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Phenanthrolinic analogs of quinolones show antibacterial activity against *M. tuberculosis*.

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KEYWORDS

Tuberculosis; Fluoroquinolones; Dihydrophenanthrolinones;

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ABSTRACT

Several phenanthrolinic analogs of quinolones have been synthesized and their antibacterial activity tested against *Mycobacterium tuberculosis*, other mycobacterial species and bacteria from other genera. Some of them show high activity (of the range observed for rifampicin) against *M. tuberculosis* replicating *in vitro* and *in vivo* (infected macrophages) conditions. These derivatives show the same activity with all or several *M. tuberculosis* complex bacterial mutants resistant to fluoroquinolones (FQ). This opens the way to the construction of new drugs for the treatment of FQ resistant bacterial infections, including tuberculosis. Several compounds showed also activity against *Staphylococcus aureus* and probably other species. These compounds do not show major toxicity. We conclude that the novel phenanthrolinic derivatives described here are potent hits for further developments of new antibiotics against bacterial infectious diseases including tuberculosis in particular those resistant to FQ.

Introduction

Tuberculosis (TB) remains one of the most life-threatening infectious diseases worldwide with 8 million new cases and 1.5 million deaths per year. The standardized 6 months short term treatment is not always followed appropriately and results in relapses and drug resistances. Multi-drug resistant (MDR), defined as resistance to at least rifampicin (RMP) and isoniazid (INH) and extensively drug resistant (XDR-TB), defined as MDR-TB plus resistance to any fluoroquinolone (FQ) and at least one second line injectable agent - kanamycin (KM), amikacin (AMK) or capreomycin (CPM), are causing increasing concern with about 500 000 new MDR cases among which 10% are XDR-TB.[1]

Among the second line drugs used for the treatment of MDR-TB, FQ and KM or AMK are key drugs. Resistances to these drugs raise major treatment problems because the number of other drugs showing efficacy without major side effects is very limited.[2]

During the last ten years, intensive efforts in drug screening or drug repurposing has led to limited new possibilities of regimen. Only one new scaffold was discovered and used for the treatment of MDR-TB, bedaquiline (also named SIRTURO or TMC207).[3] This new drug shows important side effects that should be taken into account before extensive utilization.[4] Most of the new leads discovered using different approaches has resulted essentially in molecules targeting the cell wall of the mycobacteria.[5] One can wonder whether the mycobacterial cell wall has been a major barrier for the penetration of molecules active on central metabolic pathways and present in the chemical libraries that had been screened. An alternative explanation might reside in the specificities of enzymes naturally resistant to molecules acting on homologous enzymes in other bacterial genera.

Similar problems were encountered in the past after the discovery of quinolones as molecules acting only on Gram negative bacteria but not on *Pseudomonas* and Gram positive ones.[6] Chemical synthesis of derivatives had resulted in very efficient fluoroquinolones.[7] This class of molecules is targeting the bacterial topoisomerase II also called gyrase without effect against the analogue eukaryotic enzyme. This makes this target very attractive with no obvious expected side effect in the host devoid of the target enzyme. The FQ inhibits the gyrase function that results in DNA breaks inducing SOS response and ROS production that leads to bacterial cell death.[8] The gyrase is composed of a tetramer containing two GyrA and two GyrB subunits. The reaction mechanism consists in unwinding DNA supercoiling generated during replication. The quinolone intercalates into DNA at the nicks produced by the gyrase and the

quinolone C-7 ring interact with GyrB, whereas the 3-carboxyl end extends into GyrA and participates in a magnesium-water bridge that stabilizes the drug-enzyme-DNA complex. It has been proven that interactions with the fluoroquinolone happens with different forms of the complex and that the quinolone C7 ring can interact alternatively with both GyrA and GyrB.[9]

FQ are used for many purposes, either as a monotherapy in urinary tract infections or in combination with rifampicin for severe forms of staphylococcal infections. It is a key drug for the treatment of MDR-TB in association with other second line drugs. However, an increasing number of MDR-TB cases that are resistant to FQ are appearing, several of them being XDR. Although the vast majority of mutations responsible for high level resistance to FQ are located in the GyrA sub-unit of the topoisomerase II, knowledge is limited on the effect of these GyrA mutations on the resistance to FQ because these mutations are distant from the enzymatic site of the enzymatic complex.

