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Communications

Dual *Escherichia coli* DNA Gyrase A and B Inhibitors with Antibacterial Activity

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The emergence of multidrug-resistant bacteria is a global health threat necessitating the discovery of new antibacterials and novel strategies for fighting bacterial infections. We report first-in-class DNA gyrase B (GyrB) inhibitor/ciprofloxacin hybrids that display antibacterial activity against *Escherichia coli.* Whereas DNA gyrase ATPase inhibition experiments, DNA gyrase supercoiling assays, and in vitro antibacterial assays suggest binding of the hybrids to the *E. coli* GyrA and GyrB subunits, an interaction with the GyrA fluoroquinolone-binding site seems to be solely responsible for their antibacterial activity. Our results provide a foundation for a new concept of facilitating entry of nonpermeating GyrB inhibitors into bacteria by conjugation with ciprofloxacin, a highly permeable GyrA inhibitor. A hybrid molecule containing GyrA and GyrB inhibitor parts entering the bacterial cell would then elicit a strong antibacterial effect by inhibition of both the GyrA and GyrB subunits of DNA gyrase and potentially slow bacterial resistance development.

Bacterial DNA gyrase, a type IIa topoisomerase responsible for ATP-driven introduction of negative supercoils into DNA,[1] is a well-established target of antibacterials.[2] Whereas fluoroquinolones targeting the GyrA subunit of a heterodimeric A_2B_2 enzyme are widely used to treat infections with gram-positive and gram-negative bacteria,[2b,d,3] GyrB inhibitors interfering with ATP binding to subunit B have not advanced into the clinic despite intensive research over the last 50 years after the discovery of novobiocin as the first ATP-competitive GyrB inhibitor in the 1960s.[2f–h] We recently reported several structural types of low-nanomolar pyrrole-2-carboxamide GyrB inhibitors[4–6] and established the binding mode of 2-((2-(4,5-dibromo-1*H*-pyrrole-2-carboxamido)benzo[*d*]thiazol-6-yl) amino)-2-oxoacetic acid (**1a**; IC_{50} *E. coli* = 58 nM),[6] a 4,5-dibromo-pyrrole analog of **1b** (IC_{50} *E.coli* = 43 nM),[7] to GyrB from *E. coli* with X-ray crystallography. However, most of these inhibitors were devoid of in vitro antibacterial activity because of insufficient permeation and/or extrusion by bacterial efflux pumps.[6,7]

Dual targeting of GyrB and structurally similar topoisomerase IV ParE subunits has been suggested to prolong the onset of resistance in bacteria because mutations at both essential sites are less probable than single mutations at GyrB or ParE ATP-binding sites.[2g,8a] This observation evoked our interest in the design and preparation of the first dual inhibitors of GyrA and GyrB that could open new avenues for DNA gyrase inhibition and fighting bacterial resistance.[8b]

Designed multiple ligands can be obtained by linking, merging or fusing individual pharmacophores in a way tolerated by respective targets.[9] Several 4-quinolone hybrids with trimethoprim,[10a] linezolid,[10b] and tobramycin[10c] were obtained, and these as well as other studies[11] demonstrated that moieties of different sizes attached to the piperazine *NH* group of ciprofloxacin are well tolerated, with retention of DNA gyrase inhibition and antibacterial activity. Furthermore, exposure of the terminal carboxylate group of our GyrB inhibitors to bulk water observed in the crystal structure of the 4,5-dibromo-1*H*-pyrrole-2-carboxamide inhibitor **1a** bound to *E. coli* GyrB and simulated by docking for a reversed inhibitor **1d** (IC_{50} *E.coli* = 38 nM)[6] should allow functionalization in this region without losing DNA gyrase B inhibitory activity (positions in ciprofloxacin and in **1a**–**e** which should tolerate substitution without loss of DNA gyrase inhibition are colored red; Figure 1).

