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FULL PAPER

Synthesis and Evaluation of *N*-Phenylpyrrolamides as DNA Gyrase B Inhibitors

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Abstract: ATP-competitive inhibitors of DNA gyrase and topoisomerase IV (topo IV) are among the most interesting classes of antibacterial drugs that do not have any representative in the antibacterial pipeline. We have developed thirty-two new *N*-phenylpyrrolamides and evaluated them against DNA gyrase and topoisomerase IV from *Escherichia coli* and *Staphylococcus aureus*. Antibacterial activities were studied against Gram-positive and Gram-negative bacterial strains. The most potent compound displayed an IC_{50} of 47 nM against *E. coli* DNA gyrase, and a minimum inhibitory concentration (MIC) of 12.5 μM against Gram-positive *Enterococcus faecalis*. Some compounds displayed good antibacterial activities against the efflux pump deficient *E. coli* strain (MICs = 6.25 μM) and against wild type *E. coli* in the presence of efflux pump inhibitor PAβN (MIC = 3.13 μM). We describe here new findings regarding structure-activity relationship of *N*-phenylpyrrolamide DNA gyrase B inhibitors and explore factors that are important for antibacterial activity of this class of compounds.

Introduction

Infections are among the major causes of human morbidity and mortality and the pharmaceutical industry is not able to keep up with the growing needs for effective novel antibacterials. The main reason for this situation is the rapid adaptation of bacteria to antibiotics, which results in the development of resistance soon after antibacterial drugs are introduced to therapy.[1] Another problem is the low number of new approved antibacterial drugs in the last decades, which is associated with the strict regulatory requirements and relatively low cost-to-benefit ratio for antibacterial R&D compared to other pharmacological fields.[2][3]
Bacterial topoisomerases are enzymes that catalyse changes in DNA topology during DNA replication, transcription and recombination. They are essential in all bacteria but absent in higher eukaryotes, which makes them attractive targets for antibacterial drug discovery. DNA gyrase is involved primarily in the negative supercoiling of DNA, while topoisomerase IV is responsible mainly for the decatenation of the two daughter chromosomes after replication. DNA gyrase and topoisomerase IV both belong to type IIA topoisomerases. They are heterotetrameric enzymes composed of two pairs of subunits: two GyrA and two GyrB subunits in DNA gyrase, and two ParC and two ParE subunits in topoisomerase IV. The GyrA and GyrB subunits of DNA gyrase are similar in amino acid sequence to the ParC and ParE subunits of topoisomerase IV, respectively. The main function of GyrA/ParC is cleavage and reunion of DNA, whereas GyrB/ParE binds ATP, which is crucial for providing the energy for the conformational movements of the enzyme, required for the ligation process.[4]
There are two main mechanisms of action of drugs targeting DNA gyrase and topoisomerase IV. The first is stabilisation of the covalent enzyme–DNA complex, which is typical for the fluoroquinolone class of antibacterials.[5] The presence of some serious side effects together with the growing bacterial resistance to fluoroquinolones has stimulated the search for inhibitors targeting various binding sites on DNA gyrase.[6] The second possible mechanism of action of drugs targeting DNA gyrase and topoisomerase IV involves inhibition of ATP binding on the GyrB/ParE subunit. Novobiocin, a natural aminocoumarin antibiotic that was withdrawn from the clinic because of toxicity or lack of effectiveness,[7] is a classic example of ATP-competitive GyrB inhibitors. In recent decades, several new scaffolds have been identified as GyrB and/or ParE inhibitors, such as pyridylureas,[8] pyrimidinoindoles,[9] pyrrolopyrimidines,[10] benzimidazole ureas,[11] pyrrolamides,[12] and pyrazolopyridones[13] (Figure 1). Even though some of the new inhibitors have advanced to Phase I, none of them has so far reached clinical practice.[14] In the present study, we describe a new series of *N*-phenylpyrrolamide DNA gyrase (GyrB) and topoisomerase IV (ParE) inhibitors, resulting in optimised on-target and antibacterial activities.

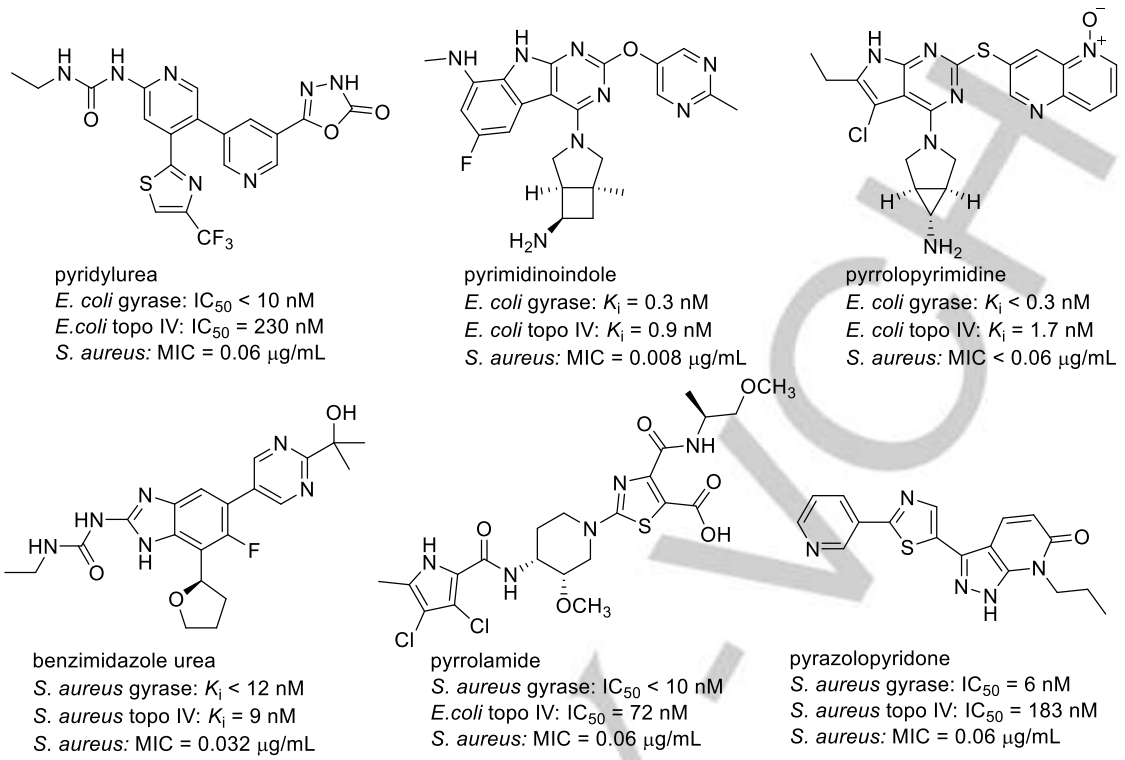


Figure 1. Representative inhibitors of DNA gyrase (GyrB) and topoisomerase IV (ParE), and their IC_{50}, *K*i and MIC (*S. aureus*) values.

Results and Discussion

Design. The design of the present series of compounds was based on the crystal structure of *N*-phenylpyrrolamide inhibitor **A** in complex with *E. coli* DNA gyrase B (Figure 2, PDB code: 4ZVI),[15] and was aimed at improving its GyrB/ParE inhibitory potency and antibacterial activity. Three structural types of compounds (type I-III, Figure 2) were prepared, in which we introduced modifications proposed by the detailed study of the binding mode of inhibitor **A** in the GyrB active site, and by molecular docking (Figure 3). In all structural types (I-III) we have retained the adjacent hydrogen bond donor (pyrrole NH) and hydrogen bond acceptor (pyrrolamide C=O) groups that are necessary for establishing the H-bonding network with Asp73 (*E. coli* numbering) and the structurally conserved water molecule (Figure 2). The amide NH of the pyrrolamide moiety was left unsubstituted because it is important for indirect contacts with Asn46 through a crystal water molecule. In some derivatives, we have replaced the 4,5-dibromo-1*H*-pyrrole group on the left-hand side of the molecules with 3,4-dichloro-5-methyl-1*H*-pyrrole. Thus far, replacing the 4,5-dibromo-1*H*-pyrrole moiety by 4-bromo-1*H*-pyrrole, 1*H*-indole, or by 4,5-dichloro-1*H*-pyrrole resulted in weaker activity.[15-16] The 3,4-dichloro-5-methyl-1*H*-pyrrole was selected because the chlorine atoms are slightly smaller than the bromine atoms and are thus proposed to bind more strongly, not only to DNA gyrase, but also to the slightly smaller binding pockets of *S. aureus* DNA gyrase and topoisomerase IV.[4b,17] Additionally, the 3,4-dichloro-5-methylpyrrole moiety is found e.g. in kibdelomycin, a complex antibiotic isolated from extracts of the soil bacterium *Kibdelosporangium* sp.,[18] and in some pyrrolamide DNA gyrase B inhibitors developed by AstraZeneca.[12d]

The right-hand side of the molecules was designed to contain groups able to form ionic interactions with Arg136 (e.g. carboxylic acid groups) or π-stacking interactions with the Glu50-Arg76 salt bridge (e.g. 4-amino-2-hydroxybenzoic acid or 1,3,4-oxadiazol-2-one ring). Whereas type I compounds were all glycine derivatives, in some type II compounds, the glycine group (n = 1, Figure 2) was modified to beta-alanine group (n = 2, Figure 2). In order to improve transport across the bacterial cell membranes, the ionizable terminal carboxylic acid functionality was replaced in some type I and type II compounds by its less acidic bioisostere 1,3,4-oxadiazol-2-one. The 1,3,4-oxadiazol-2-one ring was proposed to form similar interactions with Arg136 as does the terminal carboxylic group, but its lower acidity promises better permeability across bacterial membranes and, consequently, better antibacterial activity.[8] Additionally, some type I and type II compounds with terminal hydrazide groups were analyzed.