The attractiveness of the topoisomerase II as a target for new leads has encouraged the search for new scaffolds with activity against this major metabolic pathway.[10] Molecules with new scaffolds had been designed through the analysis of the gyrase three-dimensional structure complexed with DNA and inhibitors. Molecules with low MIC and that are efficient on *M. tuberculosis* isolates resistant to FQ were discovered and described during this study. We undertook the synthesis of novel analogs of fluoroquinolones with an extended scaffold in the 4-oxo-2,3-dihydro-1,10-phenanthroline-3-carboxylic acid series (Figure 1). These compounds are devoid of C-6 fluorine which was replaced by various halogen atoms, amino or nitro groups. The latter provided to some compounds high efficiency against *M. tuberculosis* replication *in vitro* and *in vivo* cultured macrophages. These novel compounds are not toxic for mammalian cells and are active against *M. tuberculosis* complex strains carrying the *gyrA* mutations responsible

for high level resistances to the FQ. The present work opens new ways for the synthesis of drugs active on FQ resistant bacterial including MDR and XDR tuberculosis.

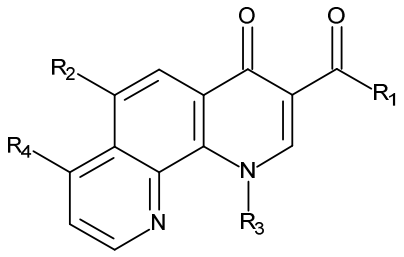


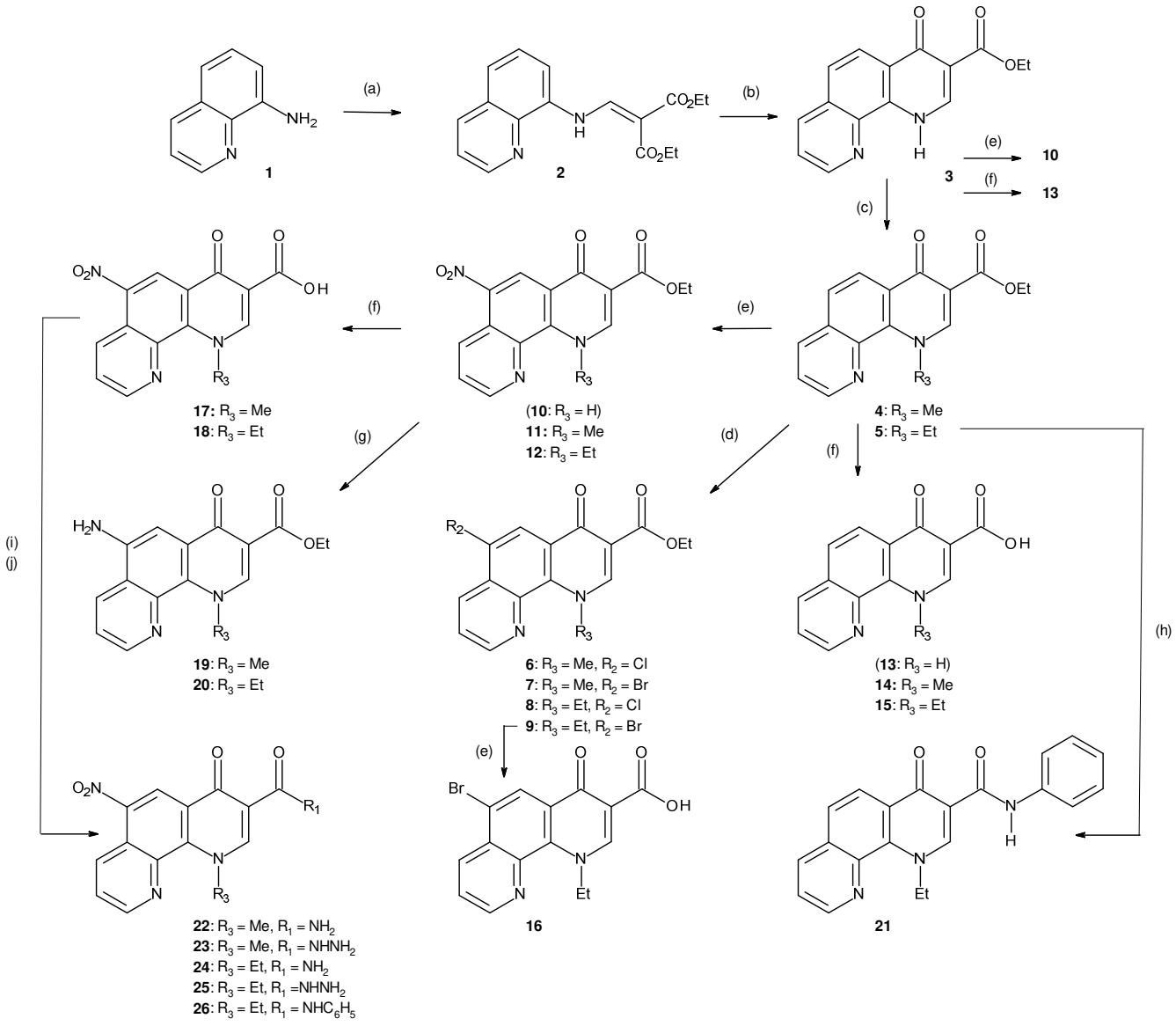
Figure 1. Targeted 4-oxo-2,3-dihydro-1,10-phenanthroline-3-carboxylic acid derivatives

