Design, synthesis and biological evaluation of 4,5-dibromo-*N*-(thiazol-2-yl)-1*H*-pyrrole-2-carboxamide derivatives as novel DNA gyrase inhibitors

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ABSTRACT

Development of novel DNA gyrase B inhibitors is an important field of antibacterial drug discovery whose aim is to introduce a more effective representative of this mechanistic class into the clinic. In the present study, two new series of *Escherichia coli* DNA gyrase inhibitors bearing the 4,5-dibromopyrrolamide moiety have been designed and synthesized. 4,5,6,7-Tetrahydrobenzo[1,2-*d*]thiazole-2,6-diamine derivatives inhibited *E. coli* DNA gyrase in the submicromolar to low micromolar range (IC_{50} values between 0.891 and 10.4 μM). Their "ring-opened" analogues, based on the 2-(2-aminothiazol-4-yl)acetic acid scaffold, displayed weaker DNA gyrase inhibition with IC_{50} values between 15.9 and 169 μM. Molecular docking experiments were conducted to study the binding modes of inhibitors.

KEYWORDS antibacterial; DNA gyrase; docking; inhibitor; thiazole

1. Introduction

Antibacterial drug resistance, especially that of 'ESKAPE' organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter* spp.), is a growing threat to human health, not only in hospitals but in the community in general [1]. Therefore, continuous discovery and development of new antibacterial agents that avoid the existing resistance mechanisms is of great importance to increase the number of drug candidates in the pipeline for treating these serious, life-threatening infections.

DNA gyrase is a well-established and validated target for antibacterial drug discovery. It offers opportunities for the development of drugs that could avoid some of the existing resistance mechanisms. It catalyzes changes in DNA topology during replication by introducing negative supercoils. It is a heterotetrameric enzyme consisting of two GyrA subunits, which are involved in cleavage and reunion of the DNA substrate, and two GyrB subunits that, by hydrolysis of ATP, provide energy for the supercoiling reaction of GyrA. Topoisomerase IV, which is involved in decatenation of daughter DNA following DNA replication, is structurally similar to DNA gyrase, being composed of two ParC and two ParE subunits that are homologous to GyrA and GyrB, respectively [2].

The structural similarity of DNA gyrase and topoisomerase IV provides the opportunity for dual targeting in most bacteria, which prolongs the onset of resistance, making these two enzymes attractive targets for antibacterial drug discovery [3, 4]. The GyrA subunits are targets of the clinically important class of antibacterial drugs, the fluoroquinolones, which stabilize the complex between DNA gyrase and DNA [5]. The increasing level of resistance to fluoroquinolones limits their therapeutic use [6] and stimulates the search either for novel structural classes of inhibitors targeting GyrA or for development of inhibitors of the GyrB ATPase activity [4, 7, 8]. Novobiocin, an

aminocoumarin antibiotic, remains the first and still the only, example of GyrB inhibitor that has been used in therapy, primarily for infections caused by Gram positive bacterial strains. Its use declined during the 1960s and early 1970s following the introduction of penicillinase-stable penicillins and the first generation of cephalosporins, and was eventually withdrawn from the market [4]. Despite extensive efforts to develop a new representative of this mechanistic class, no GyrB inhibitor has progressed beyond phase 1 clinical trials [4]. Several structural classes of GyrB and/or ParE inhibitors, such as cyclothialidines [9], ethylureas [10-14], azaindoles [15], and pyrrolamides [16-18], were investigated in the past decades by the pharmaceutical industry. The majority of these GyrB and/or ParE inhibitors possess potent enzyme inhibitory activity and antibacterial activity, but mainly against Gram positive strains [4]. In contrast, there are few examples of GyrB/ParE inhibitors displaying anti-Gram negative activity, such as pyrrolopyrimidines [19] and pyrimidinoindoles [20]. DNA gyrase inhibitors have been discovered by academic research groups, for example by virtual screening [21-23] and by structure-based design of marine alkaloid analogues [24, 25]. Although many antibacterial drug discovery projects in the pharmaceutical industry were recently terminated, the knowledge gathered over more than 50 years of research directed towards ATPase inhibitors of DNA gyrase and topoisomerase IV provides an excellent foundation for further research to finally introduce a new and effective representative of this mechanistic class in antibacterial therapy [4].

We have recently designed several structural classes of analogues of marine alkaloids based on clathrodin, hymenidin and oroidin, isolated from sponges of the genus *Agelas*, and evaluated their voltage-gated sodium channel modulatory activity [26-29], inhibition of biofilm formation [30], antimicrobial activity [31], and pro-apoptotic activity in HepG2 and THP-1 cell lines [32]. The similarity of oroidin and its analogues to the known pyrrolamide-based inhibitors [17, 18] stimulated us to evaluate selected oroidin analogues from our library

for *E. coli* DNA gyrase inhibition. Optimization of the hit compounds [24] and design of novel 4,5-dibromopyrrolamide derivatives [25] resulted in potent DNA gyrase inhibitors exhibiting IC_{50} values in the low nanomolar range, but possessing weak antibacterial activity. The X-ray crystal structure of 4,5-dibromopyrrolamide-based inhibitor in complex with *E. coli* DNA gyrase subunit B was solved, which confirmed the assumed binding mode of inhibitor to the ATP-binding site of DNA gyrase [25]. The pyrrolamide moiety was found to occupy the same binding pocket of the *E. coli* GyrB ATP-binding site as that observed for other pyrrolamide-based DNA gyrase inhibitors, such as natural antibiotics clorobiocin [33] and kibdelomycin [34], and some described synthetic inhibitors [17, 18] (Figure 1). We describe here the design and synthesis of a novel series of 4,5-dibromopyrrolamides and the evaluation of their *E. coli* DNA gyrase inhibition as well as their antibacterial activity against selected Gram positive and Gram negative bacterial strains.

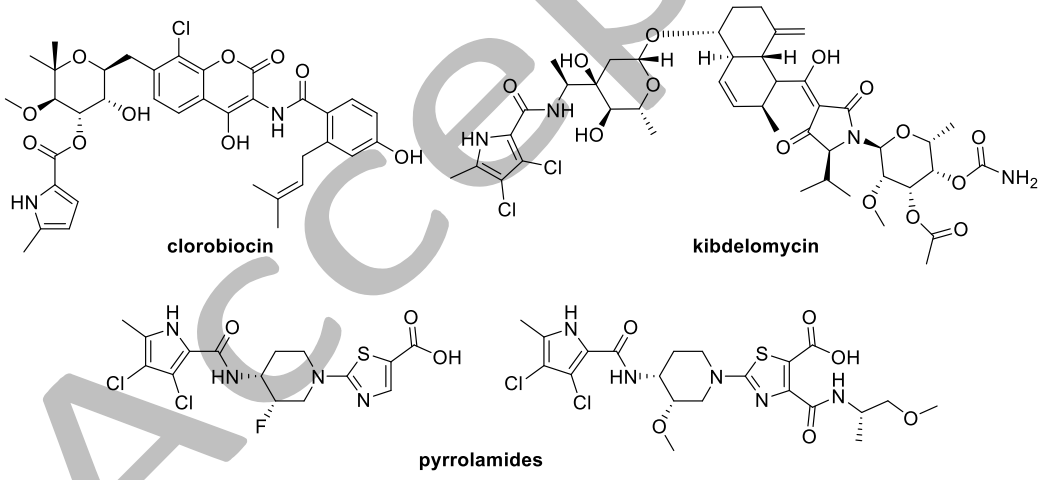


Figure 1. Pyrrolamide-based inhibitors of DNA gyrase.

2. Results and discussion

2.1. Design

X-Ray crystal structure of the 4,5-dibromopyrrolamide-based inhibitor **I** in the ATP-binding site of *E. coli* DNA gyrase revealed three hydrogen bonds formed between the

enzyme and the pyrrolamide moiety of inhibitor [25]. A hydrogen bond is seen between a pyrrole NH group and the Asp73 side chain, while a second hydrogen bond is formed between an adjacent carbonyl group and a structurally conserved water molecule that is in contact with the Asp73 side chain (Figure 2) [25]. With this conserved binding motif of adjacent hydrogen bond donor/acceptor groups, GyrB inhibitors mimic the binding of ATP [8]. The NH of the amide group is additionally bridged by a water molecule to the side chain of Thr365 (Figure 2). The formation of several hydrophobic interactions between the dibromo substituted pyrrole moiety and the protein is crucial for achieving potent *E. coli* DNA gyrase inhibition, since 4-bromopyrrole, 4,5-dichloropyrrole and indole derivatives displayed weaker inhibition of the enzyme [24, 25]. In this new series, the 4,5-dibromopyrrolamide moiety has thus been retained. To further explore the structure-activity relationship of the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole-2,6-diamine derivatives we synthesized compounds in which the 4,5-dibromopyrrole-2-carbonyl moiety is attached to the amino group at position 2, instead of that at position 6, resulting in the "reversed" type **III** analogues (Figure 3) of the parent compounds (compound **II** and its analogues [24], Figure 3). Additional substituents designed to target the Arg76/Arg136 side chain were then introduced at the 6-amino group (Figure 3). In addition, a series of 2-(2-aminothiazol-4-yl)acetic acid derivatives (type **IV** analogues, Figure 3) were designed and synthesized. These so-called "ring-opened" compounds are more flexible than type **III** compounds and possess a smaller central scaffold, and thereby offering the opportunity for the introduction of larger substituents for potential interaction with Arg76/Arg136. The design of all these compounds was supported by molecular docking to the *E. coli* DNA gyrase ATP-binding site. The predicted binding modes are presented in Figures 4 and 5.