The presence of carboxylate groups in **1a**, **1b** and **1d** did not seem critical for GyrB inhibition since the *N*-acetyl analog of **1b** (**1c**; IC_{50} *E. coli* = 9 nM) and its reversed analog *N*-(2-acetamido-benzo[*d*]thiazol-6-yl)-3,4-dichloro-5-methyl-1*H*-pyrrole-2-carboxamide (**1e**; IC_{50} *E. coli* = 66 nM) [7] also showed good inhibition of DNA gyrase. Because DNA gyrase A-inhibiting fluoroquinolones and our pyrrole-2-carboxamide GyrB inhibitors **1a**–**e** do not share common structural features that would allow fusion of both pharmacophores, we decided to use a merging strategy to combine the GyrA inhibitor ciprofloxacin and our GyrB inhibitors in the same molecule and

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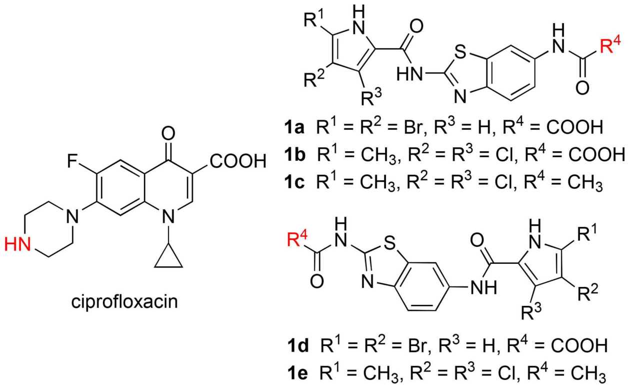
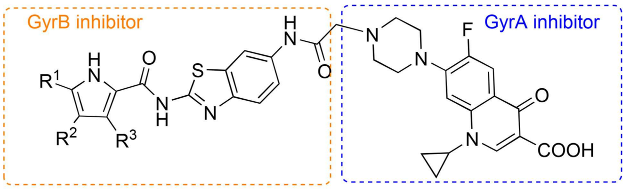
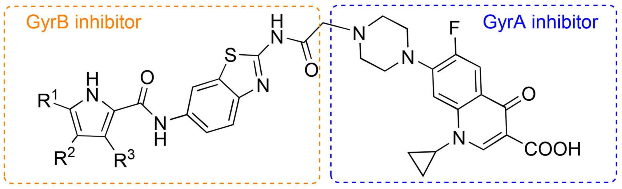


1a $R^1 = R^2 = Br, R^3 = H, R^4 = COOH$
1b $R^1 = CH_3, R^2 = R^3 = Cl, R^4 = COOH$
1c $R^1 = CH_3, R^2 = R^3 = Cl, R^4 = CH_3$
1d $R^1 = R^2 = Br, R^3 = H, R^4 = COOH$
1e $R^1 = CH_3, R^2 = R^3 = Cl, R^4 = CH_3$

Figure 1. Ciprofloxacin and GyrB inhibitors **1a**–**e**.

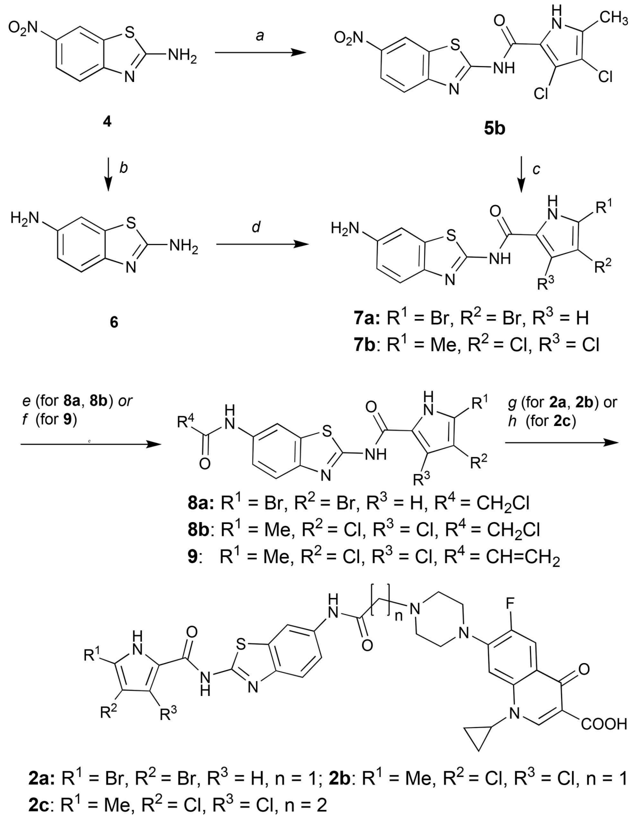


2a: $R^1 = Br, R^2 = Br, R^3 = H$; **2b**: $R^1 = Me, R^2 = Cl, R^3 = Cl$



3a: $R^1 = Br, R^2 = Br, R^3 = H$; **3b**: $R^1 = Me, R^2 = Cl, R^3 = Cl$

Figure 2. GyrA/GyrB inhibitor hybrids **2a**–**b** and **3a**–**b**.