With the type II series, we have introduced additional alkoxy substituents to the 3-position of the central benzene ring. The preliminary docking studies have indicated the possibility of additional hydrophobic contacts between these groups and the lipophilic floor of the enzyme formed by the amino acid residues Ile94, Ile78, Val120 and Val167 (Figure 3). The size of substituents was increased from methoxy, through isopropoxy, to the benzyloxy group.

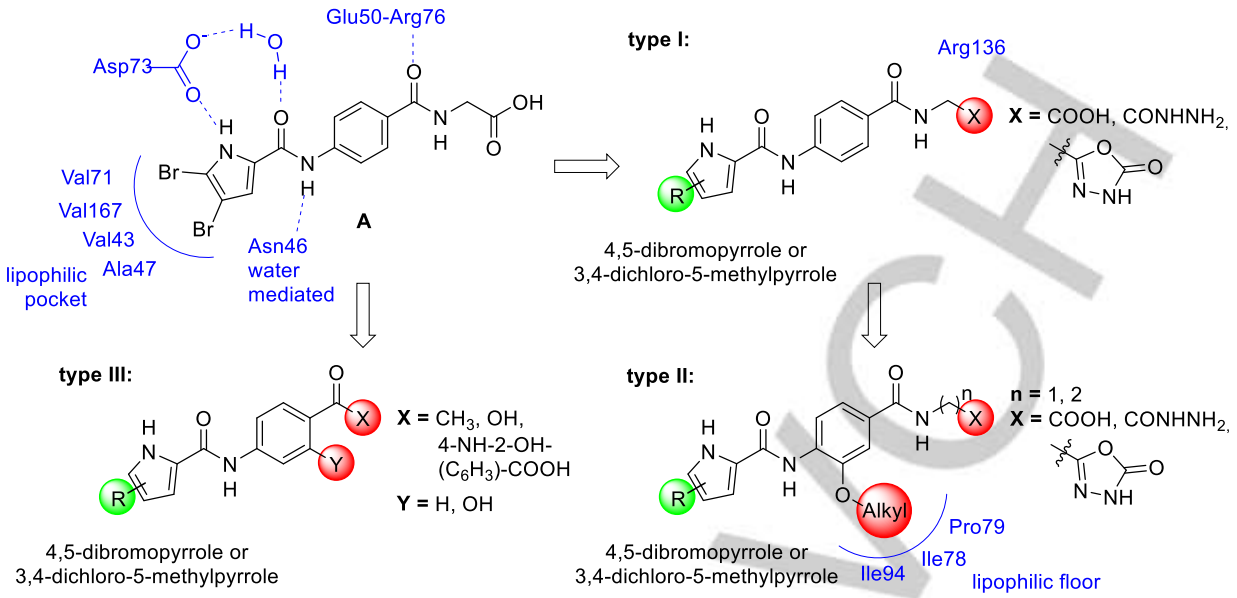


Figure 2. Design of three structural types (I-III) of *N*-phenylpyrrolamides as DNA gyrase B inhibitors, based on the co-crystal structure of the inhibitor A – GyrB (*E. coli*) (PDB code: 4ZVI[15]).

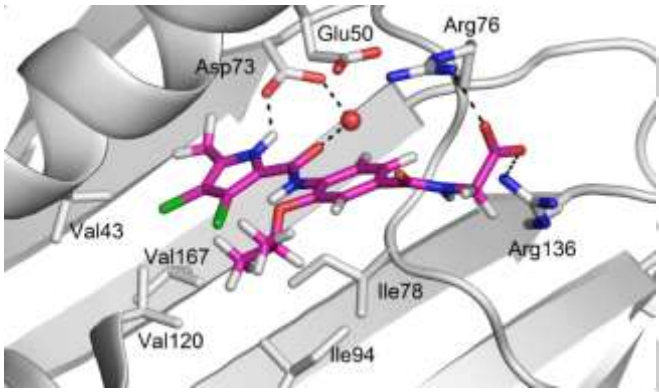


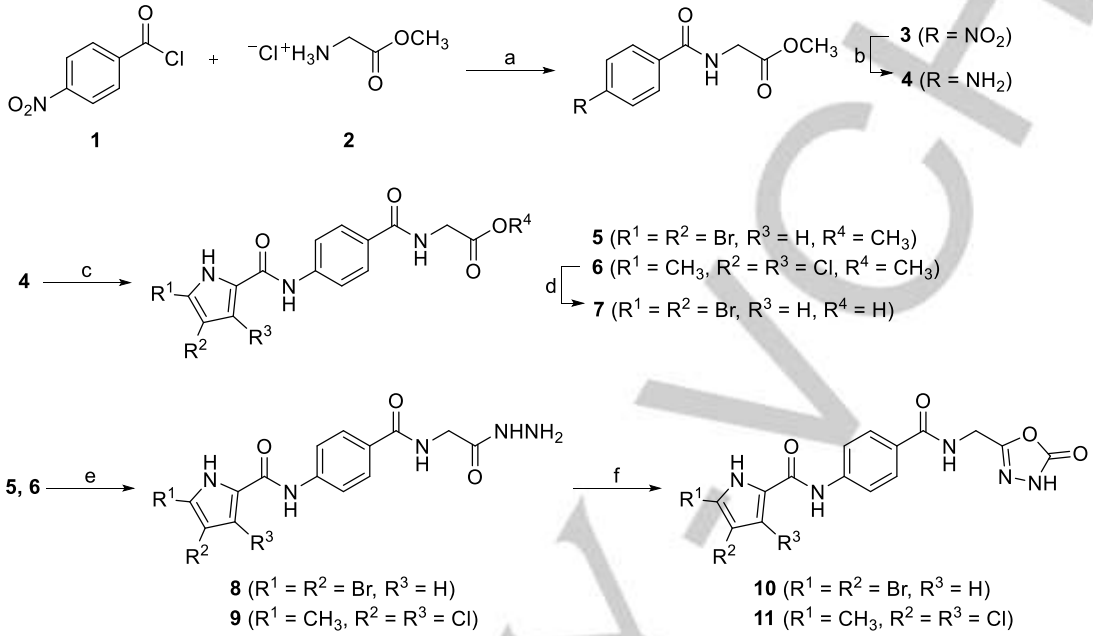
Figure 3. The docking binding mode of inhibitor **19f** (in magenta) in the ATP binding site of *E. coli* DNA gyrase B (in grey, PDB code: 4DUH[19]). The ligand and the neighbouring protein side-chains are shown as stick models, coloured according to the chemical atom type (blue, N; red, O; orange, S; green, Cl). Hydrogen bonds are indicated by black dotted lines.

Based on the relatively high activity of one of our previously reported inhibitors with only a short $COCH_3$ substituent on the *para* position of the central aminobenzene ring (i.e. *N*-(4-acetylphenyl)-4,5-dibromo-1*H*-pyrrole-2-carboxamide, IC_{50} = 1.6 μM),[16b] we decided to further explore the chemical space around this molecule by preparing a small library of its analogues (type III compounds, Figure 2). Analogues were prepared with carboxylic groups attached directly to the central phenyl ring (**X** = OH, **Y** = H, Figure 2), compounds with an additional hydroxyl group on the phenyl ring (**X** = OH, **Y** = OH, Figure 2), and compounds with an acetophenone functionality (**X** = CH_3, **Y** = H, Figure 2). To determine the influence of increased length of the molecules on gyrase B and topoisomerase IV inhibition, we prepared a compound with an additional aromatic ring (**X** = 4-NH_2-2-OH-(C_6H_3)-COOH, **Y** = H, Figure 2). The structure-activity relationships (SAR) of all the prepared compounds were studied, enabling the essential structural elements for GyrB/ParE inhibition and for antibacterial activity to be determined.

Chemistry. The synthesis of type I compounds (**7-11**) is presented in Scheme 1. First, 4-nitrobenzoyl chloride (**1**) was reacted with glycine methyl ester hydrochloride (**2**) in the presence of potassium carbonate to give compound **3**. The nitro group of **3** was reduced by catalytic hydrogenation and the obtained amine **4** was coupled with 4,5-dibromopyrrole-2-carboxylic acid using *N,N,N',N'*-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU), to yield pyrrolamide **5**, or with 3,4-dichloro-5-methylpyrrole-2-carboxylic acid chloride prepared *in situ* from the carboxylic acid using oxalyl chloride, to give pyrrolamide **6**. Compound **7** was prepared by alkaline hydrolysis of methyl ester **6** while hydrazides **8** and **9** were prepared by heating **5** or **6** with hydrazine at 78 °C. To obtain the target 1,3,4-oxadiazol-2-ones **10** and **11**, compounds **8** and **9** were reacted with 1,1'-carbonyldiimidazole (CDI).

Type II compounds (**19a-g**, **20** and **21**) were synthesized according to Scheme 2. First, methyl ester **13** was prepared from 3-hydroxy-4-nitrobenzoic acid (**12**) and thionyl chloride in methanol. The phenolic group of **13** was then alkylated with methyl (compound **14a**), isopropyl (compound **14b**) or benzyl (compound **14c**) substituents. Compounds **14a** and **14c** were prepared using methyl iodide or benzyl bromide and potassium carbonate as base, while compound **14b** was prepared using isopropanol under Mitsunobu conditions. Methyl esters **14a-c** were hydrolyzed with 1 M sodium hydroxide to give carboxylic acids **15a-c**, which were subsequently coupled with glycine methyl ester hydrochloride to give compounds **16a-c**, or with methyl 3-aminopropanoate to give compounds **16d-f**. The nitro groups of **16a-f** were reduced to amino groups by catalytic hydrogenation (for products **17a-b**, **17d-e**) or with tin(II) chloride (for products **17c** and **17f**). Pyrrolamides **18a-c** were prepared by

coupling **17a-b** or **17d** with 4,5-dibromopyrrole-2-carboxylic acid using TBTU methodology, while pyrrolamides **18d-h** were prepared from **17a-c** or **17e-f** and the corresponding pyrrole-2-carboxylic acid chloride prepared *in situ* using oxalyl chloride as reagent. Alkaline hydrolysis of compounds **18a-g** gave the final products **19a-g**, while hydrolysis of **18h** was unsuccessful. Compound **18b** was additionally reacted with hydrazine monohydrate to give hydrazide **20**, and, after heating **20** with CDI, the final product **21** was obtained.