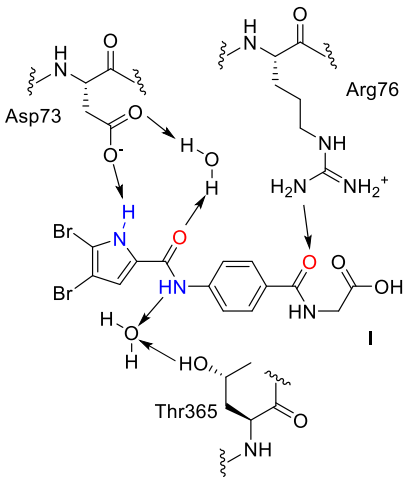


Figure 2. Schematic representation of hydrogen bonds between inhibitor **I** and *E. coli* DNA gyrase ATP-binding site residues (PDB entry: 4ZVI [25]).

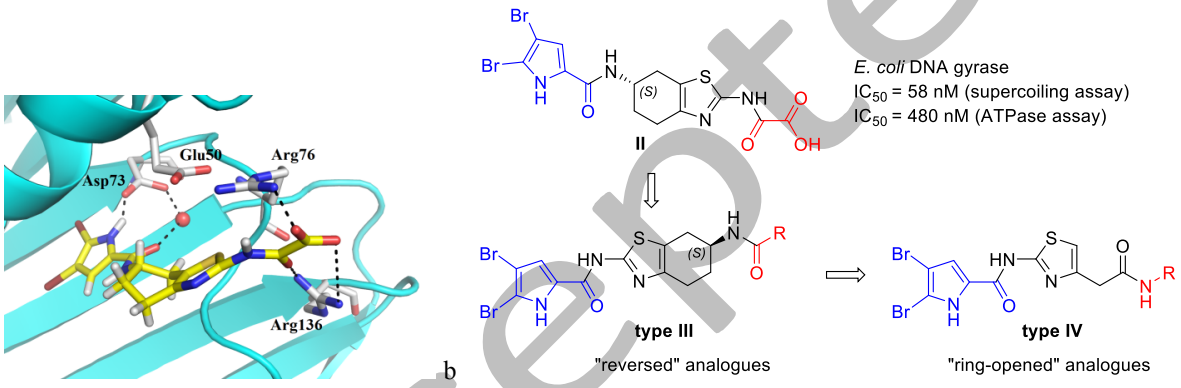
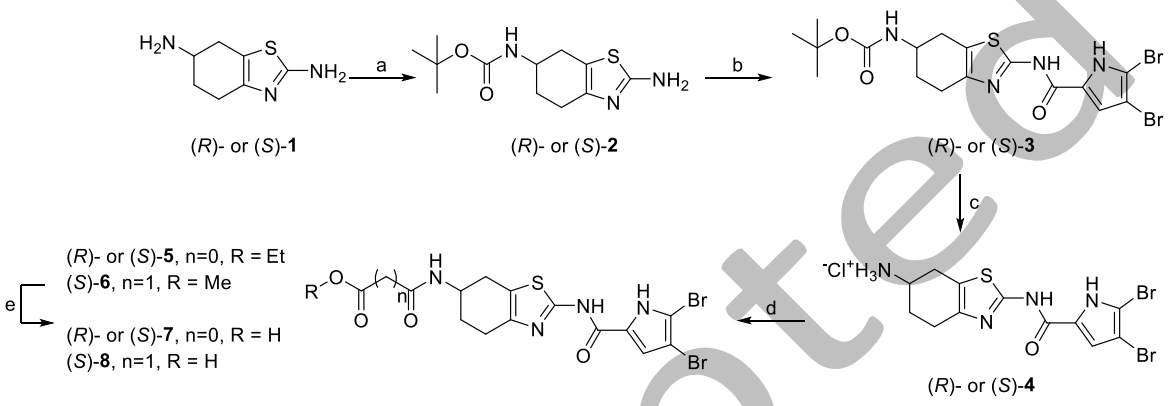


Figure 3. a) Molecular docking pose of *E. coli* DNA gyrase inhibitor **II** in the ATP-binding site of *E. coli* DNA gyrase [24]. b) Design of a novel series of 4,5-dibromopyrrolamide-based DNA gyrase inhibitors.

2.2. Chemistry

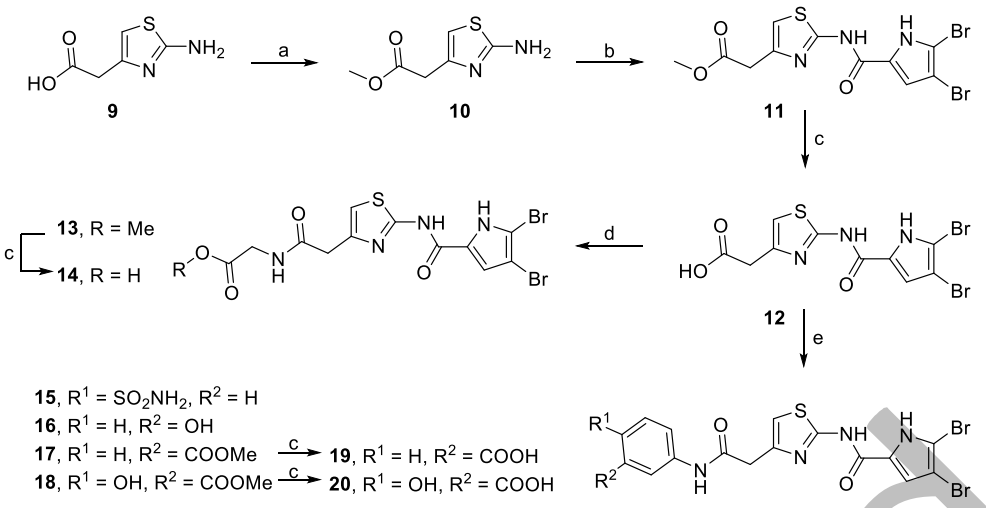
The designed 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole-2,6-diamine derivatives **5-8** were prepared according to the synthetic procedure presented in Scheme 1. Enantiomerically pure starting (*S*)- and (*R*)-4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole-2,6-diamines ((*R*)-**1** and (*S*)-**1**) were synthesized according to the reported procedure [24, 35]. In the first step, the amino group at position 6 was protected as a *tert*-butylcarbamate using di-*tert*-butyl dicarbonate in tetrahydrofuran to yield (*R*)-**2** and (*S*)-**2**. The amino group at position 2 of (*R*)-**2** and (*S*)-**2** was then acylated with 2,2,2-trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethanone in *N*,*N*-

dimethylformamide (DMF) at elevated temperature. The Boc protecting groups of the compounds (*R*)-**3** and (*S*)-**3** obtained were removed with HCl gas generated *in situ* by the slow addition of acetyl chloride to methanol. This acidolysis yielded hydrochlorides (*R*)-**4** and (*S*)-**4** which were then acylated with ethyl oxalyl chloride ((*R*)-**5** and (*S*)-**5**) or methyl malonyl chloride ((*S*)-**6**) in DMF at room temperature. In the final step, esters (*R*)-**5**, (*S*)-**5** and (*S*)-**6** were hydrolyzed to their acid derivatives (*R*)-**7**, (*S*)-**7** and (*S*)-**8** using alkaline conditions.



Scheme 1. *Reagents and conditions.* a) Boc_2O, THF, r.t., 24 h; b) 2,2,2-trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethanone, Na_2CO_3, DMF, 80 °C, 18 h; c) acetyl chloride, MeOH, 0 °C, 1 h, then r.t., 18 h; d) ethyl oxalyl chloride (for (*R*)-**5** and (*S*)-**5**) or methyl malonyl chloride (for (*S*)-**6**), DBU, DMF, r.t., 24 h; e) 1 M NaOH, $MeOH/H_2O$, r.t., 24 h.

Synthesis of the 2-(2-aminothiazol-4-yl)acetic acid derivatives **11-20** is outlined in Scheme 2. 2-(2-Aminothiazol-4-yl)acetic acid (**9**) was first esterified with methanol to give **10** and the amino group at position 2 of the thiazole moiety of **10** then acylated by 2,2,2-trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethanone, as described above, to give compound **11**. Hydrolysis of the methyl ester **11** resulted in carboxylic acid **12**, which was coupled with either glycine methyl ester hydrochloride to give **13** or substituted aniline derivatives **15-18** using EDC/HOBt-promoted amide bond formation. Further, methyl esters **13**, **17** and **18** were converted to their carboxylic acid counterparts **14**, **19** and **20** by alkaline hydrolysis.



Scheme 2. *Reagents and conditions.* a) $SOCl_2$, MeOH, reflux, 1.5 h; b) 2,2,2-trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethanone, Na_2CO_3, DMF, 80 °C, 18 h; c) 2 M NaOH, MeOH, r.t., 24 h; d) H-Gly-OMe HCl, EDC, HOBt, NMM, DMF, r.t., 18 h; e) corresponding $H_2NC_6H_4R^1R^2$, EDC, HOBt, NMM, DMF, r.t., 18 h.

2.3. Biological evaluation, structure-activity relationship and molecular modeling

The final compounds (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7**, (*S*)-**8**, **13-20** were tested for *E. coli* DNA gyrase inhibition using the DNA gyrase supercoiling assay (Tables 1 and 2). Results are presented as residual activities (RA) of the enzyme at 100 μM of the tested compound, or as IC_{50} values for the more active compounds (RA<50% at 100 μM). All prepared final compounds were screened for their antibacterial activity at 50 μM against two Gram positive (*Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923) and two Gram negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) bacterial strains. Results of the antibacterial activity evaluation are presented in Tables 1 and 2.