7a: $R^1 = Br, R^2 = Br, R^3 = H$
7b: $R^1 = Me, R^2 = Cl, R^3 = Cl$

8a: $R^1 = Br, R^2 = Br, R^3 = H, R^4 = CH_2Cl$
8b: $R^1 = Me, R^2 = Cl, R^3 = Cl, R^4 = CH_2Cl$
9: $R^1 = Me, R^2 = Cl, R^3 = Cl, R^4 = CH{=}CH_2$

2a: $R^1 = Br, R^2 = Br, R^3 = H, n = 1$; **2b**: $R^1 = Me, R^2 = Cl, R^3 = Cl, n = 1$
2c: $R^1 = Me, R^2 = Cl, R^3 = Cl, n = 2$

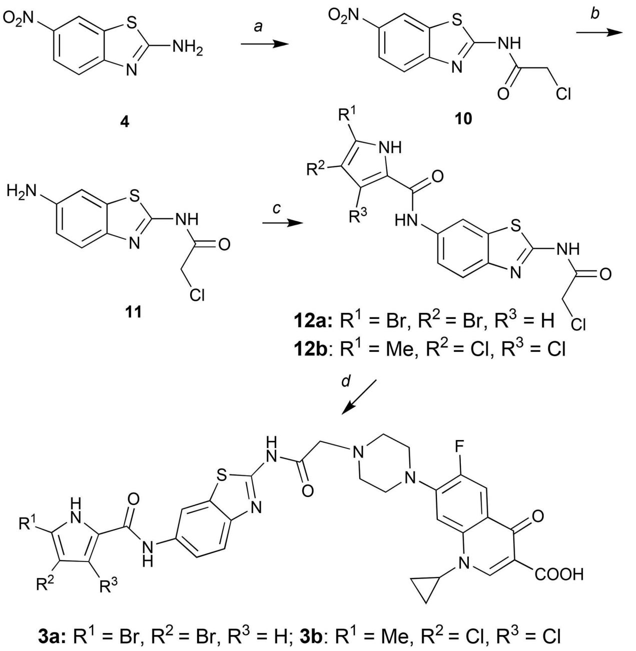
Scheme 1. (a) 3,4-dichloro-5-methyl-1*H*-pyrrole-2-carbonyl chloride, toluene, reflux, 18 h; (b) H_2, Pd/C (10%), 1.5 bar, EtOH, rt, 18 h; (c) $SnCl_2 \times 2\ H_2O$, EtOH, reflux, 24 h; (d) 4,5-dibromopyrrole-2-yl-trichloromethyl ketone, K_2CO_3, DMF, 80 °C, 20 h; (e) $ClCH_2COCl$, Et_3N, dichloromethane, rt, 24 h; (f) acryloyl chloride, K_2CO_3, THF, ice bath, 0.5 h; (g) ciprofloxacin, KI, Na_2CO_3, CH_3CN, reflux, 4 h; (h) ciprofloxacin, CH_3CN/AcOH, reflux, 72 h.

obtain hybrid compounds **2** and **3** containing both GyrA- and GyrB-inhibiting pharmacophores (Figure 2). Docking of compounds **2b** and **3b** to *E. coli* GyrB (PDB: 4DUH)[12a] and to *S. aureus* GyrA (PDB: 5CDQ)[12b,c] (data not shown) suggested that they could bind to both subunits since there was no steric clash with GyrA or GyrB binding site residues. We further anticipated that merging our GyrB inhibitors with highly permeable ciprofloxacin would facilitate entry of the hybrid GyrA/GyrB inhibitors and make them active against bacteria.

The hybrids **2a** and **2b** were prepared (Scheme 1) by acylation of the amines **7a** and **7b** with chloroacetyl chloride and subsequent substitution of chlorine in **8a** and **8b** with ciprofloxacin. Amine **7a** was obtained by selective *N2* acylation of the diamine **6** with 4,5-dibromopyrrole-2-yl trichloromethyl ketone, whereas the amine **7b** was prepared by acylation of 6-nitrobenzo[*d*]thiazol-2-amine (**4**) with 3,4-dichloro-5-methyl-pyrrole-2-carboxylic acid chloride and subsequent reduction of the nitro group in the obtained **5b**.

