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Exploring the 5-substituted 2-aminobenzothiazole-based DNA gyrase B inhibitors active against ESKAPE pathogens

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KEYWORDS: antibacterial, benzothiazole, dual inhibitor, DNA gyrase.

ABSTRACT: We present a new series of 2-aminobenzothiazole-based DNA gyrase B inhibitors with promising activity against ESKAPE bacterial pathogens. Based on the binding information extracted from the cocrystal structure of DNA gyrase B inhibitor A, in complex with *Escherichia coli* GyrB24, we expanded the chemical space of the benzothiazole-based series to the C5 position of the benzothiazole ring. In particular, compound E showed low nanomolar inhibition of DNA gyrase ($IC_{50} < 10$ nM) and broad-spectrum antibacterial activity against pathogens belonging to ESKAPE group, with MIC < 0.03 $\mu\text{g/mL}$ for most Gram-positive strains and 4 to 16 $\mu\text{g/mL}$ against Gram-negative *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. To understand the binding mode of the synthesized inhibitors, a combination of docking calculations, molecular dynamics (MD) simulations and MD-derived structure-based pharmacophore modelling was performed. The computational analysis has revealed that the substitution at position C5 can be used to modify the physicochemical properties, antibacterial spectrum and enhance the inhibitory potency of the compounds. Additionally, a discussion of challenges associated with the synthesis of 5-substituted 2-aminobenzothiazoles is presented.

1 Introduction

Overuse and misuse of antibacterials in human medicine, animal health and agriculture have led to the spread of antibiotic resistant bacteria. This is a great concern and one of the most complex global health challenges. Multidrug resistant and highly virulent pathogens from the ESKAPE group, which consists of Gram-negative *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacteriaceae* and Gram-positive *Staphylococcus aureus* and *Enterococcus faecium*, are all on the WHO global critical and high priority list (2017) to promote research, discovery and development of new antibiotics. To effectively combat antibacterial resistance, new antibacterial drugs are urgently needed.¹⁻⁴

DNA gyrase and topoisomerase IV (Topo IV), both type II topoisomerases, are attractive and well-established targets for antibacterial drug discovery with potential for dual targeting, which prolongs the onset of resistance. These enzymes catalyse reactions involving transient breaks of both strands of DNA:

relaxation of supercoiled DNA and introduction of negative supercoils into the molecule. Both of these reactions are ATP-dependent. DNA gyrase is a heterotetrameric protein consisting of two GyrA and two GyrB subunits (A_2B_2) whereas Topo IV is composed of two ParC and two ParE subunits (C_2E_2), homologous to GyrA and GyrB, respectively. Fluoroquinolones, by far the most successful antibacterials targeting DNA gyrase, interact with the GyrA and ParC subunits. The GyrB and ParE subunits contain the ATP-binding site and are the site of action for coumarin antibiotics.^{5,6} The aminocoumarin novobiocin was the first and thus far the only ATP-competitive GyrB inhibitor in clinical use, but was withdrawn due to safety issues and lack of efficacy. Despite the wide array of new structurally diverse GyrB and ParE inhibitors discovered, none are currently in clinical use.^{7,8} However, DNA gyrase remains a promising target, as fobrepodacin (SPR720) and DS-2969b are currently in clinical trials for the treatment of nontuberculous mycobacterial and *Clostridium difficile* infections respectively (Figure 1).^{9,10}

To investigate the potential of the position C5 of the benzothiazole for optimization we first solved a crystal structure of **A** in a complex with the 24 kDa fragment of *E. coli* GyrB (GyrB24) at a resolution of 1.16 Å (PDB code: 7P2N) (Figure 3). As expected, the inhibitor is bound in the ATP-binding site of GyrB24. The benzothiazole ring is involved in a cation- π -stacking interaction with the Arg76 side chain. The aromatic carboxylate group at C6 of the benzothiazole of the inhibitor **A** and the Arg136 side chain form a salt bridge. The carboxamide oxygen interacts with a conserved water molecule and the hydroxyl group in the side chain of Thr165. The 3,4-dichloro-5-methylpyrrole moiety is involved in hydrophobic interactions in the lipophilic pocket with residues Val167, Val120, Ile78, Val71, Ala47, and Val43, while the NH group of pyrrole forms a hydrogen bond with the side chain of Asp73. The hydroxy group at C5 of the benzothiazole scaffold is oriented towards the solvent-exposed area. This allows introduction of larger substituents to C5 which could influence the pharmacokinetic properties of benzothiazole based compounds and possibly broaden antibacterial activity.

