain intermediate **11**. The nitro group is reduced using tin(II) chloride dihydrate to give key amino derivative **12**, which was further derivatized. Acylation with oxalyl chloride moiety gave ester **13**. The corresponding carboxylic acid **14** is isolated after hydrolysis with 1 M sodium hydroxide. Reaction of amino derivative **12** with acetic anhydride in the presence of triethylamine in DCM gave target compound **15a**. To obtain the urea derivative **15b**, aminobenzothiazole **12** was first stirred at RT with CDI in DMF for 3 h and heated to 50°C overnight after addition of ammonia gas. Compounds **15c-e** were prepared by coupling of **12** with nicotinic acid (**15c**), Boc- $\beta$ -alanine (**15d**) or Boc-glycine (**15e**) in the presence of coupling agents EDC, HOBt or NMM, respectively in DMF. The Boc protective group of **15d-e** was finally removed with HCl in dioxane, to obtain **16a-b**.

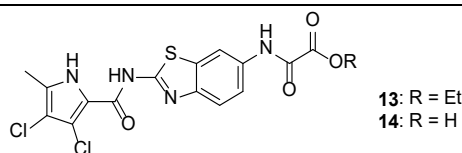
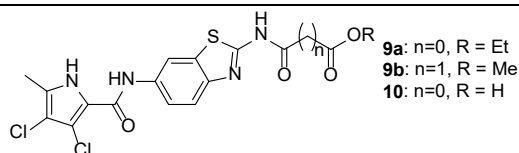
Compounds were evaluated in *E. coli* and *S. aureus* gyrase supercoiling assays as well as in *E. coli* and *S. aureus* topo IV relaxation assays. The results are presented in Tables 1-3 as IC<sub>50</sub> values or residual activity (RA) of the enzyme in a concentration of 10  $\mu$ M of the inhibitor.

The benzimidazole-based series was shown to be only weakly active (Table 1), with inhibitory activity against *E. coli* gyrase in the micromolar range. Carboxylic acids **5a-b** were approximately 10-fold more active than the corresponding esters **4a-b**, due to possible ionic interactions additional to hydrogen bonds with Arg136. The most potent compound **5a** (IC<sub>50</sub> = 0.60  $\mu$ M) showed weak activity against *S. aureus* gyrase and *E. coli/S. aureus* topo IV). Direct comparison of benzothiazole **1** with benzimidazole **5a** shows that replacement of sulphur with nitrogen resulted in 10-fold lower enzymatic inhibition against *E. coli* gyrase. Poor activity results and poor solubility of benzimidazoles pointed us back to the benzothiazole central scaffold, and the benzimidazole series was not further extended. Replacement of 4,5-dibromo-1*H*-pyrrole with 3,4-dichloro-5-methyl-1*H*-pyrrole indeed resulted in good inhibitory activity against *E. coli* gyrase but even more importantly it introduced good inhibitory activity against *E. coli* topo IV (**14**: IC<sub>50</sub> topo IV = 75 nM) and potent inhibitory activity against *S. aureus* gyrase and topo IV, which was completely absent in the case of the dibromo