2.3.1. 4,5,6,7-Tetrahydrobenzo[1,2-d]thiazoles

The *in vitro E. coli* DNA gyrase inhibitory activities of 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazoles (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7** and (*S*)-**8** (Table 1) showed weaker DNA gyrase inhibition than that of the parent compounds bearing a pyrrole moiety on the amino group at

position 6 (compound **II** and its analogues [24]). With an IC_{50} value of 4.47 μM the oxalic acid ester (*S*)-**5** was a 2-fold weaker inhibitor than the malonic acid ester (*S*)-**6** with an IC_{50} value of 2.34 μM. Hydrolysis of these esters to their carboxylic acid counterparts resulted in improved *E. coli* DNA gyrase inhibition. The oxalic acid derivative (*S*)-**7** (IC_{50} = 1.12 μM) was approximately 4-fold more potent than its parent ester (*S*)-**5** (IC_{50} = 4.47 μM), while the activity of the malonic acid derivative (*S*)-**8** was improved to the submicromolar range (IC_{50} = 0.891 μM). These results highlight the importance of the chain length, since malonic acid ester and acid (compounds (*S*)-**6** and (*S*)-**8**), with an additional methylene group, displayed more potent *E. coli* DNA gyrase inhibition than their oxalic acid-based analogues (*S*)-**5** and (*S*)-**7**. The inhibitory activity of the oxalic acid-based *R*-enantiomers (compounds (*R*)-**5** and (*R*)-**7**) was weaker (IC_{50} values of 10.4 μM and 3.58 μM, respectively) than that of their *S*-enantiomers (*S*)-**5** and (*S*)-**7**. A similar phenomenon was observed in our previous series of the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole-2,6-diamines with pyrrole moiety attached to the amino group at position 6 [24].

Molecular docking experiments using FlexX [36, 37], as available in LeadIT (BioSolveIT GmbH) [38], were conducted to study the binding modes of *E. coli* DNA gyrase inhibitors (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7** and (*S*)-**8**. We used our already described and validated docking protocol [24] using the high-resolution crystal structure of the 4,5'-bithiazole inhibitor in complex with *E. coli* DNA gyrase (PDB entry: 4DUH) as the target protein [22]. The obtained docking poses and scoring function scores cannot explain the observed differences in binding affinities. All tested inhibitors are predicted to form two hydrogen bonds in the pyrrolamide-binding pocket, one direct and one bridged by a conserved water molecule to Asp73 side chain (Figure 4). Moreover, docking predicts formation of additional hydrogen bonds with Arg76 and/or Arg136 side chains, but the binding modes suggest that there is no interaction between a salt bridge made by Glu50-Arg76 residues and

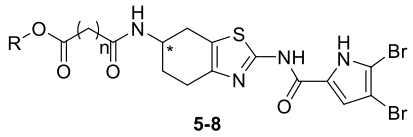
the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole ring of inhibitors (Figure 4). This could explain why oxalic acid-based compound (*S*)-**7** (IC_{50} = 1.12 μM) is less potent that its counterpart **II** (Figure 3, IC_{50} = 0.058 μM, while its benzothiazole-based analogue, in which such interaction is still possible, was shown to possess almost equipotent activity as compound **II** [39].

Compounds (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7** and (*S*)-**8** were inactive or only weakly active against selected Gram positive and Gram negative bacterial strains at 50 μM (i.e. growth inhibition less than 50% for all compounds) (Table 1). There are several possible explanations for the observed lack of correlation between *in vitro* enzyme inhibition and antibacterial activity against selected bacterial strains. Firstly, the enzymatic activity could be too low (IC_{50} values between 0.891 and 10.4 μM) to result in inhibition of bacterial growth of Gram negative *E. coli*, since subnanomolar K_i values against cytoplasmic targets could be required to generate sufficient antibacterial potency on Gram negative bacteria [20]. Pyrrolamide-based type **II** analogues (compound **II** and its analogues, Figure 3) that displayed potent *E. coli* DNA gyrase inhibition in the low nanomolar range were also devoid of antibacterial activity against Gram negative strains [24]. Secondly, type II compounds [24] and pyrrolamides presented in Figure 1 were shown to possess more potent antibacterial activity against the *ΔtolC E. coli* strain, which lacks the outer membrane tolC efflux pump, than against the wild-type *E. coli* strain [16, 17]. Therefore, active efflux of the compounds from bacterial cells could be the second reason for the inactivity of pyrrolamide-based DNA gyrase inhibitors including compounds (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7** and (*S*)-**8** *in vitro*.

We assume that the 4,5-dibromopyrrole moiety is too bulky to occupy the hydrophobic pocket of *S. aureus* DNA gyrase [24], resulting in weak inhibition of *S. aureus* DNA gyrase by 4,5-dibromopyrrolamide-based compounds and, hence, in the weak antibacterial activity against Gram positive *S. aureus* [24, 25]. To evaluate this hypothesis, compounds (S)-**7** and (S)-**8** were tested for *S. aureus* DNA gyrase inhibition and showed

weak inhibition with enzyme residual activities of 77% and 69% at 10 µM, respectively, which is probably too weak inhibition to result in antibacterial activity.

Table 1. Inhibition of *Escherichia coli* DNA gyrase and antibacterial activity of 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazoles (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7** and (*S*)-**8**.



5-8

cpd	n	R	*E. coli* DNA gyrase IC_{50} [µM]	% inhibition[a]			
				S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*
(*S*)-**5**	0	Et	4.47 µM	0	26	7	0
(*S*)-**6**	1	Me	2.34 µM	0	48	0	8
(*S*)-**7**	0	H	1.12 µM	11	4	5	0
(*S*)-**8**	1	H	0.891 µM	1	0	0	3
(*R*)-**5**	0	Et	10.4 µM	19	5	10	4
(*R*)-**7**	0	H	3.58 µM	0	3	8	6

[a]% growth inhibition at 50 µM of compound.

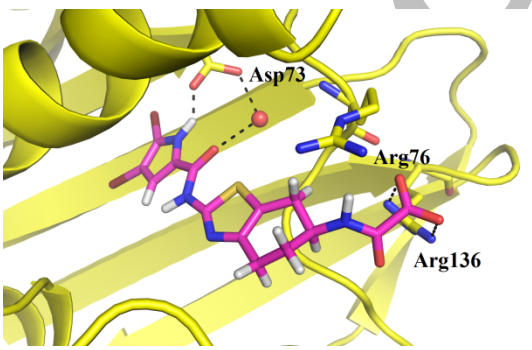


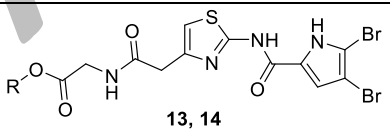
Figure 4. Molecular docking pose of inhibitor (*S*)-**7** (*magenta* sticks) in the *E. coli* DNA gyrase ATP-binding site (PDB entry: 4DUH [22]) in *yellow* cartoon representation. For clarity, only amino acid residues forming hydrogen bonds with inhibitor are presented as *yellow* sticks. The water molecule is shown as a *red* sphere. Figure was prepared by PyMOL [40].

2.3.2. 2-(2-Aminothiazol-4-yl)acetic acids

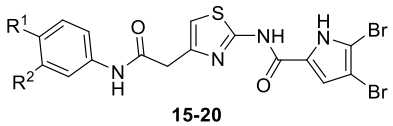
2-(2-Aminothiazol-4-yl)acetic acids **11-20** (Table 2) were designed as ring-opened analogues of the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazoles (Figure 3). In the first step, compounds **13** and **14**, which by their length mimic the structures of **5** and **7**, were prepared. Their *E. coli* DNA gyrase inhibitory activity, with IC_{50} values of 170 µM for glycine ester derivative **13** and 16 µM for its carboxylic acid **14**, are weaker than those for **5** and **7**. However, docking studies suggest similar binding modes for **14** (Figure 5) and (*S*)-**7** (Figure 4), with formation of two hydrogen bonds with Arg136.

The lack of antibacterial activity of compounds (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7**, (*S*)-**8**, **13** and **14**, as well as of previously reported type **II** analogues (compound **II** and its analogues [24]), which all possess flexible substituents addressing Arg136, suggested the introduction of more lipophilic and rigid aromatic moieties decorated with substituents with hydrogen bond acceptor properties and acidic character. Although sulfonamide **15**, phenol **16** and methyl esters **17** and **18** were all devoid of inhibitory activity, benzoic acid **19** and salicylic acid **20** derivatives inhibited *E. coli* DNA gyrase with IC_{50} values of 33 µM and 40 µM. Despite being more lipophilic (ChemBioDraw clogP = 3.37 for **19** *vs* clogP = 1.42 for **14**) and less acidic than their aliphatic analogues, their antibacterial activity was not improved, because their inhibitory activity is probably still too weak to result in potent antibacterial activity (Table 2).

Table 2. Inhibition of *Escherichia coli* DNA gyrase and antibacterial activity of 2-(2-aminothiazol-4-yl)acetic acid derivatives **13-20**.



13, 14



15-20

cpd	R^1	R^2	*E. coli* DNA gyrase IC_{50} [µM] or RA[a] [%]	% inhibition[b]			
				S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*
13	Me	-	169 µM	13	10	7	0

14	H	-	15.9 µM	15	14	7	0
15	SO_2NH_2	H	82%	0	0	6	0
16	H	OH	143 µM	0	0	3	0
17	H	COOMe	64%	8	11	25	0
18	OH	COOMe	100%	12	4	7	0
19	H	COOH	32.8 µM	6	2	8	0
20	OH	COOH	39.8 µM	0	0	3	0

[a]Residual activity of the enzyme at 100 µM of compound.

[b]% growth inhibition at 50 µM of compound.

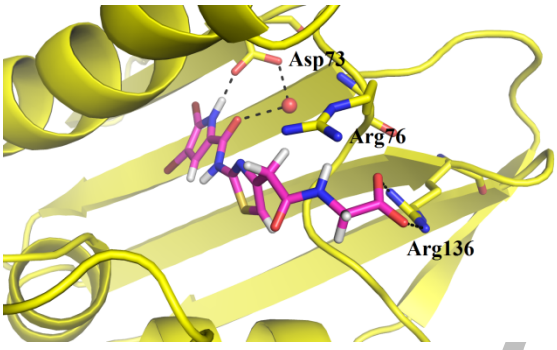


Figure 5. Molecular docking pose of inhibitor **14** (in *magenta* sticks) in the *E. coli* DNA gyrase ATP-binding site (PDB entry: 4DUH [22]; in *yellow* cartoon representation). For clarity, only amino acid residues forming hydrogen bonds with inhibitor are presented as *yellow* sticks. The water molecule is shown as a *red* sphere. The figure was prepared by PyMOL [40].