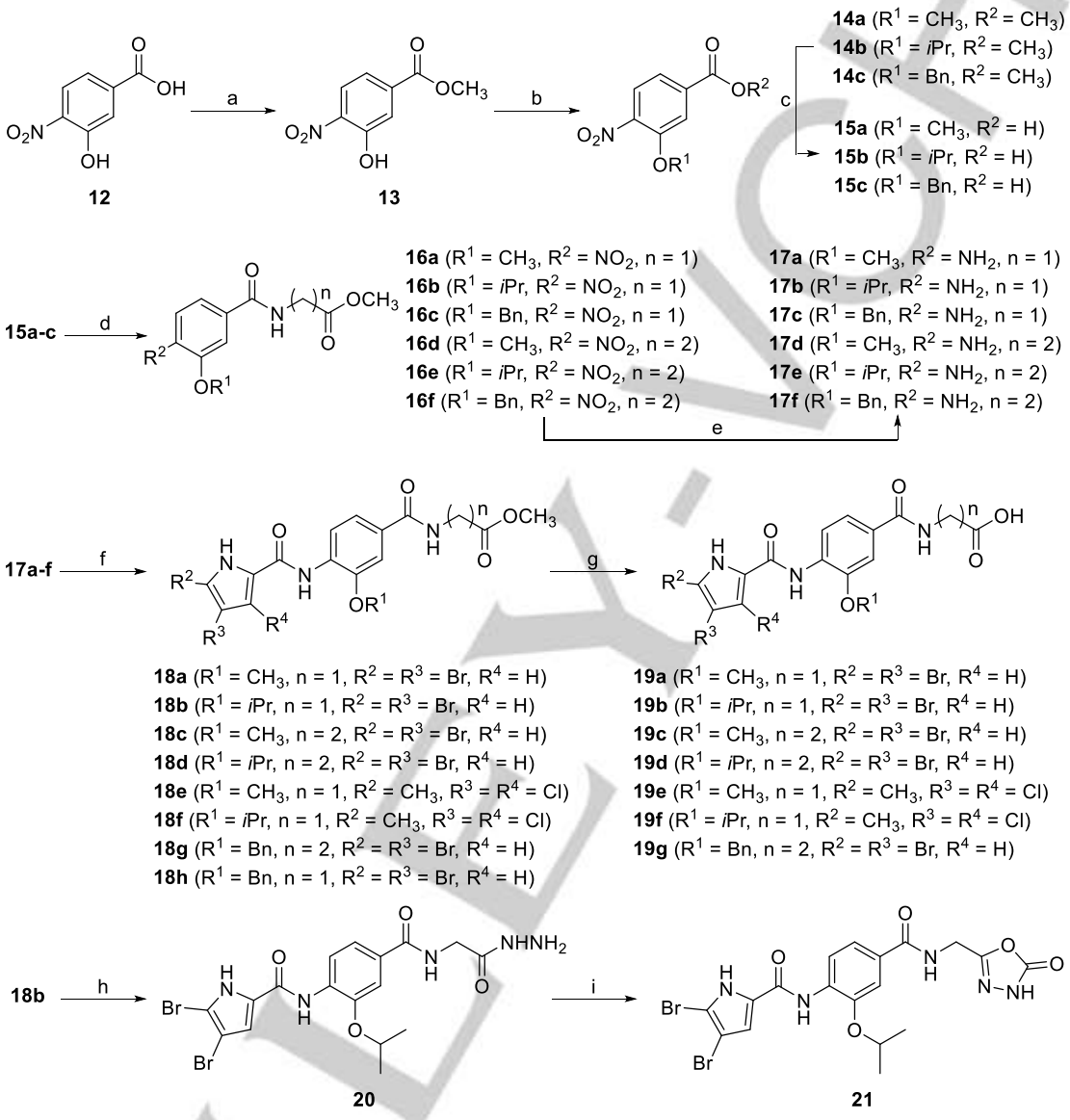
Scheme 1. *Reagents and conditions*: a) K_2CO_3, CH_3CN, 0 °C → rt, 15 h; b) H_2, Pd-C, THF, 3 h; c) 4,5-dibromopyrrole-2-carboxylic acid, TBTU, NMM, CH_2Cl_2, 50 °C, 15 h (for the synthesis of **5**), *i*) 3,4-dichloro-5-methylpyrrole-2-carboxylic acid, oxalyl chloride, CH_2Cl_2, rt, 15 h, then *ii*) **4**, pyridine, CH_2Cl_2, rt, 15 h (for the synthesis of **6**); d) 1 M NaOH, THF, rt, 15 h; e) hydrazine monohydrate, EtOH, reflux, 15 h; f) CDI, 1,4-dioxane, 101 °C, 15 h (for the synthesis of **10**), CDI, DMF, 50 °C, 15 h (for the synthesis of **11**).

Compounds **25a-c** and **29** were prepared according to Scheme 3. In the first step, the carboxylic acids **22a** and **22b** were converted to methyl esters using thionyl chloride and methanol. The obtained esters **23a** and **23b** were then coupled with 4,5-dibromopyrrole-2-carboxylic acid or with 3,4-dichloro-5-methylpyrrole-2-carboxylic acid, as described above, to give compounds **24a-d**. The final products **25a-c** were obtained after alkaline hydrolysis of methyl esters **24a-c**. For the synthesis of compound **29**, first, 4-nitrobenzoyl chloride (**1**) was reacted with methyl 4-aminosalicylate (**23b**) in the presence of pyridine to give the amide **26**. After reduction of the nitro group of compound **26** by catalytic hydrogenation, the obtained amine **27** was coupled with 4,5-dibromopyrrole-2-carboxylic acid to give compound **28** which was submitted to alkaline hydrolysis to give the final compound **29**.

Inhibitory Activities against DNA Gyrase and Topoisomerase IV. Thirty-two new compounds were prepared and evaluated against *E. coli* DNA gyrase in a supercoiling inhibition assay. The results are presented in Tables 1-3 as residual activities (RA) of the enzyme in the presence of 10 µM of compounds or as concentrations of compounds that inhibit the enzyme activity by 50% (IC_{50} values). The most active compounds were additionally tested against topoisomerase IV from *E. coli*, and against DNA gyrase and topoisomerase IV from *S. aureus*.

In total, twelve compounds displayed IC_{50} values lower than 0.5 µM against *E. coli* DNA gyrase, three of which (**11**, **19f** and **19g**) showed inhibitory activities in the low nanomolar range (IC_{50} < 0.1 µM) and their enzymatic inhibition was thus better than that of novobiocin (IC_{50} = 0.17 µM). Generally, compounds containing 3,4-dichloro-5-methylpyrrole groups on the left-hand side of the molecules displayed higher activities against *E. coli* DNA gyrase than compounds containing 4,5-dibromopyrrole moieties. For example, in the type I series, 3,4-dichloro-5-methylpyrrolamide **11** (IC_{50} = 0.080 µM) displayed a four-fold higher activity than its 4,5-dibromopyrrolamide analog **10** (IC_{50} = 0.30 µM). Similarly, compound **9** (IC_{50} = 0.43 µM) was five-times more active than its 4,5-dibromopyrrolamide analog **8** (IC_{50} = 2.4 µM). Similar differences in activity could be observed between 4,5-dibromopyrrolamides and 3,4-dichloro-5-methylpyrrolamides of the type II (Table 2) and type III (Table 3) series. These results indicate that both pyrroles can occupy the hydrophobic binding pocket of the enzyme and form hydrophobic interactions with Val71, Val167, Val43 and Ala47, but the smaller methyl and chlorine atoms fit better into the enzyme's binding site. A further observation from Tables 1-3 is that compounds containing methyl ester groups on the right-hand side show weaker activities than their carboxylic acid counterparts, probably because they cannot form ionic interactions with Arg136. This can be seen by comparing the esters **6** (IC_{50} = 0.41 µM), **18a** (IC_{50} = 3.7 µM) and **24c** (IC_{50} = 1.6 µM) on the one hand, with the corresponding carboxylic acids **7** (IC_{50} = 0.28 µM), **19a** (IC_{50} = 0.31 µM) and with **25c** (IC_{50} = 0.79 µM) on the other. When comparing the activities of compounds with carboxylic acid groups with their hydrazide