analogue **1**. When comparing compounds with the pyrrole attached to position 2 (compound **14**) to a regioisomer with the pyrrole attached to position 6 (compound **10**), the inhibitory activity on *E. coli* gyrase is favourable for compound **10** and even more favourable regarding *E. coli* topo IV inhibitory activity as well *S. aureus* gyrase and topo IV. Overall, compound **14** has superior enzymatic activity against all four tested enzymes compared to novobiocin. Benzothiazole with 3,4-dichloro-5-methyl-1*H*-pyrrole on position 2 was thus selected to explore the possible replacements of the unfavourable oxalyl moiety. The results of the *E. coli* gyrase inhibitory activity assays reveal that the anionic centre is not required for potent inhibitory activity. Compounds with the acetyl moiety, the urea derivative and the glycine derivative with a free primary amino group all possess *E. coli* gyrase inhibitory activity in the low nanomolar range (10-25 nM). Having an aromatic moiety (**15c**) pointed to the water environment (and possibly having  $\pi$ -cationic interactions with Arg136) is clearly not optimal for this series of compounds, although such an approach was successful in tricyclic inhibitors of GyrB (PDB: **4KFG**).<sup>30</sup> Investigation of the Boc-protected amino acid derivatives **15d** and **16a** reveals that the bulky lipophilic moiety can have favourable binding to *E. coli* GyrB. Although this might seem contradictory, it is known from thermodynamic evaluations that the binding of compound with “unfavourable” lipophilic moieties extending into a water environment can be beneficial as more polar/ionized groups can pay a high desolvation penalty, which contributes to net unfavourable binding.<sup>31</sup> The amino compound **12**, lacking the carbonyl group, is a very weak binder, which indicates that a carbonyl moiety is a prerequisite for potent enzyme binding. Acetyl derivative **15a** with lowest molecular weight in the series and single digit nanomolar binding with IC<sub>50</sub> = 9.5 nM seemed very interesting, therefore regioisomer **9c** with a pyrrole attached to position 6 and acetyl to position 2 of benzothiazole was prepared. The trend observed already from previous compounds was the same in this series: the isomer **15a** with pyrrole attached to position 2 of benzothiazole was 7-fold more potent than regioisomer with pyrrole attached to position 6.

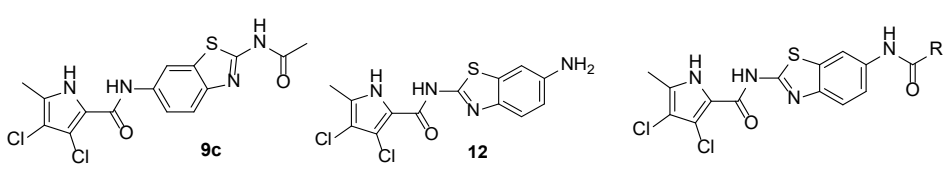
**Table 2** Inhibitory activity of benzothiazole compounds with acidic terminal functional groups.

Cmpd	n	R	IC <sub>50</sub> (nM) or RA (%) <sup>a</sup>			
			<i>E. coli</i> gyrase	<i>E. coli</i> topo IV	<i>S. aureus</i> gyrase	<i>S. aureus</i> topo IV
<b>9a</b>	0	Et	290 ± 170 nM	100%	69%	100%
<b>9b</b>	1	Me	200 ± 180 nM	100%	59%	48%
<b>10</b>	0	H	29 ± 16 nM	6 400 ± 3 000 nM	250 ± 130 nM	910 ± 340 nM
<b>13</b>	0	Et	48 ± 12 nM	100%	100%	100%
<b>14</b>	0	H	4.8 ± 2.1 nM	75 ± 28 nM	38 ± 16 nM	290 ± 180 nM
<b>1</b>			58 nM	13 000 nM	>100 $\mu$ M	10 000 nM
<b>novobiocin</b>			168 nM	11 000 nM	41 nM	27 000 nM



a Residual activity of the enzyme at 10  $\mu\text{M}$  concentration of the compound.

**Table 3. Inhibitory activity of benzothiazole compounds with neutral or basic terminal functional groups.**



Cmpd	R	IC <sub>50</sub> (nM) or RA (%) <sup>a</sup>			
		<i>E. coli</i> gyrase	<i>E. coli</i> topo IV	<i>S. aureus</i> gyrase	<i>S. aureus</i> topo IV
<b>9c</b>		66 ± 8 nM	100%	35 400 nM	100%
<b>12</b>		16 000 ± 4 000 nM	100%	60%	100%
<b>15a</b>	-CH <sub>3</sub>	9.5 ± 2.5 nM	4 600 ± 100 nM	400 ± 120 nM	1 600 ± 300 nM
<b>15b</b>	-NH <sub>2</sub>	26 ± 8 nM	5 200 ± 2 700 nM	1 300 ± 600 nM	5 800 ± 3 100 nM
<b>15c</b>	-pyridine-4-yl	2 500 ± 1 500 nM	100%	100%	100%
<b>15d</b>	-CH <sub>2</sub> CH <sub>2</sub> NHBoc	110 ± 20 nM	100%	780 nM	100%
<b>15e</b>	-CH <sub>2</sub> NHBoc	29 ± 15 nM	60%	260 ± 120 nM	100%
<b>16a</b>	-CH <sub>2</sub> CH <sub>2</sub> NH <sub>3</sub> <sup>+</sup> Cl <sup>-</sup>	110 ± 50 nM	10 000 ± 2 000 nM	1 500 ± 600 nM	290 ± 180 nM
<b>16b</b>	-CH <sub>2</sub> NH <sub>3</sub> <sup>+</sup> Cl <sup>-</sup>	280 ± 10 nM	100%	380 nM	100%