3. Conclusions

Two series of novel *E. coli* DNA gyrase inhibitors possessing 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole (compounds (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7**, (*S*)-**8**) or 2-(2-aminothiazol-4-yl)acetic acid as a central core (compounds **13-20**) were designed and synthesized. Compounds were designed based on our recently reported series of potent DNA gyrase inhibitors [24] in order to further explore the SAR and to improve their antibacterial activity. The results of *in vitro* enzymatic activity measurements using the *E. coli* DNA

supercoiling assay showed that active compounds (*R*)-**5**, (*S*)-**5**, (*S*)-**6**, (*R*)-**7**, (*S*)-**7**, (*S*)-**8**, **13**, **14**, **19** and **20** inhibit *E. coli* DNA gyrase in the sub-micromolar to low micromolar range, however they display weak activity against Gram positive *S. aureus* and *E. faecalis* and Gram negative *E. coli* and *P. aeruginosa*. Nevertheless, the described results for this novel structural class of DNA gyrase inhibitors provides valuable information for the discovery of improved DNA gyrase B inhibitors.

4. Experimental section

4.1. Chemistry

4.1.1. General procedures

Chemicals were obtained from Acros Organics (Geel, Belgium), Sigma-Aldrich (St. Louis, MO, USA) and TCI Europe N.V. (Zwijndrecht, Belgium) and used without further purification. Analytical TLC was performed on silica gel Merck 60 F_{254} plates (0.25 mm), using visualization with UV light and spray reagents. Column chromatography was carried out on silica gel 60 (particle size 240–400 mesh). HPLC analyses were performed on Agilent Technologies 1100 instrument with G1365B UV-VIS detector, G1316A thermostat and G1313A autosampler using Agilent Eclipse Plus C18 column (5 μm, 4.6 × 150 mm) using Method A: mobile phase: 0.1% trifluoroacetic acid in water (A) and methanol (B); gradient: 90% A to 10% A in 20 min, then 5 min 10 % A; flow rate 1.0 mL/min; injection volume: 10 μL; Method B: mobile phase: 0.1% trifluoroacetic acid in water (A) and acetonitrile (B); gradient: 2 min 95 % A, 90% A to 10% A in 12 min, 10% A to 5% A in 1 min, then 5 min 5% A; flow rate 1.0 mL/min; injection volume: 10 μL. All tested compounds were ≥95% pure by HPLC. Melting points were determined on a Reichert hot stage microscope and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker AVANCE III 400 spectrometer (Bruker Corporation, Billerica, MA, USA) in DMSO-

d_6 or $CDCl_3$ solutions, with TMS as the internal standard. IR spectra were recorded on a Thermo Nicolet Nexus 470 ESP FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectra were obtained using a VG Analytical Autospec Q mass spectrometer (Fisons, VG Analytical, Manchester, UK). Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. The reported values for specific rotation are average values of 5 successive measurements using an integration time of 5 s.

*4.1.2. General procedure A. Synthesis of compounds (R)-**2** and (S)-**2***

A solution of (*S*)- or (*R*)-4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole-2,6-diamine ((*S*)- or (*R*)-**1**) (1 mmol) in tetrahydrofuran (THF) (5 mL) was cooled to 0 °C on an ice bath. Then a solution of di-*tert*-butyl dicarbonate (Boc_2O) (1.1 mmol) in THF (3 mL) was added dropwise over 20 min. The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the crude product dissolved in ethyl acetate (10 mL). Organic phase was successively washed with saturated aqueous $NaHCO_3$ solution (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. Product was used without further purification.

*4.1.2.1. tert-Butyl (S)-(2-amino-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)carbamate ((S)-**2**)*

Compound was prepared from (*S*)-**1** (2.436 g, 14.40 mmol) and Boc_2O (3.457 g, 15.84 mmol) according to the general procedure **A**. Yield: 3.892 g (100%); white solid; m.p. 147-149 °C; $[\alpha]_D$ -40.3 (*c* 0.26, MeOH); IR (ATR) ν 3363, 2976, 1684, 1634, 1514, 1445, 1365, 1307, 1282, 1249, 1229, 1167, 1090, 1050, 975, 880, 829, 783, 760, 742, 690, 609 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.38 (s, 9H, $C(CH_3)_3$), 1.54-1.65 (m, 1H, H_A-7), 1.80-1.86 (m, 1H, H_B-7), 2.31-2.47 (m, 3H, H_A-4, H-5), 2.68 (dd, 1H, J_1 = 14.8 Hz, J_2 = 5.5 Hz, H_B-4), 3.57-3.69 (m, 1H, CHNH), 6.64 (s, 2H, NH_2), 6.94 (d, 1H, J = 7.9 Hz, NH) ppm; ^{13}C NMR

(100 MHz, DMSO-d_6): δ 24.9, 28.2 (3C), 28.87, 28.94, 46.8, 77.6, 112.4, 144.1, 154.9, 166.1 ppm.

*4.1.2.2. tert-Butyl (R)-(2-amino-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)carbamate ((R)-**2**)*

Compound was prepared from (*R*)-**1** (1.000 g, 5.92 mmol) and Boc_2O (1.360 g, 6.21 mmol) according to the general procedure **A**. Yield: 1,59 g (100%); off-white solid; m.p. 153-154 °C; $[\alpha]_D$ +39.7 (*c* 0.24, MeOH); IR (ATR) ν 3361, 3122, 2976, 2934, 1684, 1514, 1443, 1367, 1307, 1251, 1228, 1171, 1090, 1047, 993, 975, 866, 827, 744 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.38 (s, 9H, $C(CH_3)_3$), 1.54-1.65 (m, 1H, H_A-7), 1.80-1.86 (m, 1H, H_B-7), 2.31-2.47 (m, 3H, H_A-4, H-5), 2.68 (dd, 1H, J_1 = 14.8 Hz, J_2 = 5.5 Hz, H_B-4), 3.57-3.69 (m, 1H, CHNH), 6.64 (s, 2H, NH_2), 6.94 (d, 1H, J = 7.9 Hz, NH) ppm.

*4.1.3. General procedure B. Synthesis of compounds (R)-**3** and (S)-3*

A solution of (*S*)- or (*R*)-**2** (1 mmol) and Na_2CO_3 (1 mmol) in *N,N*-dimethylformamide (5 mL) was stirred at room temperature for 15 min. Then 2,2,2-trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethan-1-one (1.1 mmol) was added and the mixture stirred at 80 °C overnight. Solvent was removed under reduced pressure and the crude product dissolved in ethyl acetate (10 mL). Organic phase was successively washed with 10% citric acid (10 mL), saturated aqueous $NaHCO_3$ solution (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. Product was purified by column chromatography using dichloromethane/methanol (30:1) as eluent.

*4.1.3.1. tert-Butyl (S)-(2-(4,5-dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)carbamate ((S)-**3**)*

Compound was prepared from (*S*)-**2** (0.800 g, 2.97 mmol) according to the general procedure **B**. Yield: 0.610 g (39.5%); white crystals; m.p. 176-178 °C; $[\alpha]_D$ -36.8 (*c* 0.21, MeOH); IR (ATR) ν 3366, 3120, 2844, 1680, 1616, 1556, 1457, 1379, 1335, 1280, 1160,

1090, 972, 856, 779, 750, 708, 680 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.41 (s, 9H, $C(CH_3)_3$), 1.68-1.78 (m, 1H, H_A-7), 1.91-1.99 (m, 1H, H_B-7), 2.55-2.76 (m, 3H, H_A-4, H-5), 2.91 (dd, 1H, J_1 = 15.1 Hz, J_2 = 4.5 Hz, H_B-4), 3.69-3.79 (m, 1H, CHNH), 7.01 (d, J = 7.7 Hz, CHNH), 7.40 (s, 1H, pyrrole-H), 12.18 (s, 1H, NH), 13.08 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.5, 28.2 (3C), 28.4, 28.8, 46.5, 77.7, 98.7, 107.6, 115.1, 119.7, 123.3, 125.97, 126.01, 154.7, 160.2 ppm; HRMS (ESI^-) m/z for $C_{17}H_{19}N_4O_3SBr_2$ ($[M-H]^-$): calcd 516.9545, found 516.9541.

*4.1.3.2. tert-Butyl (R)-(2-(4,5-dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)carbamate ((R)-**3**)*

Compound was prepared from (*R*)-**2** (1.000 g, 3.72 mmol) according to the general procedure **B**. Yield: 0.645 g (33.4%); white crystals; m.p. 174-176 °C; $[\alpha]_D$ +35.2 (*c* 0.23, MeOH); IR (ATR) ν 3227, 2973, 1735, 1655, 1541, 1442, 1406, 1377, 1315, 1291, 1215, 1178, 1090, 1046, 1015, 992, 975, 858, 744 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.40 (s, 9H, $C(CH_3)_3$), 1.67-1.77 (m, 1H, H_A-7), 1.90-1.99 (m, 1H, H_B-7), 2.55-2.76 (m, 3H, H_A-4, H-5), 2.91 (dd, 1H, J_1 = 15.7 Hz, J_2 = 4.4 Hz, H_B-4), 3.69-3.79 (m, 1H, CHNH), 7.03 (d, J = 7.9 Hz, CHNH), 7.38 (s, 1H, pyrrole-H), 12.21 (s, 1H, NH), 13.11 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.5, 28.2 (3C), 28.4, 28.8, 46.5, 77.7, 98.7, 107.6, 115.1, 119.7, 123.3, 125.97, 126.01, 154.7, 160.2 ppm; HRMS (ESI^-) m/z for $C_{17}H_{19}N_4O_3SBr_2$ ($[M-H]^-$): calcd 516.9545, found 516.9540.

*4.1.4. General procedure C. Synthesis of compounds (R)-**4** and (S)-**4***

Methanol (10 mL) was cooled on an ice bath and then acetyl chloride (10 mmol) was added dropwise. The mixture was stirred at 0 °C for 30 min and then solution of (*R*)-**3** or (*S*)-**3** (1 mmol) in methanol (10 mL) was added. Reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight.

