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Short communication

In vitro activities of a new fluoroquinolone derivative highly active against *Chlamydia trachomatis*

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ABSTRACT

Chlamydia trachomatis is a bacterial human pathogen responsible for the development of trachoma, an infection leading to blindness, and is also the cause of the main bacterial sexually transmitted infection worldwide. We designed a new inhibitor of this bacterium with, however, some prerequisites using (i) the iron dependency of the bacterium, (ii) a commercially available broad-spectrum antibiotic and (iii) a short synthetic pathway. The corresponding 8-hydroxyquinoline-ciprofloxacin conjugate was evaluated against a panel of pathogenic bacteria, including *C. trachomatis* but also the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa and Enterobacter species*). Its anti-*Chlamydia* activity is higher than that of ciprofloxacin and seems to be related to the fluoroquinolone moiety of the molecule, which is also responsible for the complexation of iron(III), as demonstrated by spectrophotometric titration.

1. Introduction

Chlamydia trachomatis (*C. trachomatis*), an obligate intracellular Gram-negative bacterium, is responsible for the most common sexually transmitted bacterial infection in the world (131 million new cases in 2012, WHO data) [1], which causes severe complications leading to serious sequelae in men and women, including infertility. This infection also facilitates other sexually transmitted infections (STI), including HIV [2,3], and increases the incidence and persistence of human papillomavirus (HPV) infection [4,5]. *C. trachomatis* is also responsible for trachoma, the most common infectious cause of blindness [6]. Treatment of *C. trachomatis* infection requires special considerations related to the bacterial cycle. Indeed, infectious elementary bodies are metabolically inert and insensitive to antibiotics which target bacterial replication (fluoroquinolones) and translation (macrolides, tetracyclines). Effective antibiotics must target the metabolically active reticulate bodies, which replicate in an intracellular inclusion. Lipophilic antibiotics such as azithromycin (octanol–water partition coefficient, log P = 4.02) [7] reach very high intracellular concentrations, which explains their good bactericidal activity against Gram-negative bacteria such as *C. trachomatis* [8,9].

Drug-resistant *C. trachomatis* has rarely been reported [10]. However, under the unfavorable conditions produced, for example, by treatment, bacteria can enter into a persistent form, which is viable, non-replicative and less sensitive to antibiotics; it has not yet been isolated *in vivo* [11]. This persistent form can remain for several months/years in infected tissues, causing recurrent infections, chronic inflammation and tissue fibrosis. Consequently, treatment failure, which is observed in 5–23% of cases, could be the result of the re-emergence of the persistent infection [12]. Moreover, the persistent form of *C. pneumoniae* (responsible for respiratory infections) is suspected to spread to other tissues and to be responsible for arthritis, atherosclerosis, endocarditis and asthma [13]. Thus, incomplete antibiotic efficacy may be due to a lack of sensitivity of persistent forms of *Chlamydiaceae* but also to modest intracellular concentrations of the conventionally prescribed drugs. All these elements highlight the

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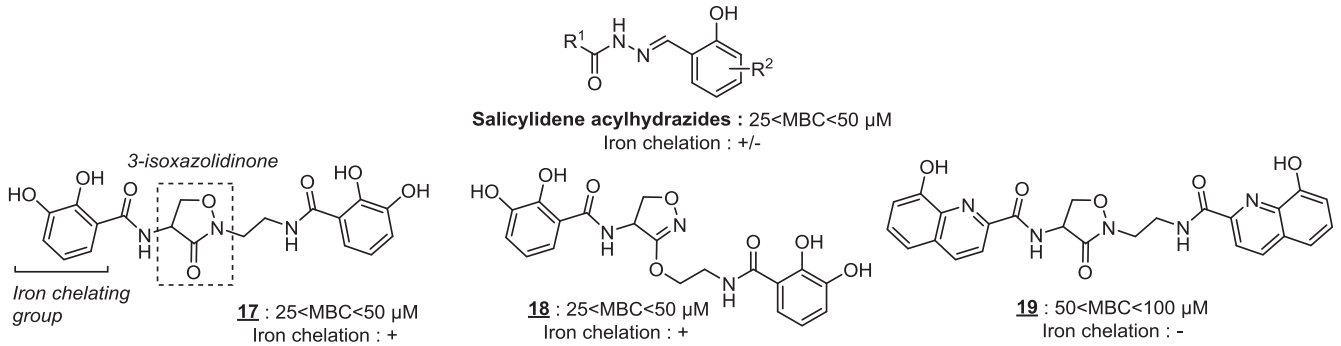


Fig. 1. Previously described inhibitors of *C. trachomatis* [17].

importance of seeking new antibiotics against *Chlamydiaceae*.