<sup>a</sup> Residual activity of the enzyme at 10  $\mu\text{M}$  concentration of the compound.

To confirm binding to the ATP-pocket of GyrB and to gain insight into molecular interactions, crystallisation of selected ligands with the 24 kDa fragment (N-terminal sub-domain) of GyrB was attempted. Among crystallization attempts with **15a**, **16a** and **9c**, co-crystals of **16a** with *E. coli* GyrB24 were obtained and the structure was solved to a resolution of 1.60 Å (Figure 2). The 3,4-dichloro-5-methyl-1*H*-pyrrole moiety of **16a** is bound to the adenine-binding pocket, making a hydrogen bond between the pyrrole NH group and Asp73 side chain, while interaction of the Asp73 side chain and pyrrolamide carbonyl oxygen is bridged by coordinated water molecule (NHCO-H<sub>2</sub>O H-bond distance is 2.61 Å). Comparison with the GyrB crystal structure of kibelomycin (PDB: **4URM**)<sup>32</sup> and Astra Zeneca's pyrrolamide (PDB: **3TTZ**),<sup>33</sup> reveals identical interactions of the 3,4-dichloro-5-methyl-1*H*-pyrrole moiety. There is a strong  $\pi$ -cation interaction (3.28 Å) between the aromatic ring and Arg76. The carbonyl moiety of  $\beta$ -alanine interacts with Arg136 through a H-bond

(2.87 Å), while the primary amino group of  $\beta$ -alanine is pointed towards the water environment, making no interactions with the protein. While interactions of the oxalyl moiety of **1** with Arg136 were not apparent (PDB code **5L3J**, Resolution: 2.83 Å), improved resolution now offers clear insight into the binding mode. Hydrogen bonding of Arg136 with the carbonyl oxygen of  $\beta$ -alanine is evident, thus demonstrating that a carboxylic acid moiety is not prerequisite for binding and explains strong enzymatic inhibitory activity of compounds having a neutral side chain.

Compounds were assayed on 4 wild type bacterial strains to determine their antibacterial activity (*E. coli*, *S. aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*). Additionally, we included two other *E. coli* strains, an *E. coli* strain with impaired outer

**Table 4. Antibacterial activity of selected compounds.**

Cmpd	MIC ( $\mu\text{M}$ ) <sup>a</sup>					
	<i>E. coli</i> (ATCC 25922)	<i>S. aureus</i> (ATCC 29213)	<i>E. faecalis</i> (ATCC 29212)	<i>P. aeruginosa</i> (ATCC 27853)	<i>E. coli</i> (JD17464) <sup>b</sup>	<i>E. coli</i> (JW5503) <sup>c</sup>
<b>4a, 4b, 5a, 5b, 9a, 9c, 10, 14, 15c, 15d</b>	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$
<b>9b</b>	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	1.56 $\mu\text{M}$
<b>13</b>	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	50 $\mu\text{M}$
<b>15a</b>	>50 $\mu\text{M}$	>50 $\mu\text{M}$	3.13 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	0.78 $\mu\text{M}$
<b>15b</b>	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	1.56 $\mu\text{M}$
<b>15e</b>	>50 $\mu\text{M}$	>50 $\mu\text{M}$	3.13 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	0.78 $\mu\text{M}$
<b>16a</b>	>50 $\mu\text{M}$	>50 $\mu\text{M}$	12.5 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	12.5 $\mu\text{M}$
<b>16b</b>	>50 $\mu\text{M}$	25 $\mu\text{M}$	6.25 $\mu\text{M}$	>50 $\mu\text{M}$	>50 $\mu\text{M}$	3.13 $\mu\text{M}$