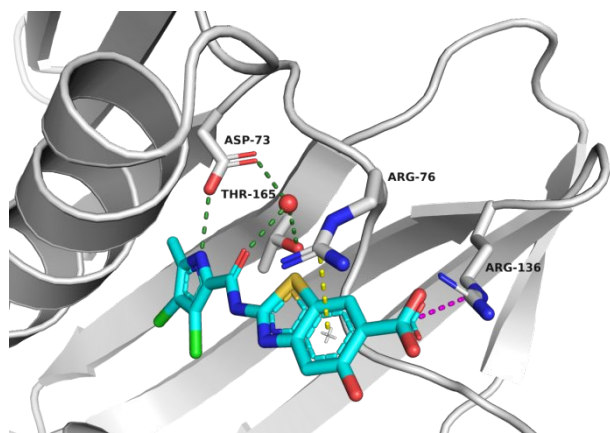


Figure 3: Cocrystal structure of compound **A** (in cyan sticks) in the ATP-binding site of *E. coli* DNA gyrase B (in grey cartoon; PDB ID 7P2N). The ligand and the amino acid residues that interact with it are shown as stick models, and coloured according to atom type (blue, N; red, O; light green, Cl; brown, S). The water molecule is presented as a red sphere, hydrogen bonds are indicated by green dotted lines, the salt bridge is indicated by the magenta dotted line and the cation- π interaction by a yellow dotted line.

Based on the data obtained from the crystal structure and favourable on-target activity of the inhibitor **A**, we decided to further explore the chemical space by the introduction of a fluorine atom and amine substituents at the C5 position (Figure 2). To probe potential interactions of new substituents with the enzyme, docking calculations and MD simulations were performed, using the cocrystal structure of *E. coli* GyrB containing the flexible loop Leu98-Gly117, which is absent in the crystal structure of **A** and GyrB (PDB ID 7P2N).

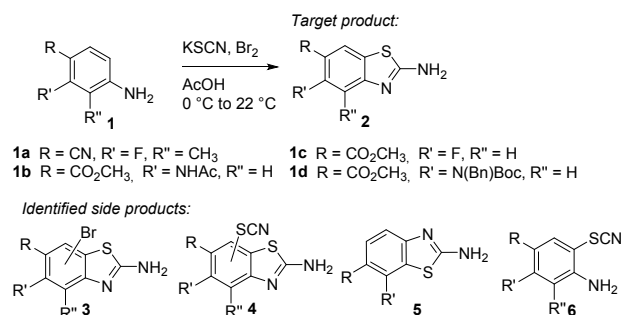
Our objective was to synthesize novel compounds that would retain potent inhibitory activity against the target enzymes, improve antibacterial activity against Gram-positive strains and at the same time broaden the spectrum of activity to Gram-negative bacterial strains.

2.2 Chemistry

For the synthesis of the inhibitors, one of the crucial synthetic steps was the formation of 5-substituted methyl 2-aminobenzo[*d*]thiazole-6-carboxylates **2** from the corresponding 2-substituted methyl 4-aminobenzoates **1** (Scheme 1). We first attempted the synthesis of the 2-aminobenzothiazoles **2** using our general synthetic protocol involving potassium thiocyanate (4 equivalents) and bromine (2 equivalents) in glacial acetic acid.²¹ Starting from 4-amino-2-fluoro-3-methylbenzonitrile (**1a**), methyl 2-acetamido-4-aminobenzoate (**1b**) or methyl 4-amino-2-fluorobenzoate (**1c**), the formation of the target benzothiazoles **2** was very low-yielding due to formation of side products with a similar solubility profile and chromatographic retention factor, which made isolation difficult. The identified side products **3–6** are presented in Scheme 1. For detailed side-product analysis, see Schemes S1–S4 and Figures S1–S4.