*4.1.4.1. (S)-2-(4,5-dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-aminium chloride ((S)-**4**)*

Compound was prepared from (*S*)-**3** (0.505 g, 0.97 mmol) according to the general procedure **C**. The precipitate was filtered off and dried. Additional amount of (*S*)-**4** was obtained from mother liquor, which was concentrated *in vacuo* and dried. Yield: 0.443 g (100%); off-white solid; m.p. >300 °C; $[\alpha]_D$ -60.7 (*c* 0.20, MeOH); IR (ATR) ν 3394, 2828, 1692, 1631, 1567, 1519, 1382, 1338, 1199, 1183, 1073, 970, 904, 845, 733, 616 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.87-1.97 (m, 1H, H_A-7), 2.11-2.19 (m, 1H, H_B-7), 2.71-2.80 (m, 3H, H_A-4, H-5), 3.10 (dd, 1H, J_1 = 16.0 Hz, J_2 = 5.1 Hz, H_B-4), 3.51-3.54 (m, 1H, CHNH), 7.40 (d, 1H, *J* = 2.7 Hz, pyrrole-H), 8.28 (d, 3H, *J* = 4.4 Hz, NH_3^+), 12.30 (s, 1H, NH), 13.14 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.5, 26.4, 26.6, 46.4, 98.8, 107.7, 115.4, 117.8, 124.3, 125.9, 143.2, 156.2 ppm; HRMS (ESI^-) m/z for $C_{12}H_{11}N_4OSBr_2$ ($[M-H]^-$): calcd 416.9020, found 416.9030.

*4.1.4.2. (R)-2-(4,5-dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-aminium chloride ((R)-**4**)*

Compound was prepared from (*R*)-**3** (0.600 g, 1.15 mmol) according to the general procedure **C**. The precipitate was filtered off and dried. Additional amount of (*R*)-**4** was obtained from mother liquor, which was concentrated *in vacuo* and dried. Yield: 0.527 g (100%); off-white solid; m.p. >300 °C; $[\alpha]_D$ +58.7 (*c* 0.21, MeOH); IR (ATR) ν 3355, 3312, 3226, 3127, 2980, 1697, 1651, 1547, 1508, 1436, 1409, 1375, 1292, 1219, 1175, 1115, 1089, 1010, 979, 855, 825, 781, 736, 704 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.86-1.96 (m, 1H, H_A-7), 2.09-2.18 (m, 1H, H_B-7), 2.71-2.80 (m, 3H, H_A-4, H-5), 3.10 (dd, 1H, J_1 = 15.7 Hz, J_2 = 5.0 Hz, H_B-4), 3.51-3.54 (m, 1H, CHNH), 7.41 (s, 1H, pyrrole-H), 8.23 (s, 3H, NH_3^+), 12.29 (s, 1H, NH), 13.13 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.5, 26.4, 26.6,

46.4, 98.8, 107.7, 115.4, 117.8, 124.3, 125.9, 143.2, 156.2 ppm; HRMS (ESI^-) m/z for $C_{12}H_{11}N_4OSBr_2$ ([M-H]$^-$): calcd 416.9020, found 416.9030.

*4.1.5. General procedure D. Synthesis of compounds (S)-**5**, (R)-**5** and (S)-**6***

To a solution of amine (1 mmol) in *N*,*N*-dimethylformamide (DMF) (5 mL) cooled to 0 °C, 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) (3 mmol) and ethyl 2-chloro-2-oxoacetate or methyl 3-chloro-3-oxopropanoate (1.3 mmol) were added dropwise. Reaction mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure and the oily residue dissolved in ethyl acetate (20 mL). Organic phase was successively washed with 10% citric acid (2 × 10 mL), saturated aqueous $NaHCO_3$ solution (2 × 10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and the solvent removed under reduced pressure.

*4.1.5.1. Ethyl (S)-2-((2-(4,5-dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)amino)-2-oxoacetate ((S)-**5**)*

Compound was prepared from (*S*)-**4** (0.115 g, 0.25 mmol) according to the general procedure **D**. Yield: 0.080 g (61.1%); off-white solid; m.p. 160-162 °C; $[\alpha]_D$ -19.8 (*c* 0.11, MeOH); IR (ATR) ν 3228, 2937, 1734, 1668, 1535, 1443, 1405, 1378, 1318, 1284, 1262, 1211, 1110, 1017, 973, 933, 859, 745, 699, 639, 620 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.28 (t, 3H, *J* = 7.1 Hz, $CH_2C\underline{H}_3$), 1.87-1.98 (m, 2H, H-7), 2.69-2.76 (m, 3H, H_A-4, H-5), 2.93 (dd, 1H, J_1 = 15.8 Hz, J_2 = 5.5 Hz, H_B-4), 4.07-4.14 (m, 1H, C<u>H</u>NH), 4.25 (q, 2H, *J* = 7.1 Hz, $C\underline{H}_2CH_3$), 7.41 (s, 1H, pyrrole-H), 9.04 (d, 1H, *J* = 8.1 Hz, CON<u>H</u>CH), 12.23 (s, 1H, NH), 13.12 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.8, 24.5, 27.5, 28.0, 45.7, 62.0, 98.8, 107.7, 115.2, 119.5, 130.8, 131.5, 141.0, 155.8, 156.9, 160.9 ppm; HRMS (ESI^-) m/z for $C_{16}H_{15}N_4O_4SBr_2$ ([M-H]$^-$): calcd 516.9181, found 516.9186. HPLC: method B, t_r 12.91 min (95.1% at 254 nm).

*4.1.5.2. Ethyl (R)-2-((2-(4,5-dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)amino)-2-oxoacetate ((R)-**5**)*

Compound was prepared from (*R*)-**4** (0.150 g, 0.33 mmol) according to the general procedure **D**. Crude product was purified by column chromatography using dichloromethane/methanol (40:1) as eluent. Yield: 0.021 g (11.5%); yellow solid; m.p. 161-164°C; $[\alpha]_D$ +20.1 (*c* 0.12, MeOH); IR (ATR) ν 3356, 3226, 2936, 1735, 1696, 1651, 1538, 1442, 1407, 1376, 1315, 1290, 1214, 1177, 1091, 1014, 992, 976, 858, 826, 742 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.28 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$), 1.85-1.99 (m, 2H, H-7), 2.67-2.76 (m, 3H, H_A-4, H-5), 2.93 (dd, 1H, J_1 = 15.6 Hz, J_2 = 4.9 Hz, H_B-4), 4.04-4.14 (m, 1H, C<u>H</u>NH), 4.25 (q, 2H, J = 7.1 Hz, $C\underline{H}_2CH_3$), 7.41 (s, 1H, pyrrole-H), 9.04 (d, 1H, J = 8.1 Hz, CON<u>H</u>CH), 12.23 (s, 1H, NH), 13.12 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.8, 24.5, 27.5, 28.0, 45.7, 62.0, 98.8, 107.7, 115.2, 119.5, 130.8, 131.5, 141.0, 155.8, 156.9, 160.9 ppm; HRMS (ESI^-) m/z for $C_{16}H_{15}N_4O_4SBr_2$ ($[M-H]^-$): calcd 516.9181, found 516.9186. HPLC: method B, t_r 12.90 min (95.3% at 254 nm).

*4.1.5.3. Methyl (S)-3-((2-(4,5-dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)amino)-3-oxopropanoate ((S)-**6**)*

Compound was prepared from (*S*)-**4** (0.100 g, 0.22 mmol) according to the general procedure **D**. Yield: 0.062 g (46.3%); white solid; m.p. 205-207 °C; $[\alpha]_D$ -17.5 (*c* 0.13, MeOH); IR (ATR) ν 3261, 3124, 2952, 1721, 1641, 1538, 1438, 1384, 1324, 1352, 1281, 1226, 1177, 1122, 1018, 991, 975, 902, 856, 832, 773, 740, 696 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.74-1.84 (m, 1H, H_A-7), 1.87-1.95 (m, 1H, H_B-7), 2.51-2.57 (m, 2H, H_A-4, H_A-5), 2.67-2.73 (m, 1H, H_B-5), 2.94 (dd, 1H, J_1 = 15.8 Hz, J_2 = 5.0 Hz, H_B-4), 3.24 (d, 2H, J = 1.9 Hz, $COCH_2CO$), 3.59 (s, 3H, CH_3), 4.02-4.10 (m, 1H, C<u>H</u>NH), 7.38 (s, 1H, pyrrole-H), 8.23 (d, 1H, J = 7.6 Hz, CON<u>H</u>CH), 12.21 (s, 1H, NH), 13.08 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.6, 27.7, 28.2, 42.2, 44.8, 51.8, 98.7, 107.7, 115.2, 119.4, 126.0, 132.5, 143.6,

156.6, 164.7, 168.4 ppm; HRMS (ESI$^-$) m/z for $C_{16}H_{15}N_4O_4SBr_2$ ([M-H]$^-$): calcd 516.9181, found 516.9188. HPLC: method B, t_r 12.26 min (95.5% at 254 nm).

*4.1.6. General procedure E. Synthesis of compounds (S)-**7**, (R)-**7** and (S)-**8***

To a solution of ester (1 mmol) in methanol (5 mL), 1 M NaOH (5 mmol) was added and the reaction mixture stirred at room temperature overnight. Methanol was evaporated under reduced pressure and reaction mixture extracted with ethyl acetate (10 mL). Water phase was acidified with 1 M HCl to pH ~ 2, precipitate was filtered off and purified by flash column chromatography using dichloromethane/methanol (1:1) as eluent.