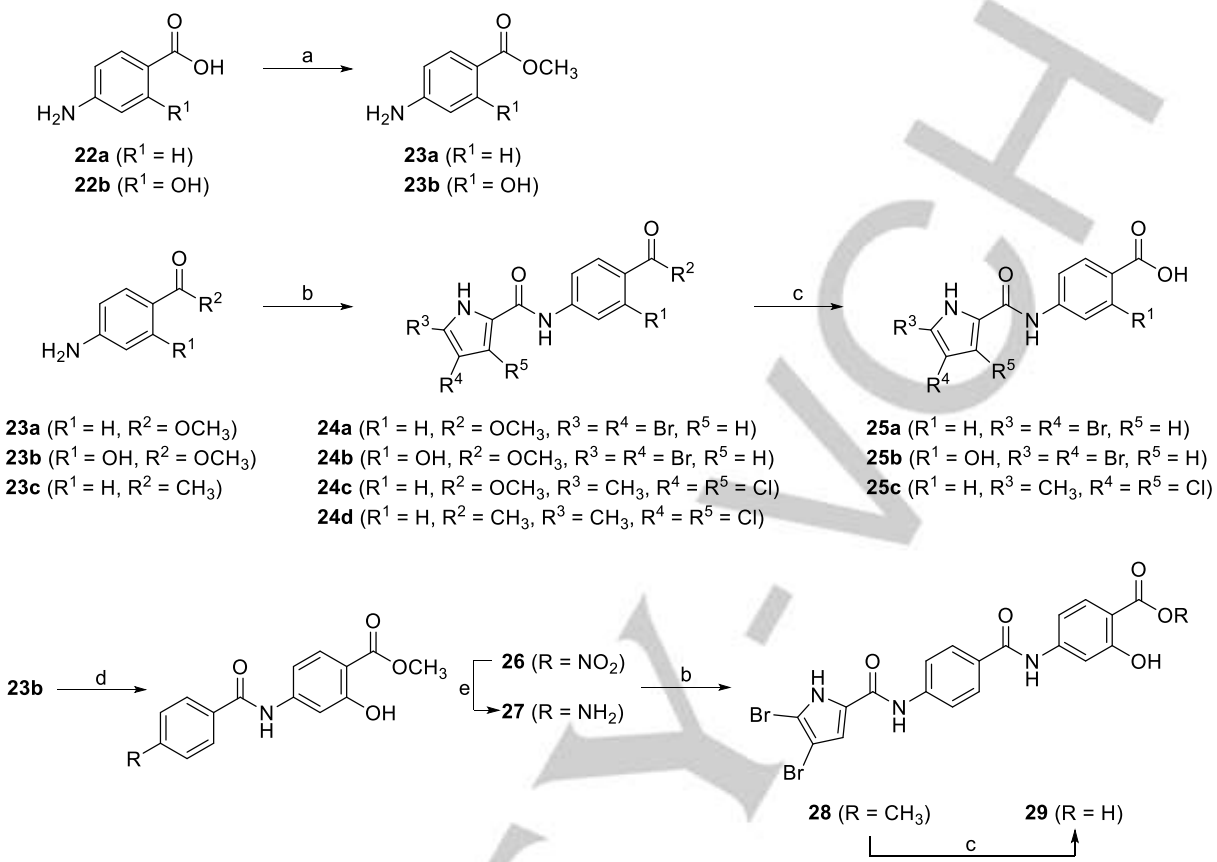
analogs, the former were generally more active. For example, carboxylic acid **7** (IC_{50} = 0.28 μM) displayed greater activity than its hydrazide analogue **9** (IC_{50} = 0.43 μM), compound **A** (IC_{50} = 0.45 μM, reported previously)[16b] was more active than the corresponding hydrazide **8** (IC_{50} = 2.4 μM), and the carboxylic acid **19b** (IC_{50} = 0.16 μM) was more potent than the hydrazide **20** (IC_{50} = 1.6 μM). These differences are, most probably, due to the loss of ionic interactions with Arg136 in the case of hydrazide groups.



Scheme 2. *Reagents and conditions*: a) thionyl chloride, MeOH, 0 °C → rt, 15 h; b) MeI, K_2CO_3, DMF, rt, 15 h (for the synthesis of **14a**), isopropanol, triphenylphosphine, DIAD, THF, rt, 15 h (for the synthesis of **14b**), K_2CO_3, benzyl bromide, CH_3CN, rt → 60 °C, 3 h (for the synthesis of **14c**); c) 1 M NaOH, MeOH, rt, 15 h; d) glycine methyl ester hydrochloride (for the synthesis of **16a-c**) or methyl 3-aminopropanoate (for the synthesis of **16d-f**), TBTU, NMM, CH_2Cl_2, rt, 2-15 h; e) H_2, Pd-C, MeOH, rt, 1-4 h (for the synthesis of **17a-b** and **17d-e**), $SnCl_2$, EtOAc, EtOH, 55 °C, 24 h (for the synthesis of **17c** and **17f**); f) *i*) 4,5-dibromopyrrole-2-carboxylic acid, TBTU, NMM, CH_2Cl_2, rt, 30 min, then *ii*) **17a** or **17b** or **17d**, DMAP, 60 °C, 15 h (for the synthesis of **18a-c**), *i*) 4,5-dibromopyrrole-2-carboxylic acid, oxalyl chloride, CH_2Cl_2, rt, 15 h, then *ii*) **17c** or **17e** or **17f**, pyridine, CH_2Cl_2, rt, 15 h (for the synthesis of **18d** and **18g-h**), *i*) 3,4-dichloro-5-methylpyrrole-2-carboxylic acid, oxalyl chloride, CH_2Cl_2, rt, 15 h, then *ii*) **17a** or **17b**, pyridine, CH_2Cl_2, rt, 15 h (for the synthesis of **18e-f**); g) 1-2 M NaOH, MeOH/THF, rt, 15 h; h) hydrazine monohydrate, EtOH, reflux, 2 d; i) CDI, 1,4-dioxane, 101 °C, 15 h.

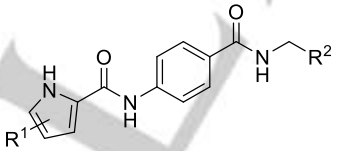
There were no significant differences in activity between compounds that contain the glycine (n = 1, Table 2) and those containing the longer beta-alanine substituent (n = 2, Table 2) on the right-hand side of the molecules. Although in the carboxylic acid subset (compounds **19a-g**, Table 2), the glycine derivative **19b** (IC_{50} = 0.16 μM) was slightly more active than the beta-alanine derivative **19d** (IC_{50} = 0.36 μM), compounds **19a** (IC_{50} = 0.31 μM) and **19c** (IC_{50} = 0.35 μM) showed almost no difference in activities. Also, by observing the methyl ester subset compounds (**18a-h**, Table 2) the optimal length of the molecules could not be determined with certainty. These results can be due to the high flexibility of this side chain in solution, as recently

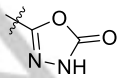
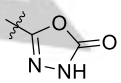
proposed by molecular dynamics simulation.[15] Probably both, the glycine and the beta-alanine groups can adopt conformations in which the terminal carboxylate group is either in contact with Arg136 or with the solvent.



Scheme 3. *Reagents and conditions*: a) thionyl chloride, MeOH, 0 °C → 70 °C, 15 h; b) *i*) 4,5-dibromopyrrole-2-carboxylic acid (for the synthesis of **24a-b** and **28**) or 3,4-dichloro-5-methylpyrrole-2-carboxylic acid (for the synthesis of **24c-d**), oxalyl chloride, CH_2Cl_2, rt, 15 h, then *ii*) **23a** or **23b** or **23c** or **27**, pyridine, CH_2Cl_2, rt, 15 h; c) 1 M NaOH, MeOH/THF, rt → 60 °C, 30 h (for the synthesis of **25a-b** and **29**), 1 M NaOH, THF, 45 °C, 15 h (for the synthesis of **25c**); d) **1**, CH_2Cl_2, pyridine, rt 15 h; e) H_2, Pd-C, MeOH/THF, rt, 3 h.

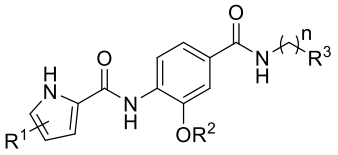
Table 1. Inhibitory activities of type I compounds **6-11** against DNA gyrase and topoisomerase IV.

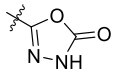


Compd	R^1	R^2	IC50 (μM)[a] or RA (%)[b] *E. coli* gyrase	*S. aureus* gyrase	*E. coli* topo IV	*S. aureus* topo IV
6	3,4-diCl-5-Me	COOMe	0.41±0.20 μM	n.d.[c]	n.d.	n.d.
7	3,4-diCl-5-Me	COOH	0.28±0.21 μM	11±0 μM	100%	100%
8	4,5-diBr	$CONHNH_2$	2.4±1.5 μM	93%	79%	95%
9	3,4-diCl-5-Me	$CONHNH_2$	0.43±0.26 μM	60%	100%	100%
10	4,5-diBr		0.30±0.09 μM	100%	98%	100%
11	3,4-diCl-5-Me		0.080±0.027 μM	23±5 μM	100%	62%
novobiocin			0.17 μM	0.041 μM	11 μM	27 μM

[a] Concentration of compound that inhibits the enzyme activity by 50%. [b] Residual activity of the enzyme at 10 μM of the compound. [c] Not determined.

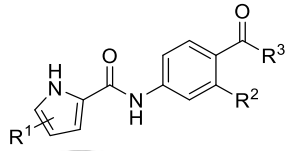
Table 2. Inhibitory activities of type II compounds **18a-h**, **19a-g**, **20** and **21** against DNA gyrase and topoisomerase IV.



Compd	R^1	R^2	R^3	n	IC_{50} (μM)[a] or RA (%)[b] *E. coli* gyrase	*S. aureus* gyrase	*E. coli* topo IV	*S. aureus* topo IV
18a	4,5-diBr	Me	COOMe	1	3.7±2.1 μM	n.d.[c]	n.d.	n.d.
18b	4,5-diBr	*i*Pr	COOMe	1	97%	n.d.	n.d.	n.d.
18c	4,5-diBr	Me	COOMe	2	4.2±0.3 μM	n.d.	n.d.	n.d.
18d	4,5-diBr	*i*Pr	COOMe	2	3.9±0.8 μM	n.d.	n.d.	n.d.
18e	3,4-diCl-5-Me	Me	COOMe	1	0.54±0.36 μM	99%	100%	100%
18f	3,4-diCl-5-Me	*i*Pr	COOMe	1	2.3±0.1 μM	n.d.	n.d.	n.d.
18g	4,5-diBr	Bzl	COOMe	2	21%	n.d.	n.d.	n.d.
18h	4,5-diBr	Bzl	COOMe	1	77%	n.d.	n.d.	n.d.
19a	4,5-diBr	Me	COOH	1	0.31±0.11 μM	17±2 μM	85%	96%
19b	4,5-diBr	*i*Pr	COOH	1	0.16±0.06 μM	22±4 μM	5.0±5.2 μM	13±10 μM
19c	4,5-diBr	Me	COOH	2	0.35±0.10 μM	67%	100%	98%
19d	4,5-diBr	*i*Pr	COOH	2	0.36±0.29 μM	28±10 μM	76%	90%
19e	3,4-diCl-5-Me	Me	COOH	1	0.12±0.10 μM	4.1±2.9 μM	99%	65%
19f	3,4-diCl-5-Me	*i*Pr	COOH	1	0.047±0.021 μM	2.3±0.5 μM	1.4±0.5 μM	2.3±0.7 μM
19g	4,5-diBr	Bzl	COOH	2	0.087±0.052 μM	20±5 μM	100%	98%
20	4,5-diBr	*i*Pr	$CONHNH_2$	1	1.6±0.0 μM	n.d.	n.d.	n.d.
21	4,5-diBr	*i*Pr		1	1.7±0.0 μM	65%	100%	99%
novobiocin					0.17 μM	0.041 μM	11 μM	27 μM

[a] Concentration of compound that inhibits the enzyme activity by 50%. [b] Residual activity of the enzyme at 10 μM of the compound. [c] Not determined.