C. trachomatis needs iron to grow. Salicylidene acylhydrazides (Fig. 1), inhibitors of the Chlamydial type III secretion system (T3SS) with iron-chelating properties, inhibit *C. trachomatis* growth through a mechanism partially involving iron restriction, with minimal bactericidal concentrations (MBC) lower than 50 µM [14]. The absence of cytotoxicity to HeLa 229 cells enables their use as vaginal microbiocides [15,16]. We previously synthesized 3-isoxazolidinone derivatives, with iron-chelating properties, as equally effective inhibitors of bacterial growth (compounds **17–19**; 25 < MBC < 50 µM; Fig. 1), without toxicity at 200 µM. However, since the antibacterial activity of **19** is not reversed by excess iron(III), metal chelation cannot be the only mechanism of action of these compounds [17].

In medicinal chemistry, the search for dual therapeutic activity is commonly encountered in the literature, to try to counter the phenomenon of bacterial resistance, for instance. Indeed, despite the growing need for antibiotics to fight infectious diseases, few new molecules are available on the market. The modification or combination of pre-existing drugs is therefore an interesting strategy, making it possible to increase the therapeutic potential of the parent molecules. The search for hybrids has, for instance, been used successfully by Sunduru et al. to obtain inhibitory compounds of both *C. trachomatis* and the T3SS of gram-negative bacteria [18].

In the present work, we synthesize a new inhibitor of this bacterium with, however, some prerequisites, using: (i) the iron dependency of the bacterium, (ii) a commercially available broad-spectrum antibiotic and (iii) a short synthetic pathway. We opted for a fluoroquinolone, ciprofloxacin, with the objective of obtaining more active derivatives than the parent antibiotic and, therefore, potentially useful for the treatment of *C. trachomatis* infection. We have selected conventionally described metal-chelating entities such as catechol, which forms extremely stable complexes with iron(III) [19]. This very high stability explains why the catechol group is present in many siderophores, molecules which are synthesized by microorganisms to trap iron in the external environment in order to facilitate its intracellular transport.

The 8-hydroxyquinoline entity, present in *O*-Trensox, a potent synthetic iron chelator [20], also attracted our attention. This heterocycle has also already been shown to be effective in the search for bacterial T3SS inhibitors also active against *C. trachomatis* [21]. However, no metal chelation studies appear in this work.

Both cathechol and 8-hydroxyquinoline were used *via* the corresponding carboxylic acids (Fig. 2).

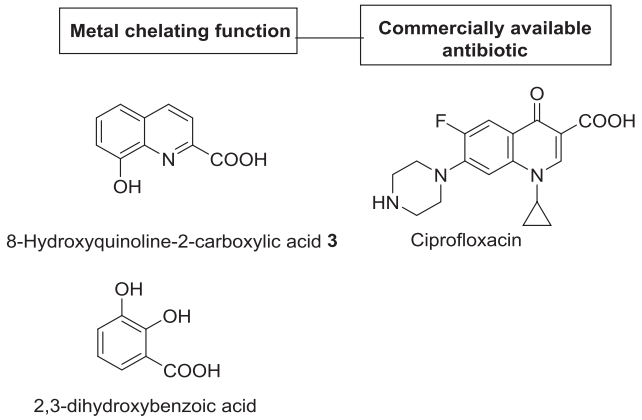


Fig. 2. Starting materials.

2. Results and discussion

2.1. Chemistry

The coupling of ciprofloxacin and 8-hydroxyquinoline-2-carboxylic acid **3** by means of TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate) and DIEA (*N,N*-diisopropylethylamine) led to compound **1** (Scheme 1). The catechol-ciprofloxacin conjugate **2** was prepared as previously described [22].