Results

Chemistry

The dihydrophenanthrolinones **3-26** have been synthesized according to the chemical pathway depicted in scheme 1. The synthesis of the phenanthrolinone core is achieved according to a two steps, previously reported [11,12], sequence during which 8-aminoquinoleine is reacted with diethyl ethoxymethylenemalonate to give the diethylenamine **2**. The latter is then heated in diphenylether to afford, after cooling, a precipitate of **3** in 59% overall yield. *N* alkylation of **3** is then performed using methyl or ethyl iodide to give **4** and **5** with 74% and 60% yield respectively. The halogenation of **4** and **5** was selectively achieved in 6 position using either *N*-chloro or *N*-bromosuccinimide in acetonitrile to give **14-17** with 48-56% yield. In a similar manner the nitration of **3-5** took selectively place in 6 position, using nitric acid in acetic anhydride, and **10-12** were respectively obtained with 40-58 yield. Saponification of the esters **3-5,9,11,12** in hydrochloric acid yielded the carboxylic acids **13-18** in quantitative yield. On the other hand, the hydrogenation of the nitro group of **11,12** was achieved using iron in acetic acid and **19** and **20** were obtained with 60 and 70% yield respectively. Finally, some amides and

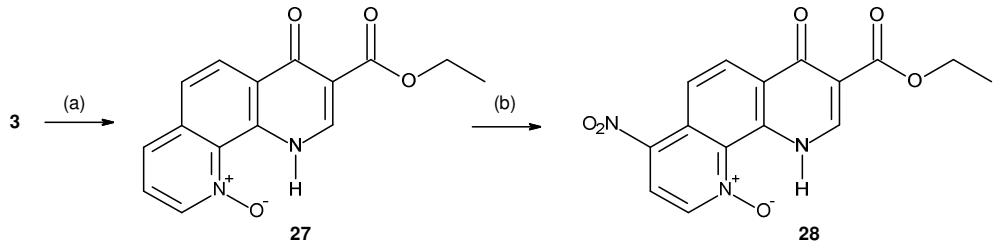
hydrazides were synthesized either starting from the ester **5**, which was reacted with aniline in diphenylether to give **21** with 48% yield, or starting from the nitroacids **17,18** which were firstly activated by oxalyl chloride in the catalytic presence of DMF, the resulting acid chlorides being then reacted with ammoniac, hydrazine or aniline in acetonitrile to give **22-26** with 41-60% yield.