Table 3. Inhibitory activities of type III compounds **24a-d**, **25a-c**, **28** and **29** against DNA gyrase and topoisomerase IV.



Compd	R^1	R^2	R^3	IC_{50} (μM)[a] or RA (%)[b] *E. coli* gyrase	*S. aureus* gyrase	*E. coli* topo IV	*S. aureus* topo IV
24a	4,5-diBr	H	OMe	5.8±1.7 μM	n.d.[c]	n.d.	n.d.
24b	4,5-diBr	OH	OMe	94%	n.d.	n.d.	n.d.
24c	3,4-diCl-5-Me	H	OMe	1.6±0.5 μM	100%	94%	100%
24d	3,4-diCl-5-Me	H	Me	0.72±0.09 μM	100%	100%	100%
25a	4,5-diBr	H	OH	2.9±0.4 μM	n.d.	n.d.	n.d.
25b	4,5-diBr	OH	OH	2.7±0.1 μM	n.d.	n.d.	n.d.
25c	3,4-diCl-5-Me	H	OH	0.79±0.02 μM	62%	11±2 μM	45±4 μM
28	4,5-diBr	H	4-NH-2-OH-(C_6H_3)-$COOCH_3$	80%	n.d.	n.d.	n.d.
29	4,5-diBr	H	4-NH-2-OH-(C_6H_3)-COOH	0.60±0.37 μM	n.d.	n.d.	n.d.
novobiocin				0.17 μM	0.041 μM	11 μM	27 μM

[a] Concentration of compound that inhibits the enzyme activity by 50%. [b] Residual activity of the enzyme at 10 μM of the compound. [c] Not determined.

With the introduction of methoxy (compounds **18a**, **18e**, **19a** and **19e**, Table 2), isopropoxy (compounds **18b**, **18d**, **18f**, **19b**, **19d**, **19f**, **20** and **21**, Table 2) or benzyloxy substituents (compounds **18g**, **18h** and **19g**, Table 2) at the 3-position of the central benzene ring, we intended to make additional contacts with the lipophilic floor of the enzyme. Among the 3,4-dichloro-5-methylpyrrolamides, the most active was the isopropoxy compound **19f** (IC_{50} = 0.047 μM), followed by the methoxy compound **19e** (IC_{50} = 0.12 μM) and the unsubstituted compound **7** (IC_{50} = 0.28 μM). In the 4,5-dibromopyrrolamide series the relationship was similar, as the isopropoxy compound **19b** (IC_{50} = 0.16 μM) and the methoxy compound **19a** (IC_{50} = 0.31 μM) were both more potent than the unsubstituted analog **A** (IC_{50} = 0.45 μM). Even though the benzyloxy-substituted compound **18g** (RA = 21%) did not display significant activity, its carboxylic acid derivative **19g** (IC_{50} = 0.087 μM) was among the most potent of the series. Overall, the results indicate that lipophilic substituents at the 3-position of the benzene ring are favorable for enhancing interactions with the enzyme.

With the type III series of compounds, we studied the activities of analogues without the glycine or beta-alanine groups on the right-hand side of the molecules (compounds **24a-d** and **25a-c**, Table 3). Some prepared compounds lacking the glycine or beta-alanine groups showed good inhibitory activities, e.g. compound **24c** had an IC_{50} value of 1.6 μM and its carboxylic acid analogue **25c** an IC_{50} of 0.79 μM against *E. coli* DNA gyrase. These results are particularly interesting when taking into account the simple, fragment-like structures of these molecules that could still be

optimized. Furthermore, two longer molecules, **28** and **29** (Table 3) have been prepared with an additional 4-aminobenzoyl group, but their activities were weaker, probably because the terminal carboxylic acid group in these molecules is too far from Arg136 to form interactions.

Overall, the activities against *S. aureus* DNA gyrase, *E. coli* topoisomerase IV, and *S. aureus* topoisomerase IV were lower than those against *E. coli* DNA gyrase (Tables 1-3). The two compounds with the greatest activity against *S. aureus* DNA gyrase were **19f** with an IC_{50} of 2.3 μM and **19e** with an IC_{50} of 4.1 μM. Six other compounds (**7**, **11**, **19a**, **19b**, **19d** and **19g**) displayed *S. aureus* DNA gyrase inhibitory activities in the low micromolar range, but none of the compounds have reached a comparable inhibition to that of novobiocin with an IC_{50} value of 41 nM. Seven of these compounds contained a free carboxylic acid group, which thus appears crucial for the activity. A possible reason for the weaker activities of compounds against *S. aureus* gyrase is that the adenine-binding pocket of this enzyme is slightly smaller than that of *E. coli* gyrase, which would indicate that smaller groups are more effective in binding to the hydrophobic binding site, as discussed recently.[16b] In line with this, the two most active compounds against *S. aureus* gyrase, **19e** (IC_{50} = 4.1 μM) and **19f** (IC_{50} = 2.3 μM), contain smaller 3,4-dichloro-5-methylpyrrole moieties, while the activities of their 4,5-dibromopyrrolamide analogs **19a** (IC_{50} =17 μM) and **19b** (IC_{50} = 22 μM) were slightly lower. The most active compound against topoisomerase IV enzyme was again compound **19f** with an IC_{50} values of 1.8 μM against *E. coli* topo IV and 2.7 μM against *S. aureus* topo IV. The weaker activities on *E. coli* topoisomerase IV than on *E. coli* DNA gyrase are probably due to the previously discussed differences in the binding sites of these enzymes.[16b]

Antibacterial Activity. Compounds were evaluated against two Gram-positive (*S. aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) and two Gram-negative (*E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) wild type bacterial strains at 50 μM concentration. Results are presented in Table 1S (Supporting information) as percent inhibition of growth after 24 h incubation. In general, the activities against Gram-positive bacteria were greater than those against Gram-negative bacteria. This could be due to difficulties in permeation of compounds through the Gram-negative bacterial cell wall, or to efflux of inhibitors from the bacteria. In total, nine compounds (**6**, **9**, **11**, **18f**, **19f**, **19g**, **21**, **25c** and **29**) inhibited the growth of Gram-positive *E. faecalis* by more than 50% at 50 μM concentration, four of which (**6**, **11**, **19f** and **25c**) displayed more than 70% inhibition of growth. Compound **11** also showed 64% inhibition of growth of *S. aureus* and 51% inhibition of growth of Gram-negative *E. coli* (Table 1S). The results of the minimum inhibitory concentration (MIC) experiments against *S. aureus*, *E. faecalis* and *E. coli* for the most interesting compounds are presented in Table 4. The MIC values for compounds **11** and **19f** against *E. faecalis* were 12.5 μM and 50 μM (MIC for ciprofloxacin = 3.02 μM). The MIC value for compound **11** against *S. aureus* was 75 μM (MIC for ciprofloxacin = 1.51 μM). With other tested compounds, the MIC level (≥ 90% inhibition) was not reached even at the highest concentration of 125 μM (Table 4). The highest antibacterial activity of compound **11** is in line with our design strategy, that the bioisosteric replacement of the terminal carboxylic acid group with the 1,3,4-oxadiazol-2-one ring might lead to easier entry of inhibitors into the bacterial cell due to their lower acidity and polarity.

Fifteen compounds (**6**, **7**, **9**, **11**, **18f**, **19f**, **24a-d**, **25a**, **25b**, **25c**, **28** and **29**) were additionally tested against two *E. coli* mutant strains, JD17464 and JW5503. *E. coli* JD17464 is an *lpxC* deletion mutant with impaired outer membrane, while *E. coli* JW5503 is a *tolC* deletion mutant with a defective efflux pump. Inhibition of growth was measured at 50 μM concentration of compounds after 24 h incubation (Table 2S). MIC values for six selected compounds (**6**, **9**, **11**, **19f**, **24a** and **25c**) against *E. coli* JW5503 are presented in Table 5. The activities against the mutant with impaired outer membrane (JD17464) were comparable with those against wild type *E. coli*. On the other hand, activities against the mutant with a defective efflux pump (JW5503) were greater for the majority of the tested compounds. The most active compound was oxadiazolone **11** with an MIC value of 6.25 μM, followed by a type III compound **25c** with 25 μM MIC. Compounds **6**, **9**, **19f** and **24a** had an MIC of 50 μM. These results indicate that the efflux of inhibitors is probably the major factor causing the lower activities of these compounds against the Gram-negative bacteria. To confirm these findings, compounds **6**, **9**, **11**, **19f** and **25c** were additionally evaluated against wild type *E. coli* in the presence of 100 μg/mL concentration of efflux inhibitor phenylalanine-arginine β-naphthylamide (PAβN)[20] (Table 5). The most active compound was again compound **11**, with an MIC of 3.13 μM, followed by **25c** with an MIC of 12.5 μM, and compounds **6** (MIC = 25 μM), **19f** (MIC = 25 μM) and **9** (MIC = 50 μM). Observing the inhibitory activity of compound **11** against wild type *E. coli* with and without PAβN at different time-points (4, 8 and 24 h) clearly demonstrates that this compound is able to affect the growth in the logarithmic growth phase also without PAβN, but that at the 24 h end-point the inhibitory activity no longer reaches the MIC (Figure 1S). In the presence of PAβN, stronger inhibition is seen at all time points. These results confirm that, in Gram-negative bacteria, the prepared compounds are most probably substrates for efflux pumps, which should be taken into account in the design of all future series of *N*-phenylpyrrolamide inhibitors. As has been demonstrated for the pyrrolopyrimidine series of DNA gyrase inhibitors,[10] despite the susceptibility to efflux, it is nevertheless possible to achieve good MICs against Gram-negative bacteria.