To potentially reduce the number of side products **3** and **4**, the excess of KSCN and Br₂ was reduced (from 4 and 2 equivalents to 3 and 1.5 equivalents respectively) and some modifications in the reaction protocol were implemented. In particular, instead of mixing KSCN and the starting aniline **1**, followed by adding Br₂ dropwise at 0 °C, the thiocyanogen reagent, (SCN)₂, was pre-formed from KSCN and Br₂ in acetic acid at 22 °C and added dropwise to the solution of aniline **1** in a small volume of acetic acid. This solved the issue of brominated side products **3**.

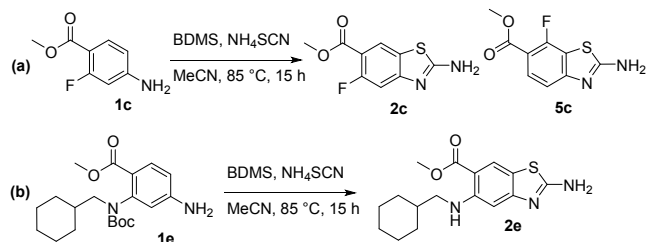
Scheme 1: Target products **2** and identified side products: products with additional Br or SCN on benzothiazole scaffold **3** and **4**, respectively, incorrectly cyclized product **5**, and acyclic side product **6**.



Instead of highly toxic and corrosive molecular Br₂ that is hazardous and difficult to handle, alternative sources of Br₂ for in-situ formation of thiocyanogen have been described, such as the solid benzyltrimethylammonium dichloroiodate,²² and bromodimethylsulfonium bromide (BDMS).²³ In our hands, using the first one resulted in no conversion, while using a combination of BDMS and ammonium thiocyanate for the conversion of aniline **1c**, the formation of side products **3c**, **4c** and **6c** was prevented. Only the regioisomers **2c** and **5c** were formed and we managed to isolate the analytically pure desired product **2c** after chromatographic purification, although the isolated yield was less than 5% (Scheme 2a). The same protocol was used to prepare the (cyclohexylmethyl)amino analog **2e** from the Boc-protected **1e** (Scheme 2b). In this case, the formation of the unwanted regioisomer **5e** was prevented by the bulky *N*-(cyclohexylmethyl)Boc-amino substituent. During reaction or workup, the Boc protection was cleaved and diamine

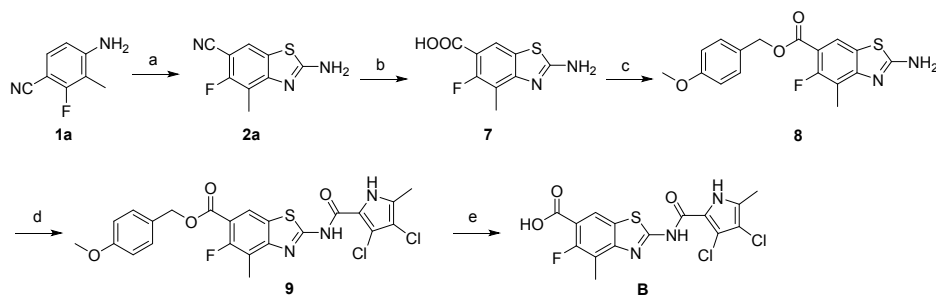
2e was isolated in 14% yield, which was not suitable for further elaboration to the target topoisomerase inhibitors.

Scheme 2: (a) Synthesis of target product **2c** and its regioisomer **5c**, and (b) synthesis of compound **2e** where the formation of its regioisomer is prevented.