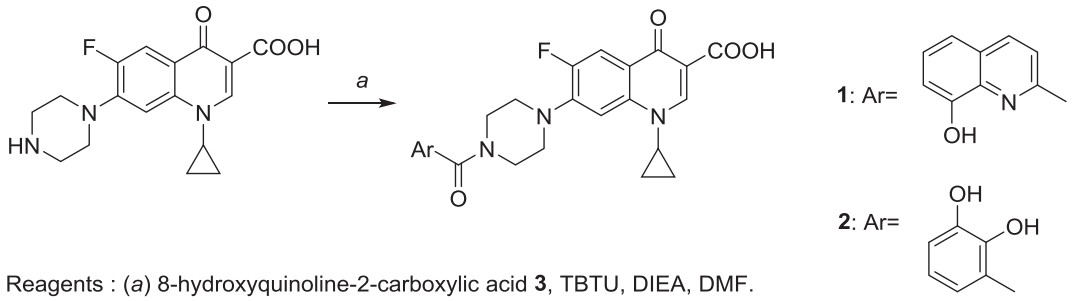
2.2. Biological evaluation

2.2.1. Anti-Chlamydia activity

The compounds were first screened for cell toxicity. Host cell viability was monitored by Trypan Blue exclusion in the presence of the compounds at different concentrations. No cell toxicity was observed at the concentrations tested (0–200 µM) on either HeLa cells, a tumour cell line, or primary cell cultures of mouse fibroblasts, a non-cancerous mammalian cell line. Compounds were then screened for their capacity to inhibit *C. trachomatis* growth in HeLa cells. The cells were infected by *C. trachomatis* serovar L2 strain as previously described [23]. Infection was performed with or without the test molecules (0–50 µM) and with or without iron citrate (200 µM). 72 h post-infection, cell lysates were processed and used to infect new HeLa cells. The reinfection capacity was scored by calculating the Inclusion Forming Unit (IFU) of each cellular lysate. Ciprofloxacin (black bars) was used as external control. The results presented in Fig. 3 show that the Minimal Bactericidal Concentration of ciprofloxacin (MBC > 16.5 µg/mL or > 50 µM) is similar to that described in the literature (> 10 µg/mL) [24] while compound **1** (grey bars) presents a MBC of 2–5 µg/mL (5–10 µM). Therefore, functionalization of the fluoroquinolone nitrogen by an 8-hydroxyquinoline entity does not inhibit its antibacterial activity. The resulting compound is even more active than the parent molecule, probably due to a gain in lipophilicity. Indeed, its calculated octanol-water partition coefficient (cLog) is higher than that of ciprofloxacin (cLogP(ciprofloxacin) = 1.32 vs cLog P(**1**) = 3.09) [25].

Under the same conditions, the catechol analogue **2** is inactive (data not shown).

Iron is an essential element for *C. trachomatis*. However, to date, no siderophores or siderophore receptors have been described in *Chlamydiaceae* [26]. Since catechol is one of the most powerful iron-



Scheme 1.

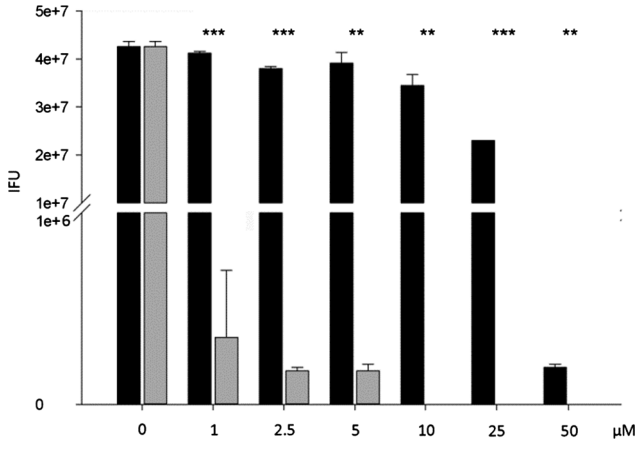


Fig. 3. Inhibitory effects on *C. trachomatis* infectious capacity of compounds tested *in cellulo* (grey bars: compound **1**; black bars: ciprofloxacin. Statistically significant differences are noted as follows: $^{**}p < 0.01$, $^{***}p < 0.001$).

chelating agents, the inactivity of compound **2** against *C. trachomatis* suggests that the inhibition induced by compound **1** is not mainly due to iron chelation.