It is likely, that weaker antibacterial activity of the presented *N*-phenylpyrrolamides with respect to some other published DNA gyrase B inhibitors is due to their lower on-target activity. For example, the representative pyrrolamide developed at AstraZeneca (Figure 1) with *S. aureus* MIC of 0.06 μg/mL displayed an IC_{50} of less than 10 nM against *S. aureus* DNA gyrase, and the representative pyrazolopyridones from Cubist Pharmaceuticals (Figure 1) with *S. aureus* MIC of 0.06 μg/mL displayed an IC_{50} of 6 nM against *S. aureus* DNA gyrase. Therefore, to obtain higher antibacterial potency, DNA gyrase activity of *N*-phenylpyrrolamides will likely need to be improved to low nanomolar range.

Table 4. Minimum inhibitory concentrations (MICs) of compounds **6**, **11**, **19f**, **19g**, **21**, **25c** and **29** against *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 29212) and *E. coli* (ATCC 25922).

Compd	MIC (μM)[a]		
	S. aureus (ATCC 25923)	*E. faecalis* (ATCC 29212)	*E. coli* (ATCC 25922)
6	n.d.[b]	>125	n.d.
11	75	12.5	>125
19f	n.d.	50	n.d.
19g	>125	>125	n.d.
21	>125	>125	n.d.
25c	n.d.	>125	n.d.
29	n.d.	>125	n.d.

[a] MIC = minimum inhibitory concentration (μM) that inhibited the growth of bacteria by ≥ 90%. MIC values of the positive control ciprofloxacin against *S. aureus*, *E. faecalis* and *E. coli* were 1.51, 3.02 and 0.05 μM. [b] Not determined.

Table 5. Minimum inhibitory concentrations (MIC) of compounds **6**, **9**, **11**, **19f** and **25c** against the efflux pump deficient strain *E. coli* JW5503, and against wild type *E. coli* in the presence of efflux inhibitor PAβN.

Compd	MIC (μM)[a]	
	E. coli (JW5503)[b]	*E. coli* (ATCC 25922) with PaβN[c]
6	50	25
9	50	50
11	6.25	3.13
19f	50	25
24a	50	n.d.[d]
25c	25	12.5

[a] MIC = minimum inhibitory concentration that inhibited the growth of bacteria by ≥ 90%. Ciprofloxacin was used as a positive control. [b] *E. coli tolC* deletion mutant with defective efflux pump. [c] Phenylalanine-arginine β-naphthylamide; an efflux pump inhibitor. The tests were performed in the presence of 100 μg/mL concentration of PAβN. [d] Not determined.

Conclusions

Despite the huge amount of research work, there are currently no inhibitors of the ATPase activities of DNA gyrase and/or topoisomerase IV in clinical use. To optimise and further develop the promising class of *N*-phenylpyrrolamide DNA gyrase B inhibitors, we have designed and prepared thirty-two new compounds of this class and evaluated their activities against DNA gyrase and topoisomerase IV from *E. coli* and *S. aureus*. The most potent compounds, **11**, **19f** and **19g**, had IC_{50} values against *E. coli* DNA gyrase in the low nanomolar range (IC_{50} < 100 nM). The activities against DNA gyrase from *S. aureus* and against topoisomerase IV were lower. The most potent compound, **11**, had an MIC value of 12.5 μM against Gram-positive *E. faecalis*. Even though the activities against wild type Gram-negative *E. coli* were lower, interesting results were obtained against the efflux pump deficient and against the wild type *E. coli* strains in the presence of efflux pump inhibitor PAβN, where the MICs of compound **11** were 6.25 μM and 3.13 μM, respectively. These results indicate that major factor responsible for the lower activity of this type of compounds against the Gram-negative bacteria is their efflux from the cells. These results should be taken into account in the design of future series of this class of GyrB inhibitors.

Experimental Section

Determination of Inhibitory Activities on *E. coli* and *S. aureus* DNA Gyrase. Inhibitory activities were determined in an assay from Inspiralis on streptavidin-coated 96-well microtiter plates from Thermo scientific Pierce. First, the plates were rehydrated with buffer (20 mM Tris-HCl with pH 7.6, 0.01% w/v BSA, 0.05% v/v Tween 20, 137 mM NaCl) and the biotinylated oligonucleotide was then immobilized. After washing off the unbound oligonucleotide, the enzyme test was performed. The reaction volume of 30 μL in buffer (35 mM Tris × HCl with pH 7.5, 4 mM $MgCl_2$, 24 mM KCl, 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* or *S. aureus*, 0.75 μg of relaxed pNO1 plasmid, and 3 μL solution of the inhibitor in 10% DMSO and 0.008% Tween 20. Reaction solutions were incubated at 37 °C for 30 min. After that, the TF buffer (50 mM NaOAc with pH 5.0, 50 mM NaCl and 50 mM $MgCl_2$) was added to terminate the enzymatic reaction. After additional incubation for 30 min at rt, during which biotin-oligonucleotide-plasmid triplex was formed, the unbound plasmid was washed off using TF buffer and SybrGOLD in T10 buffer (10 mM Tris HCl with pH 8.0 and 1 mM EDTA) was added. The fluorescence was measured with a microplate reader (BioTek Synergy H4, excitation: 485 nm, emission: 535 nm). Initial screening was done at 100 or 10 μM concentration of inhibitors. For the most active inhibitors IC_{50} was determined using seven concentrations of tested compounds. GraphPad Prism software was used to calculate the IC_{50} values. The result is given as the average value of three independent measurements. As the internal standard novobiocin (IC_{50} = 0.168 μM (lit.[21] 0.08 μM) for *E. coli* gyrase and IC_{50} = 0.041 μM (lit.[22] 0.01 μM) for *S. aureus* gyrase) was used.

Determination of Inhibitory Activities on *E. coli* and *S. aureus* Topoisomerase IV. IC_{50} values were determined in an assay from Inspiralis on streptavidin-coated 96-well microtiter plates from Thermo scientific Pierce. First, the plates were rehydrated with buffer (20 mM Tris-HCl with pH 7.6, 0.01% w/v BSA, 0.05% v/v Tween 20, 137 mM NaCl) and biotinylated oligonucleotide was then immobilized. After washing off the unbound oligonucleotide, the enzyme test was performed. The reaction volume of 30 μL in buffer (40 mM HEPES KOH with pH 7.6, 100 mM potassium glutamate, 10 mM magnesium acetate, 10 mM DTT, 1 mM ATP, 0.05 mg/mL albumin) contained 1.5 U of topoisomerase IV from *E. coli* or *S. aureus*, 0.75 μg of pNO1 supercoiled plasmid, and 3 μL solution of the inhibitor in DMSO (10%) and Tween 20 (0.008%). Reaction mixtures were incubated at 37 °C for 30 min and after that, the TF buffer (50 mM NaOAc with pH 5.0, 50 mM NaCl and 50 mM $MgCl_2$) was added to terminate the enzymatic reaction. After additional incubation for 30 min at rt, during which triplex (biotin-oligonucleotide-plasmid) was formed, the unbound plasmid was washed off using TF buffer and SybrGOLD in T10 buffer (10 mM Tris HCl with pH 8.0 and 1 mM EDTA) was added. The fluorescence was measured with a microplate reader (BioTek Synergy H4, excitation: 485 nm, emission: 535 nm). Initial screening was done at 100 or 10 μM concentration of inhibitors. For the most active inhibitors IC_{50} was determined using seven concentrations of tested compounds. GraphPad Prism software was used to calculate the IC_{50} values. The result is given as the average value of three independent measurements. As the internal standard novobiocin (IC_{50} = 11.1 μM (lit.[21] 10 μM) for *E. coli* topoisomerase IV and IC_{50} = 26.7 μM (lit.[21] 20 μM) for *S. aureus* topoisomerase IV) was used.

Determination of Antibacterial Activity. Clinical microbiology control strains of *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923) were obtained from Microbiologics Inc. (St. Cloud, Minnesota, USA). Single-gene knock-out mutant strains of *E. coli,* JW5503 (*tolC* knock-out)[22] and JD17464 (*lpxC* knock-out), were obtained from the NBRP-*E.coli* collection at the National Institute of Genetics (NIG, Japan). To determine antibacterial activity, broth microdilution assays in 96-well

plates were carried out by following the CLSI guidelines.[23] For selected compounds minimum inhibitory concentrations (MIC) were determined by dose-response experiments (reported values are from at least two independent experiments, each with three replicates per concentration). In the experiments with the efflux inhibitor, the assay media was supplemented with 100 µg/mL of Phe-Arg-β-naphthylamide (PAβN; Sigma-Aldrich, Switzerland).

Molecular Modeling.

***Protein and Ligand Preparation*.** 3D compound models were built using ChemBio3D Ultra 16.0.[24] MMFF94 force field[25] was used for the optimization of geometries and partial atomic charges were added. Energy was minimized to less than 0.001 kcal/(mol Å) gradient value. The structure was refined with GAMESS interface using PM3 method, QA optimization algorithm and Gasteiger Hückel charges for all atoms for 100 steps.[24] GOLD Suite v5.4[26-27] was used for molecular docking calculations. GOLD graphical user interface was used for receptor preparation. To the protein hydrogen atoms were added and correct tautomers and protonation states were assigned. Except for HOH614, all water molecules and ligands were deleted from the crystal structure. Amino acid residues within 7 Å around the ligand (PDB entry: 4DUH[19]) were selected as the binding site.