After these preliminary synthetic attempts it became clear that bulky substituents at the meta position of anilines **1** can prevent formation of the regioisomeric products **5** and that pre-forming thiocyanogen or use of alternative oxidising agents can prevent formation of bromo and thiocyno side-products **3** and **4**. These

Scheme 3. Synthesis of compound B.



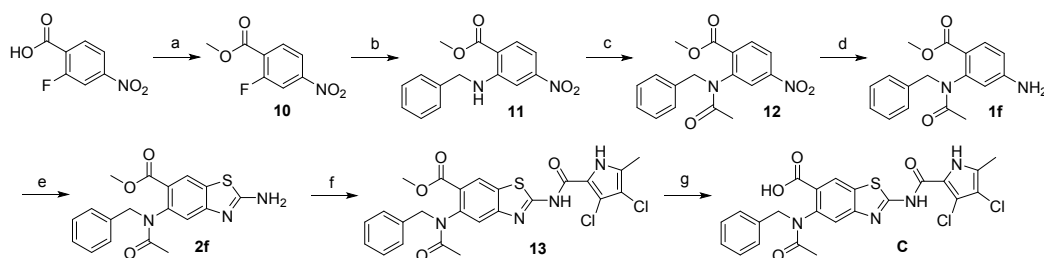
Reagents and conditions: (a) KSCN, Br₂, acetic acid, 22 °C, overnight; (b) H₂O/H₂SO₄/acetic acid = 1:1:1, 130 °C, overnight; (c) *p*-methoxybenzyl chloride, K₂CO₃, dry DMF, 40 °C, overnight; (d) 2-trichloroacetyl-3,4-dichloro-5-methyl-1*H*-pyrrole, Na₂CO₃, DMF, 60 °C, overnight; (e) 1 M HCl/ acetic acid, then 4 M HCl in 1,4-dioxane, 22–50 °C, 30 h.

Compound **C** was synthesized according to Scheme 4. 2-Fluoro-4-nitrobenzoic acid was first converted to methyl ester **10** using H₂SO₄ in methanol. The fluorine atom was displaced by phenylmethanamine to give the secondary amine **11** in a nucleophilic aromatic substitution reaction. The amine was acylated with acetyl chloride in the presence of 4-dimethylaminopyridine (4-DMAP) and *N,N*-

synthetic tools were eventually adequate to prepare the designed inhibitors of DNA gyrase and Topo IV **B–E**. To evaluate the influence of the fluorine atom at C5 on biological activity, we prepared compound **B** with a methyl group at C4 (Scheme 3). Because of its small size, the methyl group should not affect the on-target or antibacterial activity of the final compound, but it does improve synthetic feasibility by blocking the formation of the corresponding regioisomer **5a**. First, **1a** was dissolved in glacial acetic acid and cooled to 0 °C. (SCN)₂, prepared ex situ by mixing KSCN (4 equiv) and Br₂ (2 equiv) in glacial acetic acid, was added dropwise to the solution of the starting compound and stirred overnight at 22 °C to get **2a**. Hydrolysis of the cyano group of **2a** gave **7**. The newly formed carboxylic group was protected as *p*-methoxybenzyl ester. The resulting amine **8** was coupled with 2-trichloroacetyl-3,4-dichloro-5-methyl-1*H*-pyrrole in DMF in the presence of Na₂CO₃ at 60 °C to obtain **9**. In the last step, the carboxylic group was deprotected by acidolysis to yield the final product **B**.

diisopropylethylamine (DIPEA) to obtain **12**. The nitro group was then reduced using iron in glacial acetic acid to give **1f**. The above described cyclization procedure was applied to obtain the desired product **2f**, which was coupled with 2-trichloroacetyl-3,4-dichloro-5-methyl-1*H*-pyrrole to yield the pyrrolamide **13**, which was then subjected to alkaline hydrolysis of methyl ester to give the final compound **C**.

Scheme 4. Synthesis of compound C.