ciprofloxacin

0.05  $\mu\text{M}$ 1.51  $\mu\text{M}$ 3.02  $\mu\text{M}$ 3.02  $\mu\text{M}$ 0.121  $\mu\text{M}$ 0.015  $\mu\text{M}$ 

<sup>a</sup> MIC (minimum inhibitory concentration that inhibits the growth of bacteria by  $\geq 90\%$ ) values against *E. coli* and *S. aureus*. <sup>b</sup> *E. coli* strain with impaired outer membrane, *lpxC* deletion mutant. <sup>c</sup> *E. coli* strain with defective efflux pump, *tolC* deletion mutant.

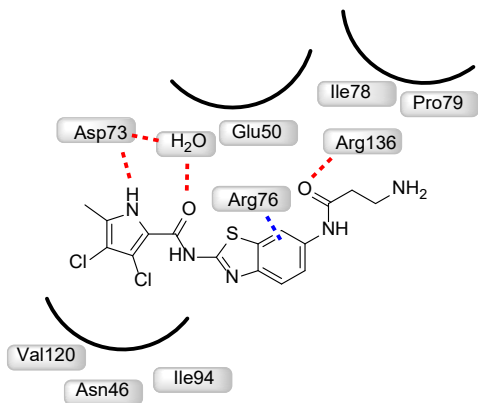
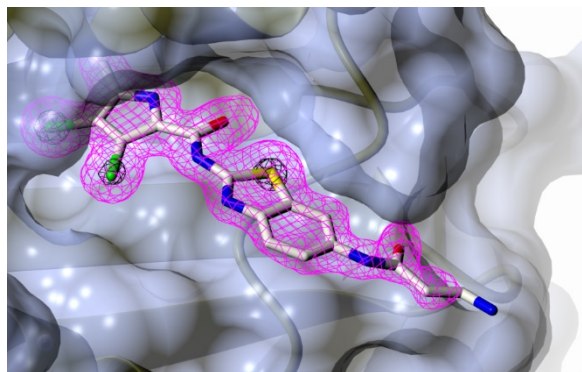


Figure 2. Crystal structure of the complex formed between **16a** and *E. coli* GyrB 24 kDa fragment (PDB: **6YD9**). The protein is depicted in cartoon representation covered by a semi-transparent molecular surface. Omit  $mF_{\text{obs}} - DF_{\text{calc}}$  positive difference electron density for the ligand at 1.6 Å resolution is depicted at two contour levels:  $3\sigma$  (magenta mesh) and  $12\sigma$  (black mesh), with the latter highlighting the locations of the electron-dense chlorine and sulphur atoms. Shown below are the interactions between amino acid residues with ligand (red line: hydrogen bond, blue line:  $\pi$ -cation interactions).

membrane (*lpxC* deletion mutant) and an *E. coli* strain with a defective efflux pump (*tolC* deletion mutant) to evaluate if weak antibacterial activity on wild type could be due to weak permeability and/or strong efflux. Both benzimidazole and benzothiazole inhibitors with the oxalyl moiety (**5a**, **10** and **14**) were inactive in all bacterial strains (MIC > 50  $\mu\text{M}$ ). Ester derivative **9b** which had good enzymatic inhibitory activity ( $IC_{50} = 200$  nM) showed also a good MIC (1.56  $\mu\text{M}$ ) in the *E. coli* strain with a defective efflux pump, indicating that the ester moiety is beneficial for entry into bacteria; however, such compounds are often the substrates for efflux pumps. Ester derivative **13** which had very good enzymatic inhibitory activity ( $IC_{50} = 48$  nM) showed only weak MIC (50  $\mu\text{M}$ ) in the *E. coli* strain with a defective efflux pump.