Taking into account the iron-chelating properties of both entities, 8-hydroxyquinoline and the fluoroquinolone, we evaluated the ability of iron(III) to reverse the inhibitory effect of compound **1** by adding exogenous iron citrate (200 µM). The results presented in Fig. 4 show that the inhibitory effect of compound **1** is only partially reversed by excess Fe^{3+} (20%, 35% and 60% decrease at 1, 2.5 and 5 µM, respectively), confirming that iron chelation is not its main antibacterial mechanism.

The World Health Organization Sexually Transmitted Infections guidelines suggest treatment of *C. trachomatis* infection with one of the

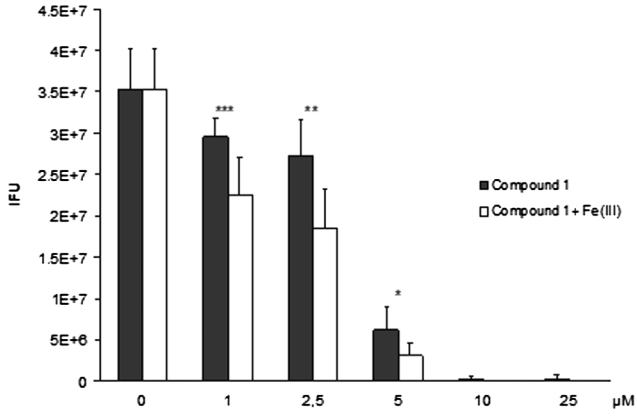


Fig. 4. Compound **1** inhibitory effect in the presence of excess iron citrate (200 µM). Statistically significant differences are noted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

following drugs: azithromycin (1 g orally as a single dose) or doxycycline (100 mg twice a day for 7 days). The *in cellulo* anti-chlamydia bactericidal activities of both compounds have been published: doxycycline and azithromycin have MBCs of 2.5–5.0 µg/mL and 10–50 µg/mL, respectively [27]. Compound **1** is therefore at least as effective *in cellulo* as these two molecules.

2.2.2. Other antimicrobial activities

Ciprofloxacin is a broad-spectrum antibiotic. We therefore looked at the ability of compound **1** to inhibit other human pathogens, including Gram-negative and Gram-positive bacteria from the ESKAPE group, an acronym including pathogenic bacteria present in the hospital environment and difficult to treat (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species*). *Staphylococcus aureus* is the most common staphylococcus strain responsible for human diseases, particularly nosocomial infections, along with *Escherichia coli*. The minimal inhibitory concentrations (MIC) of the derivatives were then determined (Table 1). Many microbes, including *P. aeruginosa* strains, are well known to synthesize siderophores to scavenge iron from their environment. In order to facilitate the transport of antibiotics into bacteria, siderophore-conjugates have been described and used in a Trojan-horse strategy [28]. Compound **2** was previously described as a

Table 1
In vitro antibacterial activities (IC_{50} or MIC) of compounds **1–2**, ciprofloxacin and acid **3**.

Organism	Gram[a]	Ciprofloxacin	1	2	3
M. tuberculosis	–	12–17[b,c]	> 128[b]	> 128[b]	> 128[b]
E. coli	N	1–2.9[b]	8 – 16[b]	4 – 15[b]	> 128[b]
E. coli ATCC-25922	N	≤0.06[d]	≤0.06[d]	*nd*	> 8[d]
K. pneumoniae ATCC-700603	N	0.25[d]	4[d]	*nd*	> 8[d]
P. aeruginosaATCC-27853[e]	N	0.25[d]	4[d]	> 128[f]	> 8[d]
A. baumannii CIP-7010	N	0.125[d]	2[d]	nd	> 8[d]
S. aureus HG001 (laboratory strain)	P	0.125[d]	0.0625[d]	5[d]	*nd*
S. aureus ATCC-25923 (clinical isolate)	P	0.25[d]	0.125[d]	*nd*	> 8[d]
S. aureus ATCC-700699 (resistant isolate)	P	> 8[d]	> 8[d]	*nd*	> 8[d]
S. epidermis ATCC-14990	P	0.125[d]	0.25[d]	*nd*	> 8[d]
ATCC-35984	P	≤0.06[c]	≤0.06[d]		> 8[d]
E. faecalis JH2-2	P	2[d]	8[c]	*nd*	> 8[d]
UCN41	P	1[d]	8[d]	*nd*	> 8[d]
E. faecium ATCC-19434T	P	1[d]	8[d]	*nd*	> 8[d]
BM-4147	P	4[d]	> 8[d]	*nd*	> 8[d]