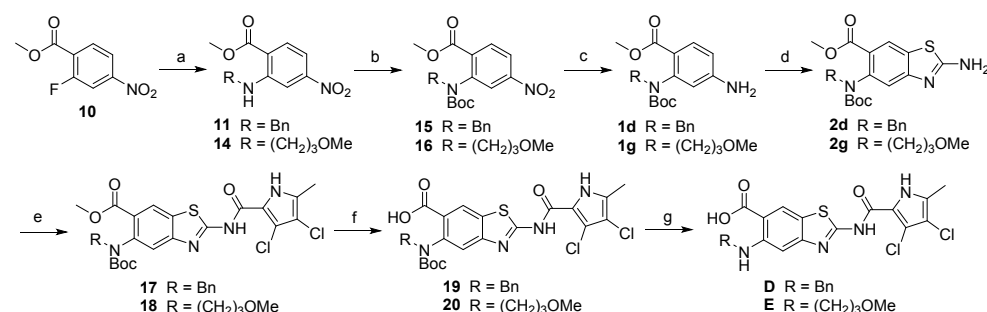


Reagents and conditions: (a) H₂SO₄, MeOH, 65 °C, overnight; (b) benzylamine, K₂CO₃, CH₃CN, 60 °C, overnight; (c) acetyl chloride, 4-DMAP, DIPEA, dry DCM, 22–35 °C, overnight; (d) Fe⁰, acetic acid, 22 °C, 2 h; (e) KSCN, Br₂, acetic acid, 22 °C, overnight; (f) 2-trichloroacetyl-3,4-dichloro-5-methyl-1*H*-pyrrole, Na₂CO₃, dry DMF, 22–60 °C, overnight; (g) 2 M NaOH, MeOH, 60 °C, 3 days.

Compounds **D** and **E** were synthesized according to Scheme 5. 2-Fluoro-4-nitrobenzoic acid was first converted to the methyl ester **10** using H₂SO₄ in methanol. The fluorine atom was displaced by the corresponding amine (phenylmethanamine or 3-methoxypropan-1-amine) to give a secondary amine **11** or **14** in a nucleophilic aromatic substitution reaction. The amino group of **11** or **14** was Boc-protected to obtain **15** or **16**. The nitro group was then reduced to amino using iron in glacial

acetic acid to give **1d** or **1g**. The cyclization procedure described above was applied to **1d** and **1g** to obtain the desired product **2d** and **2g**, respectively. These were coupled with 3,4-dichloro-5-methylpyrrole-2-carbonyl chloride, to yield pyrrolamides **17** and **18**, which were subjected to alkaline hydrolysis of methyl ester to give **19** and **20**. Boc-deprotection then yielded the final compounds **D** and **E**.

Scheme 5. Synthesis of compounds **D** and **E**.



Reagents and conditions: (a) R-NH₂, K₂CO₃, CH₃CN, 60 °C, overnight; (b) Boc₂O, 4-DMAP, THF, 40 °C, overnight; (c) Fe⁰, acetic acid, 22 °C, 2 h; (d) KSCN, Br₂, acetic acid, 22 °C, overnight; (e) 3,4-dichloro-5-methylpyrrole-2-carbonyl chloride, toluene, 130 °C, overnight; (f) 2 M NaOH, MeOH, 60 °C, 3 days; (g) 4 M HCl in 1,4-dioxane, 22 °C, 2 h.

2.3 In vitro enzyme inhibition and antibacterial activity

Compounds **B**, **C**, **D** and **E** were evaluated against DNA gyrase from *E. coli* a supercoiling and against Topo IV in relaxation assay. The results are presented in Table 1 and Figure S5 as concentrations of compounds that inhibit the enzyme activity by 50% (IC₅₀ values). All new inhibitors were found to have nanomolar inhibition of DNA gyrase with IC₅₀ values < 71 nM. Replacement of the hydroxyl group (**A**; IC₅₀ < 10 nM) at C5 of the benzothiazole ring with a fluorine atom (**B**; IC₅₀ = 40 nM), and *N*-benzylacetamido group (**C**; IC₅₀ = 71 nM), resulted in weaker inhibition of gyrase whereas compounds with secondary amine substituents, a benzylamino group in **D** and a 3-methoxypropylamino group in **E**, retained low nanomolar inhibition (IC₅₀ < 10 nM). Larger differences were observed for activity against Topo IV, where all substitutions implemented resulted in weaker activity, with **B** and **C** being inactive (having IC₅₀ values of 2000 nM and 4000 nM respectively). Compounds **D** and **E** showed IC₅₀ values of 293 nM and 210 nM respectively and can be considered to act as dual targeting DNA gyrase/Topo IV inhibitors. Compounds **B**, **C**, **D** and **E** were tested for antibacterial activity against Gram-positive and Gram-negative bacteria, including ESKAPE pathogens.