4.1.6.1. (S)-2-((2-(4,5-Dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)amino)-2-oxoacetic acid ((S)-7)

Compound was prepared from (*S*)-**5** (0.050 g, 0.097 mmol) according to the general procedure **E**. Yield: 0.032 g (67.7%); off-white solid; m.p. 236-238 °C; $[\alpha]_D$ -48.6 (*c* 0.18, MeOH); IR (ATR) ν 2941, 1657, 1555, 1408, 1383, 1325, 1223, 1171, 1079, 973, 864, 829, 742, 662, 618 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.89-1.97 (m, 2H, H-7), 2.45-2.47 (m, 1H, H-4/H-5), 2.70-2.77 (m, 2H, H-4/H-5), 2.92 (dd, 1H, J_1 = 15.6 Hz, J_2 = 5.1 Hz, H_B-4), 4.03-4.12 (m, 1H, CHNH), 7.40 (s, 1H, pyrrole-H), 8.95 (d, 1H, *J* = 8.4 Hz, CONHCH), 12.23 (s, 1H, NH), 13.11 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.5, 27.6, 28.0, 45.7, 98.8, 107.6, 115.2, 119.4, 126.0, 137.1, 143.2, 156.5, 158.0, 162.3 ppm; HRMS (ESI$^-$) m/z for $C_{14}H_{11}N_4O_4SBr_2$ ([M-H]$^-$): calcd 488.8868, found 488.8879. HPLC: method B, t_r 11.74 min (95.0% at 254 nm).

4.1.6.2. (R)-2-((2-(4,5-Dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)amino)-2-oxoacetic acid ((R)-7)

Compound was prepared from (*R*)-**5** (0.010 g, 0.020 mmol) according to the general procedure **E**. Yield: 0.008 g (84.2%); off-white solid; m.p. 240-242°C; $[\alpha]_D$ +47.7 (*c* 0.20, MeOH); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.89-1.97 (m, 2H, H-7), 2.44-2.47 (m, 1H, H-4/H-5), 2.70-2.78 (m, 2H, H-4/H-5), 2.92 (dd, 1H, J_1 = 15.4 Hz, J_2 = 5.2 Hz, H_B-4), 4.03-4.12 (m, 1H, CHNH), 7.40 (s, 1H, pyrrole-H), 8.94 (d, 1H, *J* = 8.2 Hz, CONHCH), 12.24 (s, 1H, NH), 13.10 (s, 1H, NH) ppm; HRMS (ESI$^-$) m/z for $C_{14}H_{11}N_4O_4SBr_2$ ([M-H]$^-$): calcd 488.8868, found 488.8872. HPLC: method B, t_r 11.744 min (100% at 254 nm).

*4.1.6.3. (S)-3-((2-(4,5-Dibromo-1H-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[1,2-d]thiazol-6-yl)amino)-3-oxopropanoic acid ((S)-**8**)*

Compound was prepared from (*S*)-**6** (0.040 g, 0.0077 mmol) according to the general procedure **E**. Yield: 0.020 g (51.3%); off-white solid; m.p. 123-125 °C; $[\alpha]_D$ -15.6 (*c* 0.12, MeOH); IR (ATR) ν 3287, 2924, 1711, 1656, 1558, 1383, 1328, 1304, 1260, 1213, 1182, 1099, 1014, 977, 921, 863, 836, 802, 740, 673, 632 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.77-1.87 (m, 1H, H_A-7), 1.90-2.00 (m, 1H, H_B-7), 2.64-2.76 (m, 3H, H_A-4, H-5), 2.97 (dd, 1H, J_1 = 15.5 Hz, J_2 = 4.7 Hz, H_B-4), 3.15 (d, 2H, *J* = 2.6 Hz, $COCH_2CO$), 4.05-4.17 (m, 1H, CHNH), 7.40 (s, 1H, pyrrole-H), 8.21 (d, 1H, *J* = 7.5 Hz, CONHCH), 12.37 (s, 1H, NH), 13.11 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.7, 27.8, 28.9, 42.6, 44.7, 98.7, 107.6, 115.1, 121.0, 126.0, 133.4, 142.5, 156.5, 157.7, 169.5 ppm; HRMS (ESI$^-$) m/z for $C_{15}H_{13}N_4O_4SBr_2$ ([M-H]$^-$): calcd 502.9024, found 502.9010. HPLC: method B, t_r 11.70 min (96.2% at 254 nm).

*4.1.7. Methyl 2-(2-aminothiazol-4-yl)acetate (**10**)*

Methanol (30 mL) was cooled to 0 °C and thionyl chloride (3.45 mL, 47.6 mmol) was added dropwise. Then 2-(2-aminothiazol-4-yl)acetic acid (**9**) (3.00 g, 19.0 mmol) was added and reaction mixture stirred under reflux for 1.5 h. The solvent was evaporated and the oily

residue triturated with diethyl ether. White precipitate (3.564 g, 89.9%) was filtered off and dried. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.66 (s, 3H, CH_3), 3.77 (s, 2H, CH_2), 6.71 (s, 1H, Ar-H), 9.30 (s, 2H, NH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.7, 52.1, 105.6, 132.4, 169.0, 169.5 ppm.

*4.1.8. Methyl 2-(2-(4,5-dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetate (**11**)*

A solution of **10** (1.044 g, 5.00 mmol) and Na_2CO_3 (0.530 g, 5.00 mmol) in DMF (20 mL) was stirred at room temperature for 15 min. 2,2,2-Trichloro-1-(4,5-dibromo-1*H*-pyrrol-2-yl)ethan-1-one (1.852 g, 5.00 mmol) was added and mixture was stirred at 80 °C overnight. Solvent was removed under reduced pressure, residue was suspended in ethyl acetate (60 mL) and successively washed with 10% citric acid (2 × 30 mL), saturated aqueous $NaHCO_3$ solution (2 × 30 mL) and brine (30 mL), dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. The crude product was recrystallized from methanol. Yield: 1.630 g (77.0%); white crystals; m.p. 200-202 °C; IR (ATR) ν 3352, 3232, 3129, 2982, 1698, 1650, 1543, 1505, 1442, 1410, 1368, 1274, 1218, 1172, 1116, 1085, 1010, 980, 886, 854, 823, 782, 729, 687 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.63 (s, 3H, CH_3), 3.75 (s, 2H, CH_2), 7.04 (s, 1H, thiazole-H), 7.45 (d, 1H, J = 2.6 Hz, pyrrole-H), 12.42 (s, 1H, NH), 13.12 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 36.4, 51.7, 98.8, 107.8, 110.8, 115.4, 125.8, 143.7, 156.6, 157.8, 170.5 ppm; HRMS (ESI$^-$) m/z for $C_{11}H_8N_3O_3SBr_2$ ([M-H]$^-$):calcd 419.8653, found 419.8650; HPLC: method A, t_r 13.09 min (95.4% at 254 nm).

*4.1.9. 2-(2-(4,5-Dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetic acid (**12**)*

To a solution of **11** (0.702 g, 1.66 mmol) in methanol (25 mL) 2 M NaOH (1.66 mL, 3.32 mmol) was added and the reaction mixture stirred at room temperature overnight. Methanol was evaporated under reduced pressure. Water phase was acidified with 2 M HCl to pH ~ 2, white precipitate filtered and dried. Yield: 0.504 g (74.2%); white crystals; m.p. 240-

242 °C; IR (ATR) ν 3353, 3230, 3129, 2983, 1699, 1652, 1545, 1505, 1442, 1408, 1367, 1292, 1217, 1174, 1115, 1087, 1010, 989, 898, 856, 823, 782, 740 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.64 (s, 2H, CH_2), 7.00 (s, 1H, thiazole-H), 7.44 (d, 1H, J = 2.7 Hz, pyrrole-H), 12.41 (s, 1H, NH), 13.13 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 97.5, 109.9, 110.0, 115.3, 127.8, 130.5, 139.9, 157.9, 160.3, 164.7 ppm, signal for $\underline{C}H_2$ overlapped with DMSO-d_6; HRMS (ESI^-) m/z for $C_{10}H_6N_3O_3SBr_2$ ($[M-H]^-$): calcd 405.8497, found 405.8488.

*4.1.10. Methyl (2-(2-(4,5-dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetyl)glycinate **(13)***

A solution of **12** (0.200 g, 0.49 mmol) in *N*,*N*-dimethylformamide (5 mL) was cooled to 0 °C and then EDC (0.113 g, 0.51 mmol) and HOBt (0.072 g, 0.51 mmol) were added. pH was adjusted to 8 with *N*-methylmorpholine and the reaction mixture stirred for 20 min at 0 °C. Then glycine methyl ester hydrochloride (0.061 g, 0.49 mmol) was added and reaction mixture stirred overnight at room temperature. The solvent was evaporated *in vacuo* and the oily residue dissolved in ethyl acetate (30 mL) and washed successively with 10% citric acid (2 × 30 mL), saturated aqueous $NaHCO_3$ solution (2 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. Yield: 0.159 g (69.4%); white crystals; m.p. 231-233 °C; IR (ATR) ν 3354, 3225, 3129, 2980, 1698, 1650, 1543, 1508, 1441, 1409, 1369, 1290, 1216, 1172, 1116, 1086, 1010, 981, 856, 824, 782, 739 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.58 (s, 2H, CH_2CO), 3.64 (s, 3H, CH_3), 3.87 (d, 2H, J = 5.8 Hz, $NHC\underline{H_2}CO$), 6.97 (s, 1H, thiazole-H), 7.45 (d, 1H, J = 2.5 Hz, pyrrole-H), 8.40 (t, 1H, J = 5.8 Hz), $N\underline{H}CH_2CO$), 12.40 (s, 1H, NH), 13.11 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 38.0, 40.7, 51.7, 98.8, 107.8, 110.2, 115.3, 125.9, 145.2, 156.5, 157.6, 169.5, 170.4 ppm; HRMS (ESI^-) m/z for $C_{13}H_{11}N_4O_4SBr_2$ ($[M-H]^-$): calcd 476.8868, found 476.8867; HPLC: method B, t_r 11.98 min (95.0% at 254 nm).