nd: not determined.
[a] P/N: positive/negative.
[b] IC_{50} (µg/mL) against wild-type DNA gyrases of *M. tuberculosis* and *E. coli*.
[c] IC_{50}s slightly higher than those previously determined [29].
[d] MIC (µg/mL).
[e] Similar MIC were obtained against *P. aeruginosa PAO1*, a laboratory strain (data not shown).
[f] *P. aeruginosa* DSM 1117 [22].

new antibiotic following this strategy [22]. Indeed, in iron-deficient culture conditions only, compound **2** is active against the *P. aeruginosa* DSM 1117 susceptible strain with a MIC of 32 μg/mL, suggesting effective transport of the corresponding iron(III) complex by bacterial iron-uptake pathways. We found that compound **2** is inactive against *P. aeruginosa* ATCC-27853, a reference strain (MIC > 128 μg/mL, Table 1), unlike compound **1** (MIC = 4 μg/mL).

While inactive against *M. tuberculosis*, compound **1** exhibits inhibitory activity against all the other Gram-negative and Gram-positive bacteria tested. For instance, it stops the growth of *E. coli* (MIC ≤ 0.06 μg/mL), probably by inhibition of its DNA gyrase, one of the bacterial targets of quinolones (IC_{50} = 8–16 μg/mL; Table 1 and Supplementary Information). In fact, this compound has a panel of interesting antibacterial activities, in that the MICs obtained are in the μg/mL range. However, this molecule is systematically less active than the parent antibiotic, ciprofloxacin, except for *S. aureus*, especially against laboratory and clinical isolates.

As already observed for *C. trachomatis*, the catechol analogue **2** is less potent than the 8-hydroxyquinoline derivative **1** on the panel of bacteria tested.

The starting acid **3** was tested in order to evaluate its contribution to the antibacterial activity of compound **1**. The results (Table 1) show that whatever the pathogen, this acid is inactive, which suggests that the fluoroquinolone part of **1** is responsible for its efficacy.

2.3. Iron-chelating properties

To further characterize compound **1**, its ability to complex Fe^{3+} was studied by spectrophotometric titration. Complexation was performed in a H_2O/DMSO (1:1; v/v) mixture to avoid precipitation of any ligand and/or complex. The pH values mentioned are those of aqueous solutions before mixing with DMSO. Compound **1** has two potential sites for metal complexation: the ciprofloxacin carboxylate and keto groups and the 8-hydroxyquinoline part. At pH 2, the addition of $FeCl_3$ to a solution of **1** leads to a bathochromic shift (red shift) of the π-π* band from 284 to 290 nm and the appearance of a ligand-to-metal charge-transfer (LMCT) band at 450 nm (Fig. 5). The latter is identical to the LMCT of a complex between iron(III) and ciprofloxacin described by Fardeau et al. [22], whereas those of the complex between Fe^{3+} and 8-hydroxyquinoline are observed at 481 and 632 nm (not shown). This result suggests that only the fluoroquinolone part of compound **1** complexes Fe^{3+} at this pH.

In order to get closer to the physiological conditions and pH values measured by Grieshaber et al. (pH of 7.28 and 7.25 for eukaryotic cell

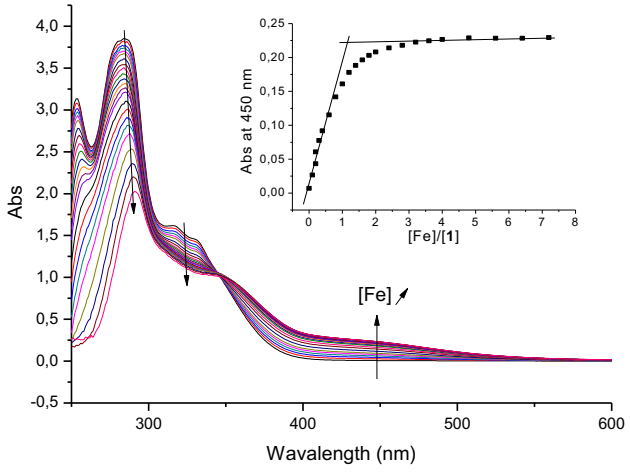


Fig. 5. Absorption spectra of **1** (10^{-4} M) in presence of increasing concentrations of $FeCl_3$ (0–10^{-3} M) at pH 2 and 25 ± 0.5 °C. Absorbance at 450 nm plotted against [Fe(III)]/[1] in inset.