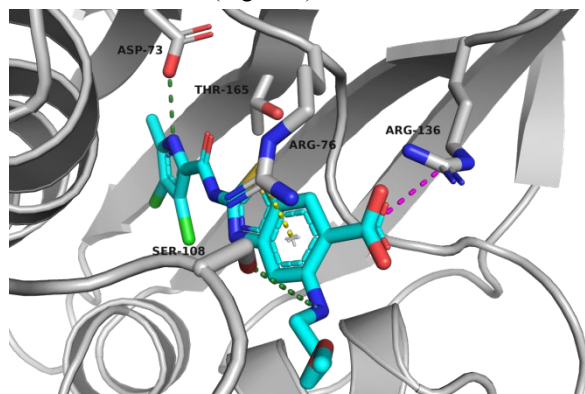
Tree compounds, **B**, **D** and **E** have, in comparison to inhibitor **A**, demonstrated improved MIC values against Gram-positives *S. aureus*, MRSA, VISA, and *E. faecium*, with excellent results for compounds **D** and **E** showing MIC < 0,03 µg/mL for most strains. Moreover, whereas **B** and **C** similarly to compound **A**, showed no activity against Gram-negative strains (MIC values > 64 µg/mL), for inhibitors **D** and **E** potent activities in the range of 4 to 16 µg/mL were detected against *E. coli*, *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*. In spite of having similar on-target activity on DNA Gyrase and being even less potent inhibitors of Topo IV than compound **A**, inhibitors **D** and **E** bearing secondary amine substituents on

position C5 show better, broad-spectrum antibacterial activity. To evaluate selectivity against a related human target, compounds **D** and **E** were tested for their inhibitory activity on human topoisomerase II α . Both compounds showed good selectivity, with enzyme residual activity of 100% for **D** and 55% for **E** at 100 µM.

2.4 Docking calculations, MD simulations, structure-based pharmacophore modelling

The binding modes of inhibitors **D** and **E** in the ATP-binding site of *E. coli* DNA gyrase were studied by a combination of docking calculations, molecular dynamics (MD) simulations and MD-derived structure-based pharmacophore modelling. For molecular modelling we used the cocrystal structure of *E. coli* GyrB in complex with phosphoaminophosphonic acid-adenylate ester (PDB ID 4WUB),²⁴ in which the flexible loop Leu98-Gly117, absent in the complex with **A** (PDB ID 7P2N), is present and allows investigation of the potential interactions between the substituent at C5 and the enzyme. Docking calculations showed that the binding mode of the core 2-(3,4-dichloro-5-methyl-1*H*-pyrrole-2-carboxamido)benzo[*d*]thiazole-6-carboxylic acid is identical to that of **A** (Figure 3). No additional interactions with GyrB and the benzylamino group of **D** were predicted by docking. However, in the case of **E**, a hydrogen bond was suggested between the oxygen atom of the 3-methoxypropylamino group and Ser108 side chain. To investigate the possible interactions between the benzylamino group of **D** or the 3-methoxypropylamino group of **E** with the flexible loop Leu98-Gly117 of *E. coli* GyrB, we performed 100 ns MD simulations starting from the docking complexes. Analysis of the interactions in the MD trajectory using structure-based pharmacophore modelling in LigandScout revealed that the 3-methoxypropylamino group of **E** rarely interacted with GyrB,