***Ligand Docking*.** Compounds were docked to the defined binding site in 25 independent genetic algorithm (GA) runs by applying different GA parameters (population size = 100, selection pressure = 1.1, number of operations = 100,000, niche size = 2, number of islands = 5, mutation frequency = 95, crossover frequency = 95, migration frequency = 10) and scoring functions (GoldScore, ChemScore, CHEMPLP). The most representative results were obtained using GoldScore as a scoring function. Ligands with RMSD value less than 1.5 Å were joined in clusters and early termination was allowed if the top 3 solutions were within 1.0 Å of the RMSD value. Proposed binding modes of the top 5 highest scored docking poses were evaluated for each ligand and used for graphical representation in PyMOL.[28]

Chemistry. Chemicals were obtained from Apollo Scientific (Stockport, UK), Sigma-Aldrich (St. Louis, MO, USA) and Acros Organics (Geel, Belgium). TLC analyses were performed on Merck 60 F_{254} plates (0.25 mm). Flash column chromatography was performed on silica gel 60 (particle size 240–400 mesh). For HPLC analyses an Agilent Technologies 1100 instrument (Agilent Technologies, CA, USA) with a G1313A autosampler, a G1316A thermostat and a G1365B UV-Vis detector was used. An Agilent Eclipse Plus C18 column was used (5 µm, 4.6 × 150 mm) with a flow rate of 1.0 mL/min. Melting points were determined on a hot-stage microscope from Reichert and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz on a Bruker AVANCE III 400 spectrometer (Bruker Corporation, MA, USA) as [D_6]DMSO or $CDCl_3$ solutions. For the IR measurements, a Thermo Nicolet Nexus 470 ESP FT-IR spectrometer (Thermo Fisher Scientific, MA, USA) was used. Mass spectra were recorded on an Advion CMS spectrometer (Advion Inc., Ithaca, USA) or VG Analytical Autospec Q mass spectrometer (Fisons, VG Analytical, Manchester, UK). All tested compounds were more than 95% pure as established by HPLC.

Synthetic Procedures.

Methyl (4-nitrobenzoyl)glycinate (3).[16b] To a suspension of glycine methyl ester hydrochloride (**2**) (0.677 g, 5.39 mmol) and potassium carbonate (2.23 g, 16.17 mmol) in acetonitrile (50 mL) cooled to 0 °C, a solution of 4-nitrobenzoil chloride (**1**) (1.00 g, 5.39 mmol) in acetonitrile (10 mL) was added dropwise. After 15 h of stirring at rt, the solvent was evaporated under reduced pressure, the residue dissolved in ethyl acetate (100 mL) and washed with water (2 × 20 mL) and brine (20 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure to afford **3** (929 mg) as yellow crystals. Yield 72% (0.929 g).

General procedure A. Synthesis of Compounds 4, 17a-b, 17d-e and 27 (with 4 as an Example). Compound **3** (0.920 g, 3.86 mmol) was dissolved in THF (50 mL), Pd/C (200 mg) was added and the reaction mixture was stirred under hydrogen atmosphere for 3 h. The catalyst was filtered off and the solvent removed under reduced pressure to obtain **4** (0.796 g) as white crystals.

Methyl (4-aminobenzoyl)glycinate (4). White crystals; yield 99% (0.796 g); mp: 129-131 °C (130-131 °C, lit.[29]).

General procedure B. Synthesis of Compounds 6, 18d-h and 24b-d (with 6 as an Example). To a suspension of 3,4-dichloro-5-methylpyrrole-2-carboxylic acid (280 mg, 1.44 mmol) in anhydrous dichloromethane (16 mL) oxalyl chloride (2 M solution in dichloromethane, 2.20 mL, 4.32 mmol) was added and the mixture stirred at rt for 15 h. The solvent was removed under reduced pressure, fresh anhydrous dichloromethane (4 mL), pyridine (2 mL) and compound **4** (300 mg, 1.44 mmol) were added and the reaction mixture was stirred at rt for 15 h. The solvent was removed under reduced pressure, the residue dissolved in ethyl acetate (20 mL) and washed with 1 M HCl (2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. To the residue ether (15 mL) was added, the obtained suspension was sonicated, filtered off, washed with ether (2 × 5 mL) and dried to afford **6** (245 mg) as a brown solid.

Methyl (4-(3,4-dichloro-5-methyl-1*H*-pyrrole-2-carboxamido)benzoyl)glycinate (6). Brown solid; yield 42% (245 mg); mp: >300 °C.

General procedure C. Synthesis of Compounds 7, 15a-c, 19e-f and 25c (with 7 as an Example). To a stirred solution of compound **6** (48 mg, 0.12 mmol) in THF (15 mL) 1 M NaOH (240 µL, 0.24 mmol) was added. The mixture was stirred at rt for 15 h, concentrated under reduced pressure and the residue was acidified with 1 M HCl to pH 1. The product was extracted with ethyl acetate (2 × 20 mL), the combined organic phases washed with water (2 × 20 mL) and brine (20 mL), dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. To the residue ether (5 mL) was added, the obtained suspension was sonicated, filtered off, washed with ether (2 × 3 mL) and dried to give **7** (25 mg) as a brown solid.

(4-(3,4-Dichloro-5-methyl-1*H*-pyrrole-2-carboxamido)benzoyl)glycine (7). Brown solid; yield 52% (25 mg); mp: >250 °C.

General procedure D. Synthesis of Compounds 8, 9 and 20 (with 8 as an Example). To the solution of compound **5** (96 mg, 0.209 mmol) in absolute ethanol (10 mL) hydrazine monohydrate (101 µL, 2.09 mmol) was added and the mixture was stirred at reflux for 15 h. The solvent was evaporated under reduced pressure, to the residue absolute ethanol (10 mL) was added, the obtained precipitate was filtered off and dried to give **8** (25 mg) as a white solid.

4,5-Dibromo-*N*-(4-((2-hydrazineyl-2-oxoethyl)carbamoyl)phenyl)-1*H*-pyrrole-2-carboxamide (8). White solid; yield 26% (25 mg); mp: 265-266 °C.

General procedure E. Synthesis of Compounds 10 and 21 (with 10 as an Example). The suspension of compound **8** (108 mg, 0.235 mmol) and 1,1'-carbonyldiimidazole (CDI, 57 mg, 0.353 mmol) in 1,4-dioxane (15 mL) was stirred at 101 °C for 15 h upon which a clear solution formed. The solvent was removed under reduced pressure and the oily residue was purified with flash column chromatography using dichloromethane/methanol (20/1) as an eluent to afford **10** (25 mg) as a white solid.

4,5-Dibromo-*N*-(4-(((5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)methyl)carbamoyl)phenyl)-1*H*-pyrrole-2-carboxamide (10). White solid, yield 22% (25 mg); mp: 261-263 °C.

General procedure F. Synthesis of Compounds 13 and 23a-b (with 13 as an Example). To the solution of 3-hydroxy-4-nitrobenzoic acid (**12**) (10.00 g, 55 mmol) in methanol (500 mL) cooled on ice bath thionyl chloride (32.20 mL, 165 mmol) was added dropwise. The mixture was stirred at rt for 15 h. The solvent was evaporated under reduced pressure to obtain **13** (10.35 g) as a yellow solid.

Methyl 3-hydroxy-4-nitrobenzoate (13). Yellow solid, yield 97% (10.53 g); mp: 87-91 °C (86-88 °C, lit.[30]).

Methyl 3-methoxy-4-nitrobenzoate (14a).[31] To a stirred suspension of compound **13** (3.43 g, 17.4 mmol) and potassium carbonate (3.61 g, 26.1

mmol) in *N,N*-dimethylformamide (30 mL) methyl iodide (2.20 mL, 34.8 mmol) was added dropwise. The mixture was stirred at rt for 15 h, concentrated under reduced pressure, the residue was dissolved in ethyl acetate (20 mL) and the organic phase was washed with water (2 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to obtain **14a** (3.01 g) as a pale yellow solid. Yield 82% (3.01 g); mp: 90-93 °C.

3-Methoxy-4-nitrobenzoic acid (15a). Synthesized according to General procedure C with methanol (50 mL) as solvent. Pale yellow solid; yield 86 % (2.38 g); mp: 229-233 °C (230-233 °C, lit.[32]).

General procedure G. Synthesis of Compounds 16a-f (with 16a as an Example). To a solution of compound **15a** (2.32 g, 11.8 mmol) and TBTU (4.90 g, 15.3 mmol) in dichloromethane (80 mL) *N*-methylmorpholine (3.9 mL, 35.2 mmol) was added and the mixture was stirred at rt for 15 min. Glycine methyl ester hydrochloride (**2**) (1.62 g, 12.9 mmol) was added and the mixture was stirred at rt for 2 h. The solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate (20 mL) and the organic phase was washed with water (2 × 20 mL), saturated aqueous $NaHCO_3$ solution (2 × 20 mL), 1 M HCl (2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to afford **16a** (2.27 g) as a yellow solid.

Methyl (3-methoxy-4-nitrobenzoyl)glycinate (16a). Yellow solid; yield 72% (2.27 g); mp: 102-105 °C.

Methyl (4-amino-3-methoxybenzoyl)glycinate (17a). Synthesized according to General procedure A with methanol (20 mL) as solvent. Brown oil; yield 99% (1.845 g).

General procedure H. Synthesis of Compounds 17c and 17f (with 17c as an Example). A mixture of compound **16c** (143 mg, 0.42 mmol) and $SnCl_2$ (394 mg, 2.08 mmol) in ethyl acetate (2 mL) and ethanol (2 mL) was stirred at 55 °C for 24 h. The mixture was poured slowly into an aqueous $NaHCO_3$ solution (20 mL) and stirred vigoroursly for 2 h, then extracted with EtOAc (20 mL) and washed with brine (20 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The residue was purified with flash column chromatography using dichloromethane/methanol (30/1 → 20/1) as an eluent to give compound **17c** (58 mg) as a brown oil.

Methyl (4-amino-3-(benzyloxy)benzoyl)glycinate (17c). Brown oil; yield 44% (58 mg).