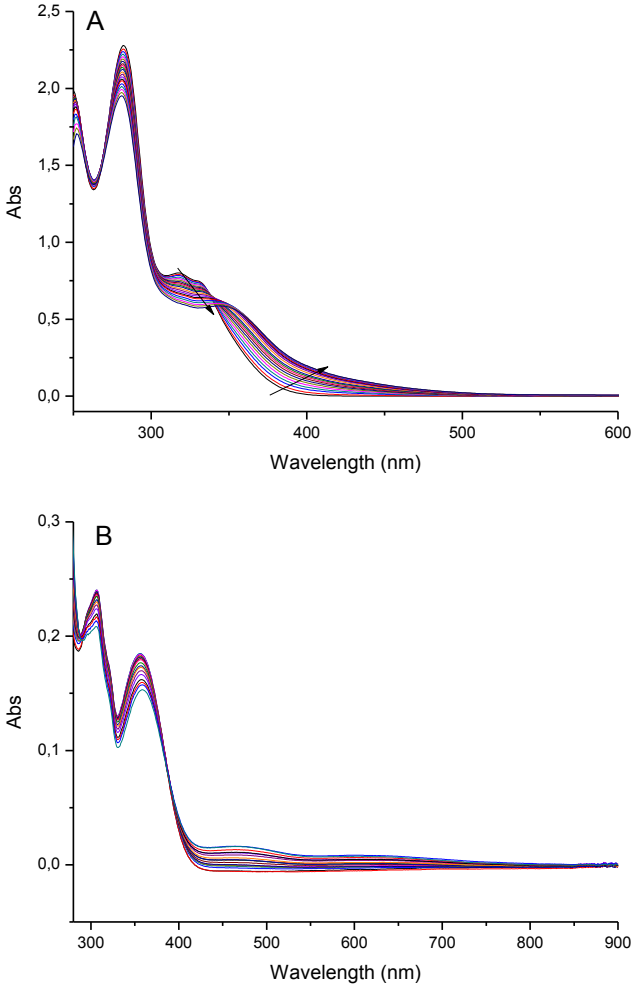


Fig. 6. Absorption spectra in the presence of Fe-NTA at pH 7.0 (A) of compound **1** (buffer: 50 mM HEPES - 150 mM KCl, solvent: DMSO; buffer/solvent, 1:1, v/v) (B) of methyl 8-hydroxyquinoline-2-carboxylate (buffer: 50 mM HEPES - 150 mM KCl, solvent: EtOH; buffer/solvent, 4:1, v/v).

cytoplasm and bacterial inclusion respectively) [30], we conducted a study at pH 7. Iron-exchange experiments between Fe-nitrilotriacetic acid (Fe-NTA), a chelating agent, and **1** or methyl 8-hydroxyquinoline-2-carboxylate, a surrogate of the 8-hydroxyquinoline moiety, show also differences in the LMCT bands (Fig. 6). Indeed, the addition of Fe-NTA to a solution of compound **1** leads to a decrease in absorbance at 282 and 319 nm. A red shift from 319 to 350 nm for the second band is also observed (Fig. 6A). In the case of methyl 8-hydroxyquinoline-2-carboxylate (Fig. 6B), there is an isosbestic point at 390 nm, and two LMCT appear at 465 and 605 nm. These LMCT bands are not observed for compound **1**, which implies that even at pH 7.0 iron is coordinated differently in these two molecules. The fluoroquinolone part of compound **1** probably chelates iron at pH 7.0.

In Fig. 5, an isosbestic point at 340 nm indicates the formation of a single iron complex. The inset in this figure presents the plot of the absorbance at 450 nm against the ratio [Fe(III)]/[**1**]: an increase in the absorbance is followed by a plateau. The two asymptotes intersect at a ratio of 1, implying a 1:1 stoichiometry (metal-ligand) for the complex. We then determined the affinity constant of the complex at pH 2, using Specfit analysis of the spectra; this is low (Log K_{11} = 2.5 ± 0.3), which confirms that iron chelation is not the main antibacterial mechanism of action of this compound.