*4.1.11. (2-(2-(4,5-dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetyl)glycine **(14)***

To a solution of **13** (0.122 g, 0.25 mmol) in methanol (5 mL) 1 M NaOH (0.42 mL, 0.42 mmol) was added and the reaction mixture stirred at room temperature overnight. Methanol was evaporated under reduced pressure. Water phase was acidified with 2 M HCl to pH ~ 2, white precipitate filtered and dried. Yield: 0.050 g (42.4%); white crystals; m.p. >300 °C; IR (ATR) ν 3355, 3228, 3128, 2982, 1699, 1651, 1543, 1512, 1441, 1408, 1376, 1294, 1220, 1175, 1090, 1011, 981, 857, 826, 738 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.56 (s, 2H, CH_2CO), 3.77 (d, 2H, J = 5.6 Hz, NHC$\underline{H_2}$CO), 6.96 (s, 1H, thiazole-H), 7.43 (s, 1H, pyrrole-H), 8.28 (t, 1H, J = 5.6 Hz), N$\underline{H}$CH$_2$CO), 12.41 (s, 1H, NH), 13.11 (s, 1H, NH) ppm, signal for COOH is not seen in the spectrum; ^{13}C NMR (100 MHz, DMSO-d_6): δ 38.0, 40.7, 98.8, 107.7, 110.1, 115.3, 125.9, 145.2, 156.6, 157.6, 169.3, 171.3 ppm; HRMS (ESI^-) m/z for $C_{12}H_9N_4O_4SBr_2$ ([M-H]$^-$): calcd 462.8711, found 462.8721; HPLC: method B, t_r 11.24 min (95.2% at 220 nm).

*4.1.12. 4,5-Dibromo-N-(4-(2-oxo-2-((4-sulfamoylphenyl)amino)ethyl)thiazol-2-yl)-1H-pyrrole-2-carboxamide (**15**)*

A solution of **12** (0.207 g, 0.51 mmol) in *N*,*N*-dimethylformamide (5 mL) was cooled to 0 °C and then EDC (0.120 g, 0.61 mmol) and HOBt (0.082 g, 0.61 mmol) were added. pH was adjusted to 8 with *N*-methylmorpholine and the reaction mixture stirred for 20 min at 0 °C. Then 4-aminobenzenesulfonamide (0.087 g, 0.51 mmol) was added and reaction mixture stirred overnight at room temperature. The solvent was evaporated *in vacuo* and the oily residue dissolved in ethyl acetate (30 mL) and washed successively with 10% citric acid (2 × 30 mL), saturated aqueous $NaHCO_3$ solution (2 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. Crude product was recrystallized from methanol. Yield: 0.044 g (15.4%); white crystals; m.p. >300 °C; IR (ATR) ν 3244, 3130, 2981, 1698, 1651, 1544, 1513, 1443, 1409, 1368, 1292, 1218, 1172, 1116, 1086, 1010, 982, 886, 855, 823, 782, 738, 660 cm^{-1}; ^1H NMR (400 MHz,

DMSO-d_6): δ 3.78 (s, 2H, CH_2), 7.03 (s, 1H, thiazole-H), 7.07 (t, 1H, J = 8.1 Hz, Ar-H), 7.27 (s, 2H, SO_2NH_2), 7.42 (s, 1H, pyrrole-H), 7.77 (s, 4H, 4 × Ar-H), 10.51 (s, 1H, CH_2CONH), 12.38 (br s, 1H, NH), 13.12 (br s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 98.7, 107.7, 110.5, 115.3, 118.6, 126.7, 138.3, 142.0, 144.8, 149.0, 156.7, 157.8, 168.5 ppm, signal for CH_2 overlapped with DMSO-d_6; HRMS (ESI$^-$) m/z for $C_{16}H_{12}N_5O_4S_2Br_2$ ([M-H]$^-$): calcd 559.8697, found 559.8687; HPLC: method B, t_r 12.31 min (95.2% at 254 nm).

*4.1.13. 4,5-Dibromo-N-(4-(2-((3-hydroxyphenyl)amino)-2-oxoethyl)thiazol-2-yl)-1H-pyrrole-2-carboxamide (**16**)*

A solution of **12** (0.200 g, 0.49 mmol) in *N*,*N*-dimethylformamide (5 mL) was cooled to 0 °C and then EDC (0.113 g, 0.59 mmol) and HOBt (0.079 g, 0.59 mmol) were added. pH was adjusted to 8 with *N*-methylmorpholine and the reaction mixture stirred for 20 min at 0 °C. Then 3-aminophenol (0.053 g, 0.49 mmol) was added and reaction mixture stirred overnight at room temperature. The solvent was evaporated *in vacuo* and the oily residue dissolved in ethyl acetate (30 mL) and washed successively with 10% citric acid (2 × 30 mL), saturated aqueous $NaHCO_3$ solution (2 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. Crude product was recrystallized from methanol. Yield: 0.110 g (45.3%); white crystals; m.p. 288-290 °C; IR (ATR) ν 3259, 1655, 1604, 1530, 1489, 1443, 1411, 1377, 1323, 1280, 1225, 1170, 1044, 980, 860, 779, 732, 686 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.71 (s, 2H, CH_2), 6.45 (ddd, 1H, J_1 = 7.9 Hz, J_2 = 2.3 Hz, J_3 = 0.9 Hz, Ar-H), 6.98 (ddd, 1H, J_1 = 8.1 Hz, J_2 = 1.8 Hz, J_3 = 0.9 Hz, Ar-H), 7.00 (s, 1H, thiazole-H), 7.07 (t, 1H, J = 8.1 Hz, Ar-H), 7.20 (t, 1H, J = 2.1 Hz, Ar-H), 7.44 (d, 1H, J = 2.6 Hz, Ar-H), 9.39 (s, 1H, OH), 10.02 (s, 1H, CH_2CONH), 12.38 (br s, 1H, NH), 13.12 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 98.8, 106.1, 107.8, 109.8, 110.3, 115.3, 125.8, 129.4, 140.2, 145.3, 156.5, 157.6, 157.7, 167.7 ppm, signal

for $\underline{C}H_2$ overlapped with DMSO-d_6; HRMS (ESI$^-$) m/z for $C_{16}H_{11}N_4O_3SBr_2$ ([M-H]$^-$): calcd 496.8919, found 496.8908; HPLC: method B, t_r 12.61 min (100% at 254 nm).

*4.1.14. Methyl 3-(2-(2-(4,5-dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetamido)benzoate (**17**)*

A solution of **12** (0.155 g, 0.38 mmol) in *N*,*N*-dimethylformamide (5 mL) was cooled to 0 °C and then EDC (0.087 g, 0.45 mmol) and HOBt (0.061 g, 0.45 mmol) were added. pH was adjusted to 8 with *N*-methylmorpholine and the reaction mixture stirred for 20 min at 0 °C. Then methyl 3-aminobenzoate (0.057 g, 0.38 mmol) was added and reaction mixture stirred overnight at room temperature. The solvent was evaporated *in vacuo* and the oily residue dissolved in ethyl acetate (30 mL) and washed successively with 10% citric acid (2 × 30 mL), saturated aqueous $NaHCO_3$ solution (2 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. Compound **17** was obtained as a white solidified oil. Yield: 0.158 g (77.1%); white solidified oil; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.79 (s, 2H, CH_2), 3.86 (s, 3H, CH_3), 7.03 (s, 1H, thiazole-H), 7.43 (s, 1H, pyrrole-H), 7.48 (t, 1H, J = 8.0 Hz, Ar-H-3), 7.65 (d, 1H, J = 8.0 Hz, Ar-H-2/4), 7.87 (d, 1H, J = 8.0 Hz, Ar-H-2/4), 8.30 (s, 1H, Ar-H-6), 10.40 (s, 1H, $CH_2CON\underline{H}$), 12.38 (s, 1H, NH), 13.12 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 52.2, 98.8, 107.8, 110.5, 115.4, 119.5, 123.5, 123.8, 125.8, 129.3, 130.1, 139.5, 145.0, 156.6, 157.7, 166.0, 168.3 ppm, signal for $\underline{C}H_2$ overlapped with DMSO-d_6; HRMS (ESI$^-$) m/z for $C_{18}H_{13}N_4O_4SBr_2$ ([M-H]$^-$): calcd 538.9024, found 538.9033; HPLC: method A, t_r 21.45 min (100% at 254 nm).

*4.1.15. Methyl 5-(2-(2-(4,5-dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetamido)-2-hydroxybenzoate (**18**)*

A solution of **12** (0.209 g, 0.51 mmol) in *N*,*N*-dimethylformamide (5 mL) was cooled to 0 °C and then EDC (0.117 g, 0.61 mmol) and HOBt (0.083 g, 0.61 mmol) were added. pH was adjusted to 8 with *N*-methylmorpholine and the reaction mixture stirred for 20 min at 0 °C. Then methyl 5-amino-2-hydroxybenzoate (0.085 g, 0.51 mmol) was added and reaction mixture stirred overnight at room temperature. The solvent was evaporated *in vacuo* and the oily residue dissolved in ethyl acetate (30 mL) and washed successively with 10% citric acid (2 × 30 mL), saturated aqueous $NaHCO_3$ solution (2 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. Crude product was recrystallized from methanol. Yield: 0.100 g (35.2%); white crystals; m.p. 257-259 °C; IR (ATR) ν 3235, 3129, 2981, 1697, 1651, 1543, 1505, 1441, 1409, 1367, 1288, 1216, 1172, 1116, 1085, 1009, 981, 899, 856, 824, 782, 737, 692 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.71 (s, 2H, CH_2), 3.90 (s, 3H, CH_3), 6.93-7.01 (m, 2H, thiazole-H, Ar-H-3), 7.44 (s, 1H, pyrrole-H), 7.67 (dd, 1H, J_1 = 9.2 Hz, J_2 = 2.6 Hz, Ar-H-4), 8.17 (d, 1H, J = 2.6 Hz, Ar-H-6), 10.17 (s, 1H, CH_2CONH or OH), 10.28 (s, 1H, CH_2CONH or OH), 12.37 (s, 1H, NH), 13.12 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 52.5, 98.8, 107.8, 110.4, 112.4, 115.4, 117.6, 120.0, 125.9, 127.2, 131.1, 145.1, 145.2, 155.9, 156.6, 167.7, 169.0 ppm, signal for CH_2 overlapped with DMSO-d_6; HRMS (ESI^-) m/z for $C_{18}H_{13}N_4O_5SBr_2$ ($[M-H]^-$): calcd 554.8973, found 554.8979; HPLC: method B, t_r 10.46 min (95.8% at 254 nm).