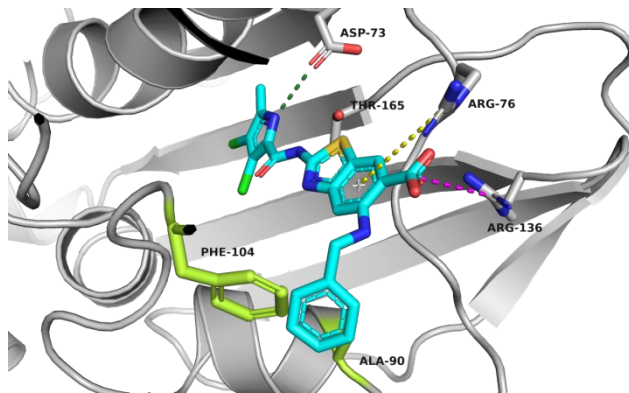
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2 as hydrogen bonding with Ser108 was present for only 1% of
3 the simulation time (Figure 4).



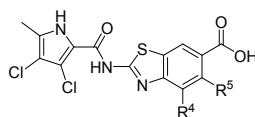
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17 **Figure 4.** Representative binding mode from molecular dynamics
18 simulation trajectory of compound **E** in the ATP-binding site of *E.*
19 *coli* DNA gyrase B (in grey cartoon; PDB ID 4WUB). The ligand
20 and amino acid residues that interact with ligands are shown as
21 stick models, according to atom types (blue, N; red, O; light green,
22 Cl; brown S). Hydrogen bonds are indicated by green dotted lines,
23 salt bridge is indicated by magenta dotted line and cation- π
24 interaction by yellow dotted line.

25 In contrast, the benzylamino group of **D** formed additional
26 interactions with the amino acid residues in the flexible loop,
27 namely a π -stacking interaction with Phe104 side chain and
28 hydrophobic interactions with Ala90, Val93 and Phe104 side
29 chains (Figure 5). From these analyses, we corroborated that the
30 substitution at position C5 can also be used to boost the

inhibitory potency of the compounds and not only affect the
physicochemical properties.



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models and coloured according to atom type (blue, N; red, O; light
green, Cl; brown S). Hydrogen bonds are indicated by green dotted
lines, salt bridge is indicated by magenta dotted line and cation- π
interaction by yellow dotted line.

Table 1. Minimum inhibitory concentrations (MIC) and IC₅₀ values for the inhibitors A–E against Gram-positive and Gram-negative bacterial strains.

Compound	A	B	C	D	E
R ⁴ =	H	CH ₃	H	H	H
R ⁵ =	OH	F			
IC ₅₀ [μM] ^a					
<i>E. coli</i> gyrase	< 0.01	0,04 ± 8 × 10 ⁻³	0,071 ± 18 × 10 ⁻³	< 0.01	< 0.01
<i>E. coli</i> Topo IV	0,095 ± 4 × 10 ⁻³	2 ± 0,9	4 ± 3 × 10 ⁻³	0,293 ± 0.155	0,21 ± 0,072
MIC [μg/mL] / [μM] ^b					
Gram-positive bacteria					
<i>S. aureus</i> ATCC29213	16/41	0.5/1.2	8/16	< 0.03/< 0.07	< 0.03/< 0.07
<i>S. aureus</i> (MRSA) ATCC43300	4/10	0.25/0.6	8/16	< 0.03/< 0.07	< 0.03/< 0.07
<i>S. aureus</i> (VISA) ATCC700699	nt	nt	nt	< 0.03/< 0.07	< 0.03/< 0.07
<i>E. faecium</i> ATCC700221	nt	nt	nt	< 0.13/< 0.26	< 0.06/< 0.14
Gram-negative bacteria					
<i>E. coli</i> ATCC25922	> 64/> 166	> 64/> 159	> 64/> 124	16/34	8/18
<i>K. pneumoniae</i> ATCC10031	8/21	> 64/> 159	64/124	4/8,4	4/9
<i>A. baumannii</i> ATCC17978	> 64/> 166	> 64/> 159	> 64/> 124	8/17	4/9
<i>P. aeruginosa</i> ATCC27853	> 64/> 166	> 64/> 159	> 64/> 124	8/17	16/35
<i>Enterobacter cloacae</i> ATCC13047	nt	> 64/> 159	> 64/> 124	> 64/> 135	> 64/> 140