For the synthesis of the isomeric hybrids **3a** and **3b** (Scheme 2) bearing a 1*H*-pyrrole-2-carboxamido moiety in position 6, **4** was acylated with chloroacetyl chloride,[13] and the obtained nitro derivative **10** was reduced to the 6-aminobenzothiazole derivative **11** by catalytic hydrogenation over 10% Pd on charcoal in ethyl acetate. Attempts to reduce the nitro group of **10** with tin(II) chloride in ethanol or with sodium sulfide nonahydrate were not successful since, in both cases, reduction of the nitro group was accompanied by amide bond cleavage, and benzo[*d*]thiazole-2,6-diamine was isolated as the main product. The amine **11** was coupled with 4,5-dibromopyrrole-2-carbonyl chloride or 3,4-dichloro-5-methyl-pyrrole-2-carbonyl chloride in the presence of triethylamine in dioxane, and the resulting intermediates **12** underwent nucleophilic substitution with unprotected ciprofloxacin to produce the target inhibitors **3a** and **3b**. The final compounds **2a**–**b** and **3a**–**b** were purified on Sephadex LH-20 to remove traces of ciprofloxacin.

A DNA gyrase supercoiling assay[4–6] demonstrated weaker inhibition of *E. coli* DNA gyrase by the hybrids **2a**–**b** and **3a**–**b** (IC_{50} values from 0.17 to 6.2 μM) than by the GyrB inhibitors **1a**–**e** (IC_{50} values from 9 to 66 nM) and slightly weaker inhibition than by the GyrA inhibitor ciprofloxacin (Table 1). The hybrids **2a** and **2b** with ciprofloxacin bound to position 6 of a benzothiazole core were better (sub-micromolar) inhibitors than **3a** and **3b** that possessed low micromolar *E. coli* gyrase



Scheme 2. (a) $ClCH_2COCl$, Et_3N, dichloromethane, rt, 12 h; (b) H_2, Pd/C (10%), EtOAc, rt, 24 h; (c) 4,5-dibromo-1*H*-pyrrole-2-carboxylic acid chloride or 3,4-dichloro-5-methyl-1*H*-pyrrole-2-carboxylic acid chloride, Et_3N, dioxane, rt, 18 h; (d) ciprofloxacin, KI, Na_2CO_3, CH_3CN, reflux, 12 h.

Table 1. Inhibition of *E. coli* DNA gyrase supercoiling and ATPase activities

Compound	MW [Da]	Supercoiling IC_{50} [μM]	ATPase IC_{50} [μM]
1a	485.85	0.058 ± 0.031	n.d.
1b	413.23	0.043 ± 0.034	n.d.
1c	383.25	0.0095 ± 0.0025	n.d.
1e	383.25	0.066 ± 0.008	n.d.
2a	787.45	0.91 ± 0.37	0.27 ± 0.01
2b	712.58	0.17 ± 0.03	0.055 ± 0.005
2c	726.61	0.16 ± 0.01	0.054 ± 0.025
3a	787.45	6.2 ± 1.7	0.35 ± 0.18
3b	712.58	5.2 ± 2.0	0.32 ± 0.19
8a	492.57	2.3 ± 0.2	n.d.
8b	417.70	0.18 ± 0.02	n.d.
12a	492.57	0.43 ± 0.02	0.38 ± 0.09
12b	417.70	0.33 ± 0.02	n.d.
novobiocin	612.62	0.17 ± 0.01	0.16 ± 0.05
ciprofloxacin	313.35	0.12 ± 0.02	n.d.

[n.d.] not determined.

IC_{50} values. Whereas increasing the size of the 6-(*N*-acetylamino) substituent of **1c**, producing **8b** (*N*-chloroacetyl), and **2b** in each case resulted in a ca. 20-fold increase in IC_{50} irrespective of the size of the *N*-substituent, increasing the size of the 2-(*N*-acylamino) substituent in the series **1e** (*N*-acetyl)→**12b** (*N*-chloroacetyl)→**3b** clearly increased the IC_{50} values at each step, increasing in total 78-fold from **1e** to **3b**. This observation indicated that appending the ciprofloxacin moiety to position 6, resulting in the compounds **2a** and **2b**, is better tolerated than its attachment to position 2, producing the compounds **3a** and **3b**. Because the observed inhibition of *E. coli* DNA gyrase in the supercoiling assay could not be undoubtedly attributed to inhibition of the GyrA or GyrB subunit, a DNA gyrase ATPase assay (Table 1) was performed to detect binding of the hybrid compounds to *E. coli* GyrB. Further, MIC assays in the *E. coli* wild-type strain and in two strains with a mutated GyrB or mutated fluoroquinolone-binding sites were performed to assess the effect of the hybrids on *E. coli* (Table 2).