General procedure I. Synthesis of Compounds 18a-c (with 18a as an Example). To a suspension of 4,5-dibromopyrrole-2-carboxylic acid (296 mg, 1.10 mmol) and TBTU (405 mg, 1.26 mmol) in dichloromethane (10 mL) *N*-methylmorpholine (350 µL, 3.15 mmol) was added and the suspension was stirred at rt for 30 min upon which a clear solution formed. Compound **17a** (145 mg, 1.05 mmol) and DMAP (14 mg, 0.115 mmol) were added and the mixture was stirred at 60 °C for 15 h. The solvent was removed under reduced pressue, to the residue ethyl acetate (10 ml) and water (10 mL) were added and the organic phase was washed with aqueous $NaHCO_3$ solution (10 mL), 1 M HCl (10 mL) and brine (2 × 10 mL), dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. The crude product was purified with flash column chromatography using ethyl acetate/petroleum ether (1/1) as an eluent to obtain a white solid which was further purified by sonicating with ether (25 mL), filtering off the precipitate and drying to give **18a** (40 mg) as a white solid.

Methyl (4-(4,5-dibromo-1*H*-pyrrole-2-carboxamido)-3-methoxybenzoyl)glycinate (18a). White solid; yield 10% (40 mg); mp: 230-233 °C.

General procedure J. Synthesis of Compounds 19a-d and 19g (with 19a as an Example). To a stirred solution of compound **18a** (34 mg, 0.070 mmol) in methanol (10 mL) 2 M NaOH (110 µL, 0.210 mmol) was added. The mixture was stirred at rt for 15 h, concentrated under reduced pressure and the residue was acidified with 1 M HCl to pH 1. The product was extracted with ethyl acetate (2 × 10 mL), the combined organic phases washed with water (2 × 10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure to afford **19a** (18 mg) as a white solid.

(4-(4,5-Dibromo-1*H*-pyrrole-2-carboxamido)-3-methoxybenzoyl)glycine (19a). White solid; yield 94% (18 mg); mp: 272-275 °C.

4,5-Dibromo-*N*-(4-((2-hydrazineyl-2-oxoethyl)carbamoyl)-2-isopropoxyphenyl)-1*H*-pyrrole-2-carboxamide (20). Synthesized according to General procedure D with the addition of further 10 eq. of hydrazine monohydrate after 15 h (34 µL, 0.700 mmol) and stirring at reflux for additional 48 h. The product was isolated without evaporating the solvent but with filtering off the white precipitate (**20**, 34 mg) formed during reaction. White solid; yield 92% (34 mg); mp: 253-257 °C.

4,5-Dibromo-*N*-(2-isopropoxy-4-(((5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)methyl)carbamoyl)phenyl)-1*H*-pyrrole-2-carboxamide (21). Synthesized according to General procedure E and additionally purifying the crude product by adding ether (10 mL) to the crude product, sonicating the obtained suspension, filtering off the solid, washing with ether (2 × 5 mL) and drying to give **21** (12 mg) as a white solid. Yield 39% (12 mg); mp: 160-162 °C.

Methyl 4-aminobenzoate (23a). Synthesized according to General procedure F with heaing the reaction mixture at 70 °C. After the completion of the reaction, the solvent was evaporated under reduced pressure, the residue was dissolved in ethyl acetate (120 mL) and washed with aqueous $NaHCO_3$ solution (3 × 40 mL) and brine (3 × 40 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to obtain **23a** (4.88g) as off-white crystals. Yield 89% (4.88 g); mp: 106-107 °C (107-110 °C, lit).

Methyl 4-(4,5-dibromo-1*H*-pyrrole-2-carboxamido)benzoate (24a). To a suspension of 4,5-dibromopyrrole-2-carboxylic acid (268 mg, 0.997 mmol) in anhydrous dichloromethane (9 mL) oxalyl chloride (392 µL, 4.48 mmol) was added and the mixture stirred at rt for 15 h. The solvent was removed under reduced pressure, fresh anhydrous dichloromethane (9 mL), pyridine (4.5 mL) and compound **23a** (126 mg, 0.833 mmol) were added and the reaction mixture stirred at rt for 15 h. The solvent was removed under reduced pressure, to the residue ethyl acetate (15 mL) and water (10 mL) were added. The undissolved solid was filtered off, phases were separated and the organic phase was washed with aqueous $NaHCO_3$ solution (3 × 10 mL) and brine (2 × 10 mL), dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. To the crude product methanol (10 mL) was added, the obtained suspension was sonicated, heated, filtered off, washed with methanol (2 × 5 mL) and dried to give **24a** (96 mg) as a pale brown solid. Yield 29% (96 mg); mp: 230-233 °C.

General procedure K. Synthesis of Compounds 25a-b and 29 (with 25a as an example). To a stirred solution of compound **24a** (70 mg, 0.174 mmol) in a mixture of methanol and THF (3.5:1, 9 mL) 1 M NaOH (696 µL, 0.696 mmol) was added. The mixture was stirred at rt for 15 h. Additional 4 eq. of 1 M NaOH (696 µL, 0.696 mmol) were added and the mixture was stirred at 60 °C for 5 h, concentrated under reduced pressure, the basic water phase was washed with ethyl acetate (10 mL) and then acidified with 1 M HCl to pH 1. The product was extracted with ethyl acetate (3 × 15 mL), the combined organic phases washed with water (3 × 10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. To the residue ether (15 mL) was added, the obtained suspension was sonicated, filtered, washed with ether (5 mL) and dried to give **25a** (42 mg) as a pale brown solid.

4-(4,5-Dibromo-1*H*-pyrrole-2-carboxamido)benzoic acid (25a). Synthesized according to General procedure K. Pale brown solid; yield 62% (42mg); mp: >300 °C.

Methyl 2-hydroxy-4-(4-nitrobenzamido)benzoate (26). The solution of 4-nitrobenzoyl chloride (**1**) (0.550 g, 2.96 mmol) and methyl 4-amino-3-hydroxybenzoate (**23b**) (495 mg, 2.96 mmol) in anhydrous dichloromethane (20 mL) and pyridine (10 mL) was stirred at rt for 15 h. The solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate (40 mL) and washed with water (2 × 15 mL), aqueous $NaHCO_3$ solution (3 × 15 mL) and brine (2 × 10 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. To the crude product ether (15 mL) was added, the obtained suspension sonicated, filtered off, washed with ether (2 × 5 mL) and dried to obtain **26** (641 mg) as a white solid. Yield 68% (641 mg), mp: 200-202 °C.

Methyl 4-(4-aminobenzamido)-2-hydroxybenzoate (27). Synthesized according to General procedure A. After evaporation of the solvent, ether (20 mL) was added to the residue, the obtained suspension sonicated,

filtered, washed with ether (2 × 5 mL) and dried to afford **27** (458 mg) as white solid. Yield 83% (458 mg); mp: 207-210 °C.

Methyl 4-(4-(4,5-dibromo-1*H*-pyrrole-2-carboxamido)benzamido)-2-hydroxybenzoate (28). To a suspension of 4,5-dibromopyrrole-2-carboxylic acid (338 mg, 1.26 mmol) in anhydrous dichloromethane (10 mL) oxalyl chloride (2 M solution in dichloromethane, 3.8 mL, 7.55 mmol) was added and the mixture was stirred at rt for 15 h. The solvent was removed under reduced pressure, fresh anhydrous dichloromethane (10 mL), pyridine (5 mL) and compound **27** (290 mg, 1.01 mmol) were added and the reaction mixture was stirred at rt for 15 h. The solvent was removed under reduced pressure, to the residue ethyl acetate (30 mL) and water (15 mL) were added. The undissolved solid was filtered off, phases were separated and the organic phase was washed with water (2 × 10 mL), aqueous $NaHCO_3$ solution (3 × 10 mL) and brine (2 × 10 mL), dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The solid residue after extraction was combined with the filtered solid and acetonitrile (20 mL) was added, the obtained suspension was sonicated, heated, filtered off, washed with acetonitrile (2 × 5 mL) and dried to give **28** (326 mg) as an off-white solid. Yield 60% (326 mg); mp: >300 °C.

4-(4-(4,5-Dibromo-1*H*-pyrrole-2-carboxamido)benzamido)-2-hydroxybenzoic acid (29). Synthesized according to General procedure K without washing the residue with ethyl acetate before acidifying it. To the crude product methanol (20 mL) was added, the obtained suspension was sonicated, filtered off, washed with methanol (2 × 10 mL) and ether (2 × 10 mL) and dried to give **29** (50 mg) as a pale yellow solid. Yield 64% (50 mg); mp: >300 °C.

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Abbreviations

ATCC, American type culture collection; ATR, attenuated total reflectance; CDI, 1,1'-carbonyldiimidazole; CFU, colony-forming unit; CLSI, Clinical and Laboratory Standards Institute; DIAD, diisopropyl azodicarboxylate; DMAP, 4-dimethylaminopyridine; DTT, dithiothreitol; GyrA, DNA gyrase A; GyrB, DNA gyrase B; MH, Mueller Hinton; NMM, *N*-methylmorpholine; ParC, topoisomerase IV subunit A; ParE, topoisomerase IV subunit B; RA, residual activity; TBTU, *N,N,N',N'*-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate; topo IV, topoisomerase IV

Conflict of interest

The authors declare no conflict of interest.

Keywords: antibacterials • DNA gyrase • GyrB • inhibitors • pyrrolamide

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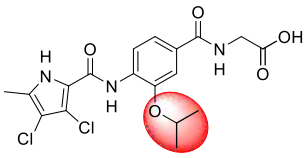

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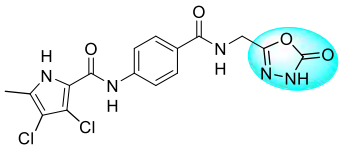

11, *E. coli* gyrase: IC_{50} = 80 nM

New *N*-phenylpyrrolamides were prepared as DNA gyrase and topoisomerase IV inhibitors and their structure-activity relationships were studied. Most potent compounds displayed IC_{50} values lower than 100 nM against *E. coli* gyrase. Minimum inhibitory concentration of 12.5 µM was determined for **11** against Gram-positive *E. faecalis*. MICs against efflux pump deficient and against the *E. coli* strains in the presence of efflux pump inhibitor were studied.