^aIC₅₀, concentration (mean ± SD of three independent experiments) that inhibits enzyme activity by 50%. ^bMIC, minimum inhibitory concentration. MIC measurements were performed according to the Clinical and Laboratory Standards Institute guidelines, with three independent measurements. nt, not tested.

3 Conclusion

Growing the 2-aminobenzothiazole-cored topoisomerase inhibitors at C5 was deemed promising based on the cocrystal structure of the inhibitor **A** in complex with *E. coli* GyrB24, and on the corresponding in silico design. The synthesis of 5-substituted 2-aminobenzothiazoles has proven to be challenging due to low reaction yields and an unfavourable impurity profile, but we have developed synthetic tools for preparation of the model dual-targeting 2-aminobenzothiazole-based DNA gyrase and Topo IV inhibitors with substitution at C5. In particular, compounds **D** and **E** showed low nanomolar inhibition of gyrase and nanomolar inhibition of Topo IV from *E. coli*. Both compounds showed broad-spectrum antibacterial activity against pathogens belonging to the ESKAPE group.

4 Experimental section

4.1 Synthetic procedures and analytical data

Synthetic procedures and analytical data including NMR, MS, HPLC data, are available within the paper supporting information files.

4.2 Determination of inhibitory activities on *E. coli* DNA gyrase, topoisomerase IV and human topoisomerase II α

Commercially available assay kits (Inspiralis Limited, Norwich, UK) were used for determination of the IC₅₀ values for test compounds for inhibition of DNA gyrase supercoiling and Topo IV and TopoII α relaxation. Assays were performed according to previously reported procedures.²⁵ For details on enzyme assays, see the Supporting Information.

4.3 Determination of antibacterial activity

Antimicrobial assays (Minimum inhibitory concentrations (MICs)) were performed by standard serial broth microdilution method. For details on antimicrobial assays, see the Supporting Information.

4.4 X-ray Crystallography

X-ray structure of compound **A** in complex with *E. coli* GyrB24 was obtained at 1.16 Å resolution (PDB code: 7P2N). For details on protein expression and purification, as well as crystallization, X-ray data acquisition, and structure solution, see Supporting Information.

4.5 Molecular modelling

For molecular modelling the cocrystal structure of *E. coli* GyrB in complex with phosphoaminophosphonic acid-adenylate ester (PDB ID 4WUB),²⁴ was used. Molecular docking calculations were performed in Schrödinger Release 2022-1 (Schrödinger, LLC, New York, NY, USA, 2022). MD simulation of compound **D** or compound **E** in complex with *E. coli* GyrB was performed using NAMD package (version 2.9)²⁶ and the CHARMM36m²⁷ force field. Pharmacophore feature analysis of *E. coli* GyrB in complex with compound **D** or compound **E** was performed using LigandScout 4.4 Expert.²⁸ For details on molecular docking, molecular dynamic simulations and structure-based pharmacophore modeling, see the Supporting Information.

ASSOCIATED CONTENT

Supporting Information.

Additional experimental data, X-ray crystallography details, NMR spectra, HPLC traces (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>

PDB Accession Codes

complex of *E. coli* GyrB24 with inhibitor **A**: 7P2N;

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare the following competing financial interest: A.M. is a Non-Executive Director, Scientific Advisor and Co-Founder of Inspiralis Ltd.

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ABBREVIATIONS

CCP4, Collaborative Computational Project No. 4; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; DTT, dithiothreitol; MIC, minimum inhibitory concentration; NAMD, Nanoscale Molecular Dynamics; NPT, Isothermal-isobaric ensemble; THF, tetrahydrofuran; TLC, thin-layer chromatography

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