All four hybrid compounds (**2a**–**b** and **3a**–**b**) displayed potent activity against the *E. coli* strains ATCC 25922 and K-12 MG1655 in the presence of the efflux pump inhibitor PaβN (MIC values between 130 and 439 ng/mL). MIC values in the absence of PaβN were in the range 1481–3333 ng/mL in the strains ATCC 25922 and K-12 MG1655, which indicates that the hybrids **2a**–**b** and **3a**--**b** are not intensively effluxed in *E. coli.* Whereas the hybrids were not active against either gram-negative *P. aeruginosa* ATCC 15692 and *A. baumannii* ATCC BAA1605 or against gram-positive *S. aureus* ATCC 700699 and *Enterococcus faecalis* ATCC 29212 strains, the antibacterial activity of the hybrids in the absence of an efflux pump inhibitor was confirmed against gram-negative *Shigella flexneri* HNCMB 20018, *Shigella sonnei* HNCMB 25021 (MICs between 1481 and 3333 ng/mL) and against *Klebsiella pneumoniae* ATCC 10031 (MICs between 293 and 658 ng/mL) as well as against gram-positive *Listeria monocytogenes* ATCC 19111 (MICs between 87 and 2863 ng/mL). These results indicate good penetration of the studied GyrA/GyrB-inhibiting hybrids through the bacterial cell wall and provide evidence that their efflux in *E. coli* and in other tested bacteria is not intensive and not detrimental for their antibacterial activity. However, the hybrid molecules **2a**–**b** and **3a**–**c** did not show any reduction in antibacterial activity in the *E. coli* K-12 MG1655 GyrB R136C mutant and suffered a substantial loss of antibacterial activity toward the *E. coli* mutant in which the fluoroquinolone-binding site was abolished by four mutations (*E. coli* K-12 MG1655 GyrA S83L, D87N; ParC S80I, E84G). This finding indicates that the observed antibacterial activity of the hybrid molecules **2a**–**b** and **3a**–**b** is mainly due to interaction with GyrA and/or ParC and not with the GyrB subunit. Although the ATPase assay demonstrated that the hybrids **2a**–**b** and **3a**–**b** inhibit the GyrB subunit, the inhibition is obviously too weak (IC_{50} values between 0.055 and 0.35 μM) to result in antibacterial activity. Assuming that increasing the flexibility of the linker between the GyrB and GyrA inhibitor moieties could increase GyrB inhibition, we synthesized the hybrid **2c**, a homolog of the hybrid **2b** that was most potent in the supercoiling and ATPase assays, possessing an additional methylene group between the ciprofloxacin and GyrB inhibitor moieties (Scheme 1). To this end, the amine **7b** was acylated with acryloyl chloride in the presence of potassium carbonate in tetrahydrofuran, and the obtained acrylamide **9** was reacted with ciprofloxacin to produce the compound **2c** with an elongated linker. However, the hybrid **2c** displayed behavior similar to that of **2b** in the supercoiling assay ($IC_{50} = 0.16$ μM), ATPase assay ($IC_{50} = 0.054$ μM) and MIC assays in *E. coli*, demonstrating that elongating the linker by one C-atom did not increase GyrB inhibition and antibacterial activity against the wild-type and mutated *E. coli* strains.