A significant improvement in MIC value was expected with the compound lacking the acidic oxalyl moiety. Compound **16b**, having a glycine moiety with a free primary amino group, was the only compound showing some antibacterial

activity on *S. aureus* wild type (MIC = 25  $\mu\text{M}$ ), confirming that good enzymatic inhibitory activity (*S. aureus* gyrase  $IC_{50} = 380$  nM) with suitable physico-chemical properties is needed for antibacterial activity. When looking at Gram-positive *E. faecalis*, the results were more encouraging, as four compounds showed good antibacterial activity. Both compounds with a free amino group (**16a** MIC = 12.5  $\mu\text{M}$  and **16b** MIC = 6.25  $\mu\text{M}$ ) had good antibacterial activity. Boc-protected analogue **15e** and compound **15a** with an acetyl moiety had even better antibacterial activity with MIC = 3.13  $\mu\text{M}$ , which also correlated with enzymatic inhibitory potency. When looking at more challenging Gram-negative *E. coli* and *P. aeruginosa*, the results were less encouraging, as none of the compounds showed antibacterial activity.

Testing on the *E. coli* strain with impaired outer membrane (*lpxC* deletion mutant), revealed that permeability is not a main issue as tested compounds did not show any antibacterial activity. On the contrary, the compounds were very active when tested on the *E. coli* strain with a defective efflux pump. The most potent compounds were **15e** and **15a** with submicromolar MIC values (MIC = 0.78  $\mu\text{M}$ ). Both are neutral molecules with lipophilic moieties (acetyl and Boc). Compounds with free amino group were also potent antibacterial compounds (**16a** and **16b**) but were not superior to the neutral compounds (**15a** and **15e**). This is somehow contrary to the recent proposal that the introduction of a primary amino group improves entry and causes accumulation in Gram-negative bacteria.<sup>34</sup> To confirm the mechanism of uptake for the reported compounds (porin pathway or direct diffusion)<sup>35</sup> additional studies should be performed in order to guide optimization.<sup>36,37</sup> Urea derivative **15b** was also a potent antibacterial compound on the efflux impaired *E. coli* strain (MIC = 1.56  $\mu\text{M}$ ), while inactive on all other strains.

In summary, three series of GyrB/ParE inhibitors were designed, synthesized and evaluated in enzymatic and antibacterial assays. Chemical space was thoroughly explored; compounds with acidic, basic and neutral properties were synthesized, possessing very potent inhibitory activity on *E. coli* and/or *S. aureus* gyrase and/or topo IV. The best compounds were active on Gram-positive bacterium *E. faecalis* with the best compound having MIC = 3.13  $\mu\text{M}$ . The compounds were inactive on Gram-negative bacteria because they are good substrates for bacterial efflux pumps, but **15a** and **15e** showed potent antibacterial activity on the efflux impaired *E. coli* strain (MIC = 0.78  $\mu\text{M}$ ). Compound **15a** with low molecular weight 383 Da, displayed potent inhibitory activity on *E. coli* gyrase ( $IC_{50} = 9.5$  nM), potent antibacterial activity on *E. faecalis* (MIC = 3.13  $\mu\text{M}$ ) and on the efflux impaired *E. coli* strain (MIC = 0.78  $\mu\text{M}$ ) and thus makes an important contribution for the development of novel gyrase and topoisomerase inhibitors in Gram-negative bacteria.

## ASSOCIATED CONTENT

### Supporting Information

(i) Experimental conditions for biochemical and microbiological assays, (ii) experimental conditions for co-crystallization, collection, and refinement statistics for compound **16a**, (iii) experimental procedures for the synthesis of all the compounds described in this manuscript and (iv) representative analytical data (PDF)

The Supporting Information is available free of charge on the ACS Publications website.

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The manuscript was written with contributions from all of the authors. ‡These authors contributed equally.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

MIC, minimal inhibitory concentration; RA, residual activity.

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