4.1.16. 3-(2-(2-(4,5-Dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetamido)benzoic acid ***(19)***

To a solution of **17** (0.113 g, 0.21 mmol) in ethanol (5 mL) 2 M NaOH (0.42 mL, 0.42 mmol) was added and the reaction mixture stirred at room temperature for 8 h. Ethanol was evaporated under reduced pressure. Water phase was acidified with 2 M HCl to pH ~ 2, white precipitate filtered and dried. Yield: 0.103 g (93.6%); white crystals; m.p. >300 °C; IR (ATR)

ν 3067, 1656, 1592, 1539, 1488, 1436, 1381, 1330, 1178, 1081, 981, 863, 833, 740, 680, 616, 544, 515 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.78 (s, 2H, CH_2), 7.04 (s, 1H, thiazole-H), 7.41-7.45 (m, 2H, pyrrole-H, Ar-H-3), 7.62 (dd, 1H, J_1 = 7.7 Hz, J_2 = 2.5 Hz, Ar-H-2/4), 7.87 (ddd, 1H, J_1 = 8.0 Hz, J_2 = 2.0 Hz, J_3 = 0.9 Hz, Ar-H-2/4), 8.28 (t, 1H, J_1 = 1.7 Hz, Ar-H-6), 10.57 (s, 1H, CH_2CONH), 12.39 (br s, 1H, NH), 13.15 (s, 1H, NH) ppm, signal for COOH is not seen in the spectrum; ^{13}C NMR (100 MHz, DMSO-d_6): δ 98.8, 107.8, 110.5, 115.4, 119.8, 123.2, 124.0, 125.9, 129.0, 131.2, 139.4, 145.0, 156.6, 157.7, 167.1, 168.2 ppm, signal for CH_2 overlapped with DMSO-d_6; HRMS (ESI^+) m/z for $C_{17}H_{13}N_4O_4SBr_2$ ($[M+H]^+$): calcd 526.9029, found 526.9028; HPLC: method A, t_r 20.10 min (95.2% at 254 nm).

*4.1.17. 5-(2-(2-(4,5-Dibromo-1H-pyrrole-2-carboxamido)thiazol-4-yl)acetamido)-2-hydroxybenzoic acid (**20**)*

To a solution of **18** (0.090 g, 0.16 mmol) in methanol (5 mL) 1 M NaOH (0.65 mL, 0.64 mmol) was added and the reaction mixture stirred at room temperature overnight. Ethanol was evaporated under reduced pressure. Water phase was acidified with 2 M HCl to pH ~ 2, white precipitate filtered and dried. Crude product was recrystallized from methanol. Yield: 0.033 g (37.8%); white crystals; m.p. >300 °C; IR (ATR) ν 3355, 3224, 3129, 2983, 1698, 1651, 1544, 1505, 1442, 1409, 1367, 1291, 1216, 1171, 1116, 1086, 1009, 988, 898, 854, 823, 781, 738, 696 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.72 (s, 2H, CH_2), 6.93 (d, 1H, J = 8.8 Hz, Ar-H-3), 7.02 (s, 1H, thiazole-H), 7.43 (d, 1H, J = 2.8 Hz, pyrrole-H), 7.70 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.6 Hz, Ar-H-4), 8.15 (d, 1H, J = 2.6 Hz, Ar-H-6), 10.22 (s, 1H, CH_2CONH or OH), 11.01 (br s, 1H, CH_2CONH or OH), 12.39 (br s, 1H, NH), 13.13 (s, 1H, NH) ppm, signal for COOH not seen in the spectrum; ^{13}C NMR (100 MHz, DMSO-d_6): δ 98.8, 101.1, 110.4, 112.4, 115.4, 117.2, 120.3, 125.9, 127.3, 131.0, 145.4, 146.6, 155.8, 157.0, 167.6, 171.7 ppm, signal for CH_2 overlapped with DMSO-d_6; HRMS (ESI^-) m/z for

$C_{17}H_{11}N_4O_5SBr_2$ ([M-H]$^-$): calcd 540.8817, found 540.8824; HPLC: method B, t_r 8.81 min (95.3% at 254 nm).

4.2. In vitro inhibitory activity screening and determination of IC_{50} values on E. coli DNA gyrase

The assay for determining IC_{50} values (Inspiralis) was performed on black streptavidin-coated 96-well microtiter plates (Thermo Scientific Pierce). The plate was first rehydrated with the wash buffer supplied (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.01% (w/v) BSA, 0.05% (v/v) Tween 20). Biotinylated oligonucleotide in wash buffer was immobilized onto the wells. The excess of oligonucleotide was then washed off and the enzyme assay carried out in the wells (5 min). The final reaction volume of 30 μL in buffer (35 mM Tris HCl (pH 7.5); 24 mM KCl; 4 mM $MgCl_2$; 2 mM DTT; 1.8 mM spermidine; 1 mM ATP; 6.5 % (w/v) glycerol; 0.1 mg/mL albumin) contained 1.5 U of DNA gyrase from *E. coli*, 0.75 μg of relaxed pNO1 plasmid, and 3 μL of inhibitors solution in 10% DMSO and 0.008% Tween® 20. Reactions were incubated for 30 min at 37 °C and, after addition of the TF buffer (50 mM NaOAc (pH 5.0), 50 mM NaCl and 50 mM $MgCl_2$), which terminated the enzymatic reaction, for another 30 min at room temperature to allow triplex formation (biotin−oligonucleotide−plasmid). The unbound plasmid was then washed off using TF buffer, and a solution of SybrGOLD stain in T10 buffer (10 mM Tris × HCl (pH 8.0) and 1 mM EDTA) was added. After mixing, the fluorescence (excitation, 485 nm; emission, 535 nm) was read using a BioTek's Synergy H4 microplate reader. Preliminary screening was performed at inhibitor concentrations of 100 μM and 10 μM. For the most potent compounds IC_{50} was determined with 7 concentrations of the inhibitors. IC_{50} values were calculated using GraphPad Prism software and represent the concentration of inhibitor where the residual activity of the enzyme is 50% in three independent measurements; the final result is given as

their average value. Novobiocin (IC_{50} = 0.17 μM (lit. 0.08 μM [41]) for *E. coli* DNA gyrase) was used as a positive control.

4.3. Determination of antibacterial activity

Clinical control strains of *Enterococcus faecalis* (Gram positive, ATCC 29212), *Staphylococcus aureus* (Gram positive, ATCC 25923), *Escherichia coli* (Gram negative, ATCC 25922) and *Pseudomonas aeruginosa* (Gram negative, ATCC 27853), were obtained from Microbiologics Inc. (St. Cloud, Minnesota, USA). Antimicrobial testing was carried out by using the broth microdilution method in 96-well plate format according to the CLSI guidelines. Briefly, bacterial suspensions for the assays were prepared into MH II broth (Becton Dickinson, Franklin Lakes, NJ, USA) from fresh slant cultures on cation-adjusted MH agar (Becton Dickinson, Franklin Lakes, NJ, USA), and incubated at 37 °C for 16–20 h at 100 rpm. Suspension yielding final inoculum of 5×10^5 CFU/mL was prepared and mixed on the plate with test compound solution diluted into assay media. After incubating the plate for 24 h at 37 °C, absorbance values were measured at 620 nm and used for evaluating the antimicrobial effects of test compounds by comparing to untreated controls and expressed as percentage inhibition of growth. Ciprofloxacin was used as a positive control every assay plate (minimum inhibitory concentration against *E. faecalis*, *S. aureus*, *E. coli* and *P. aeruginosa*, was 3.0, 1.5, 0.05 and 3.0 μM, respectively). Compounds were assayed at final concentration of 50 μM (n = 3).

4.4. Molecular modeling

4.4.1. Ligand and protein preparation

Three-dimensional models of designed compounds were built in ChemBio3D Ultra 13.0 [42]. Their geometries were optimized using MMFF94 [43] force field and partial atomic charges were added. Energy was minimized until the gradient value was smaller than 0.001 kcal/(mol Å). The optimized structure was further refined with GAMESS interface in

ChemBio3D Ultra 13.0 using the semiempirical PM3 method, QA optimization algorithm and Gasteiger Hückel charges for all atoms for 100 steps [42]. Molecular docking calculations were performed using FlexX [36, 37], as available in LeadIT [38], running on four octal core AMD Opteron CPU processors, 16 GB RAM, two 750 GB hard drives, running 64-bit Scientific Linux 6.0. Receptor was prepared in a LeadIT graphical user interface using the Receptor wizard. Amino acid residues within a radius of 7 Å around the ligand from the X-ray structure (PDB entry: 4DUH [22]) were defined as the binding site. Hydrogen atoms were added to the binding site residues and correct tautomers and protonation states were assigned. Water molecules, except HOH614, and the ligand were deleted from the crystal structure.

4.4.2. Validation of the docking protocol and ligand docking

The FlexX molecular docking program, as available in LeadIT [38], was used for ligand docking. A hybrid algorithm (enthalpy and entropy driven ligand binding) was used to place the 'base fragment'. The maximum number of solutions per iteration and the maximum number of solutions per fragmentation parameter values were increased to 1000, while other parameters were set at their default values.

In order to validate our docking protocol, crystal structure ligand was docked into the defined ATP-binding site of *E. coli* GyrB using the above described docking parameters. The protocol was able to reproduce the binding of the crystal structure ligand with an RMSD value of 1.2 Å, which highlights the docking protocol as suitable for binding mode studies of the designed DNA gyrase inhibitors that were docked using the same settings as used for docking protocol validation. Proposed binding modes and scoring function scores of the top five highest scored docking poses per ligand were evaluated and the highest ranked binding pose was used for graphical representation in PyMOL [40].

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