Table 2. MIC values (ng/mL) of the hybrids **2a**, **2b**, **2c**, **3a** and **3b**.[a]

Bacterium	**2a**	**2b**	**2c**	**3a**	**3b**	ICCP[b]
E. coli ATCC 25922+PaβN	130	130	500	130	130	4.4
E. coli K-12 MG1655+PaβN	439	439	250	293	293	6.6
E. coli ATCC 25922	2222	1481	439	1481	1481	9
E. coli K-12 MG1655	3333	1481	658	1481	2222	12
A. baumannii ATCC BAA1605	n.a.	n.a.	n.a.	n.a.	n.a.	<173
P. aeruginosa ATCC 15692	n.a.	n.a.	n.a.	n.a.	n.a.	<173
S. flexneri HNCMB 20018	3333	1481	658	1481	2222	12
S. sonnei HNCMB 25021	3333	1481	658	1481	2222	12
K. pneumoniae ATCC 10031	658	293	195	293	293	4
E. cloacae ATCC 13047	5000	2222	1481	2222	3333	26
S. aureus ATCC 700699	n.a.	n.a.	n.a.	n.a.	n.a.	15000
Enterococcus faecalis ATCC 29212	n.a.	n.a.	n.a.	n.a.	n.a.	390
Listeria monocytogenes ATCC 19111	390	87	195	2863	878	585
E. coli GyrB R136C+PaβN	439	439	250	293	293	6.6
E. coli K-12 MG1655 GyrA S83L, D87N; ParC S80I, E84G+PaβN	5000	5000	16000	5000	20000	33000

[a] n.a.: MIC $\geq$ 15000 ng/mL. [b] Ciprofloxacin.

Table 3. Frequency of resistance against a 4×MIC concentration of the hybrids **2a**–**c** and **3a**–**b** and ciprofloxacin.

Bacterium	Frequency of resistance $(\times 10^{-9})$					
	2a	**2b**	**2c**	**3a**	**3b**	CP[a]
E. coli K-12 MG1655	1.30	2.60	3.13	3.07	4.43	1.56
E. coli ATCC 25922	11.2	4.17	11.9	4.88	13.9	6.31

[a] Ciprofloxacin.

To investigate bacterial evolvability toward the dual inhibitors, as many as 10^{10} wild-type *E. coli* cells were exposed to a 4×MIC concentration of the tested compounds in a standard frequency of resistance assay.[14] The two tested *E. coli* strains showed similar potential to develop spontaneous resistance against the hybrid molecules as they did against ciprofloxacin (Table 3). This result supports other aforementioned results indicating that the observed antimicrobial activity of the tested hybrid molecules is due mainly to their interaction with GyrA and/or ParC, whereas GyrB inhibition by the hybrid molecules is limited. Therefore, resistance can arise in the form of canonical mutations against ciprofloxacin derivatives on GyrA and/or ParC.

In conclusion, the first dual DNA gyrase A and B inhibitors reported in this paper enter *Escherichia coli*, from which they are not intensively effluxed, and display a strong antibacterial activity due to the interaction of the hybrids with the GyrA and/or topoisomerase IV ParC subunits. As demonstrated by DNA gyrase ATPase and MIC assays, inhibition of GyrB by the presented hybrids, although present, is not strong enough to provide a substantial contribution to the observed antibacterial activity. In perspective, hybrids combining a benzothiazole DNA gyrase B inhibitor and the DNA gyrase A inhibitor ciprofloxacin in the same molecule connected by a cleavable linker, are a logical extension of the presented concept that could result in strong inhibition of both the DNA gyrase A and B subunits in the bacterial cell and in potent antibacterial activity.

Experimental Section

Experimental procedures are available in the Supporting Information.

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Keywords: antibiotics · ciprofloxacin · drug discovery · dual inhibitors · gyrase A · gyrase B · hybrids

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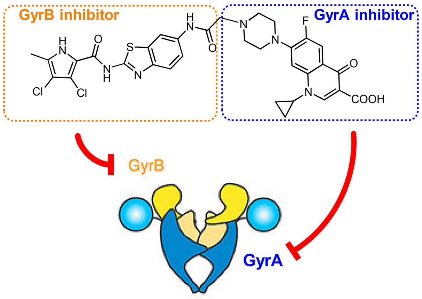
COMMUNICATIONS

The first DNA gyrase B (GyrB) inhibitor/ciprofloxacin hybrids that display antibacterial activity against *Escherichia coli* are reported. They provide a foundation for a new concept of facilitating entry of non-permeating GyrB inhibitors into bacteria by conjugation with ciprofloxacin, a highly permeable GyrA inhibitor and eliciting a strong antibacterial effect by inhibition of both the GyrA and GyrB subunits of the bacterial DNA gyrase.



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Dual *Escherichia coli* DNA Gyrase A and B Inhibitors with Antibacterial Activity