3. Conclusion

We report here the synthesis of a novel ciprofloxacin derivative by a single-step coupling of the parent antibiotic. This compound has notable antibacterial activity against Gram-negative and Gram-positive bacteria, including the obligate intracellular bacterium *C. trachomatis*. However, only its anti-chlamydial activity is higher than that of the parent antibiotic. This antibacterial effect is only partially reversed by the addition of iron(III), which is complexed by the fluoroquinolone part of the molecule.

4. Experimental

4.1. Organic chemistry

4.1.1. Material and methods

All reagents were obtained from commercial suppliers and used without further purification. To monitor the progress of a reaction, thin-layer chromatography was performed on plastic TLC sheets of silica gel 60 F254 (layer thickness 0.2 mm) from Merck. IR, ^{1}H and ^{13}C NMR spectra confirmed the structures of all compounds. IR spectra were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer and NMR spectra were recorded in DMSO-d_6 on a Bruker AC 400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The chemical shifts are given in ppm referenced to the residual solvent signal. Coupling constants (J) are given in hertz (Hz), chemical shifts in ppm, and peak multiplicities are designated as follows: s, singlet; br s: broad singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. Elemental analysis was performed by the "Service de Microanalyse" de l'Institut de Chimie des Substances Naturelles (Gif-sur-Yvette, France). High-resolution mass spectra (HRMS) was recorded at the Small Molecule Mass Spectrometry platform of IMAGIF (Centre de Recherche de Gif - www.imagif.cnrs.fr), on a Waters spectrometer using electrospray ionization-TOF (ESI-TOF).

4.1.2. Synthesis of 1-cyclopropyl-6-fluoro-7-(4-(8-hydroxyquinoline-2-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, 1

TBTU (0.85 g, 0.264 mmol) and DIEA (0.056 mL, 0.316 mmol) were added to a solution of 8-hydroxyquinolin-2-carboxylic acid (0.05 g, 0.264 mmol) in anhydrous DMF (7 mL). After 15 min of stirring under argon, ciprofloxacin (0.079 g, 0.238 mmol) was added and the resulting suspension stirred for 3 days. The solvent was then removed under vacuum. The residue was then triturated in a mixture of methanol and CH_2Cl_2, and recrystallization from EtOH (96%) provided compound **1** as a white powder. Yield = 22% (0.026 g). IR (ν cm^{-1}): 1626, 1646, 1720, 1728. ^{1}H NMR (DMSO-d_6) δ ppm: 1.18 (m, 2H), 1.31 (m, 2H), 3.41 (m, 2H), 3.51 (m, 2H), 3.75 (m, 3H), 3.96 (m, 2H), 7.15 (d, J = 7.3 Hz, 1H), 7.46–7.54 (m, 2H), 7.61 (d, J = 7.3 Hz, 1H), 7.74 (d, J = 8.5 Hz, 1H), 7.94 (d, J = 13.1 Hz, 1H), 8.46 (d, J = 8.5 Hz, 1H), 8.67 (s, 1H), 9.81 (s, 1H), 15.15 (br s, 1H). ^{13}C NMR (DMSO-d_6) δ ppm: 7.6 (2C), 35.9, 46.3, 49.2, 49.5, 106.7, 106.8, 111.0, 112.4, 117.8, 118.8, 120.7, 128.6, 128.7, 136.8, 137.4, 139.1, 144.9, 148.1, 151.6, 153.5, 154.2, 165.9, 166.8, 176.4. Anal calcd for ($C_{27}H_{23}FN_4O_5{\cdot}2.5H_2O$): C, 59.23; H, 5.15; N, 10.23. Found: C, 59.32; H, 4.75; N, 10.34. HRMS m/z calcd for $C_{27}H_{24}FN_4O_5$ $[M+H]^+$ 503.1731; found 503.1724.

4.2. Biology

4.2.1. Chlamydia trachomatis: bacteria, cell culture and biological reagents

HeLa cells were obtained from and cultured as recommended by ATCC (Manassas, VA), in 75 cm^2 tissue culture flasks for maintenance and in 24-well, 48-well or 96-well plates for assays. *C. trachomatis* serovar L2 was from ATCC. A stock of bacteria was prepared in HeLa cells as previously described and stored at −80 °C in sucrose-phosphate-glutamic acid (SPG) buffer (10 mM sodium phosphate [8 mM Na_2HPO_4 - 2 mM NaH_2PO_4], 220 mM sucrose, 0.50 mM L-glutamic acid) for later use [23]. The number of bacterial inclusion-forming units (IFU) was determined using a method described previously [17]. DMEM (Dulbecco's Modified Eagle's medium) and fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA, USA). Anti-*Chlamydia* genus-FITC (Fluorescein isothiocyanate) antibody was from Argene (Argene Biosoft 12–114, Varhiles, France). Toxicity of the molecules was assayed on mouse embryonic fibroblasts and HeLa cells. Briefly, cells were grown in 24-well plates, were incubated with different concentrations of the molecules (0–200 μM) and cultivated for 24–48 h. Cell proliferation was measured by counting the cells in each well and comparing with control cells incubated without any product. Cell death was measured by Trypan Blue exclusion 24–48 h after incubation with the molecules.

4.2.2. Inhibition of M. tuberculosis and E. coli DNA gyrases (DNA supercoiling assays, IC_{50} determination)

DNA supercoiling activity was tested with various ratios of purified *M. tuberculosis* and *E. coli* GyrA and GyrB subunits. The reaction mixture (total volume, 30 μL) contained DNA gyrase assay buffer (40 mM Tris-HCl [pH 7.5], 25 mM KCl, 6 mM magnesium acetate, 2 mM spermidine, 4 mM dithiothreitol, bovine serum albumin [0.36 mg/mL], 100 mM potassium glutamate (except for *E. coli*), 0.1 mM EDTA, 1 mM ATP) (pH 8.0) and relaxed pBR322 DNA (0.4 μg) as the substrate. Gyrase proteins were added, and the reaction mixtures were incubated at 37 °C for 1 h. Reactions were terminated by the addition of 50% glycerol containing 0.25% bromophenol blue, and the total reaction mixture was subjected to electrophoresis in a 1% agarose gel in 0.5× TBE (Tris-borate-EDTA, pH 8.3) buffer. After running for 16 h at 40 V, the gel was stained with ethidium bromide (0.7 μg/mL). One unit of enzyme activity was defined as the amount of DNA gyrase that converted 300 ng of relaxed pBR322 to the supercoiled form in 1 h at 37 °C. Inhibition of supercoiling activity of the recombinant DNA gyrases was performed by the method described previously [31]. In brief, a reaction mixture containing 1 U of purified DNA gyrase and increasing concentrations of quinolones was incubated as described above. The inhibitory effect of quinolones on DNA gyrase was assessed by determining the concentration of drug required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}). Supercoiling activity was assessed by tracing the brightness of the bands corresponding to the supercoiled pBR322 DNA with Molecular Analyst software (Bio-Rad).

4.2.3. Antimicrobial susceptibility tests for bacteria from the ESKAPE group

Drug susceptibility testing was performed as recommended by the Clinical and Laboratory Standards Institute [32], by using the conventional microdilution method (in a final volume of 100 μL per well), Mueller-Hinton broth and sterile microtiter plates.

4.2.4. Statistical analysis

Data are presented as the mean ± standard deviation of experiments, and p values were calculated using a two-tailed two-sample equal-variance Student's t test. Statistically significant differences are noted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4.3. Metal chelating properties

4.3.1. Stock solutions.

Because of its poor solubility in aqueous media, compound **1** was first dissolved in DMSO at 10^{-2} M. The complexation experiments were performed in a H_2O/DMSO mixture (1:1, v/v) at a concentration of 1.10^{-4} M of **1**, in HCl at pH 2 or HEPES buffer (50 mM HEPES, 150 mM KCl, pH 7.0). $FeCl_3$ solutions were prepared in acidic media (pH 2). FeNTA solutions were prepared as previously described [33].

4.3.2. Spectrophotometric measurements

Affinity constants were determined spectrophotometrically by the use of the SPECFIT32 Global Analysis program [34]. Spectroscopic

measurements were performed at 25.0 ± 0.5 °C on a Cary 4000 spectrophotometer.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.10.033.

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