Scheme 1. Synthetic pathways for access to compounds **2-26**. Conditions and reagents: (a) diethyl ethoxymethylenemalonate; (b) diphenylether; (c) R_3I, K_2CO_3, DMF; (d) NBX, CH_3CN;

(e) HNO_3, Ac_2O; (f) HCl, H_2O; (g) Fe, AcOH; (h) $C_6H_5NH_2$, diphenylether; (i) $(ClCO)_2$, DMF; (j) R_1H, CH_3CN.

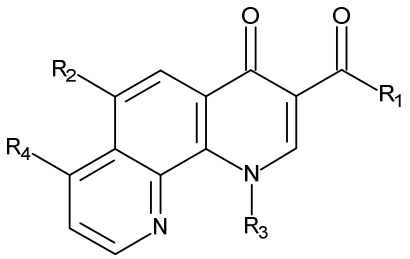
On the other hand, in order to modulate the reactivity of our dihydrophenanthrolinones, the *N*-oxide derivative **27** was synthesized in 67% yield starting from **3** and using *meta*-chloroperbenzoic acid in dichloromethane. Nitration of the latter took place, this time selectively in 7 position, and **28** was obtained in 34% yield (Scheme 2).



Scheme 2. Synthetic pathways for access to compounds **27** and **28**. Conditions and reagents: (a) *m*-CPBA, DCM; (b) HNO_3, H_2SO_4.

In vitro activity of phenanthrolinic analogs on *M. tuberculosis* and other bacterial species

The table 1 shows activities of a series of twenty-six 4-oxo-2,3-dihydro-1,10-phenanthroline-3-carboxylic acid derivatives on *M. tuberculosis*. The inhibitory effect of the molecules on bacterial growth were measured according to the dilution procedure based on cell viability using the resazurin microtiter assay (REMA) plate method. Resazurin is a blue dye, which reduces to the pink colored and fluorescent resorufin proportionally to aerobic respiration.[13,14] Molecules inhibiting the growth have been identified by a dual wavelength measurement (570 and 604 nm) at a time determined during preliminary experiments with known antibiotics like ofloxacin, rifampicin, and kanamycin. Eight derivatives exhibited MIC_{90} < 10 µM (**11**, **12**, **16**, **17**, **18**, **22**, **24** and **25**).



Cmpd	R_1	R_2	R_3	R_4	MIC_{90} (μM)
rifampicin	-	-	-	-	0.76
ofloxacin	-	-	-	-	1.73
amikacin	-	-	-	-	1.83
3	OEt	H	H	H	23.99
4	OEt	H	Me	H	182.43
5	OEt	H	Et	H	169.76
6	OEt	Cl	Me	H	328.7
7	OEt	Br	Me	H	290.14
8	OEt	Cl	Et	H	166.26
9	OEt	Br	Et	H	317.96
10	OEt	NO_2	H	H	328.76
11	OEt	NO_2	Me	H	1.21
12	OEt	NO_2	Et	H	4.99
13	OH	H	H	H	112.41
14	OH	H	Me	H	25.82
15	OH	H	Et	H	47.06
16	OH	Br	Et	H	9.39
17	OH	NO_2	Me	H	2.64
18	OH	NO_2	Et	H	5.84
19	OEt	NH_2	Me	H	92.08
20	OEt	NH_2	Et	H	41.88
21	NHC_6H_5	H	Et	H	311.59
22	NH_2	NO_2	Me	H	1.4
23	$NHNH_2$	NO_2	Me	H	20.79
24	NH_2	NO_2	Et	H	1.25
25	$NHNH_2$	NO_2	Et	H	9.84
26	NHC_6H_5	NO_2	Et	H	275.49
27	OEt	H	H	H	203.99

28	OEt	H	H	NO_2	39.48

Table 1. Antibacterial activities [MIC_{90} (μM)] of 4-oxo-2,3-dihydro-1,10-phenanthroline-3-carboxylic acid derivatives **3-28**, rifampicin, ofloxacin and amikacin on *M. tuberculosis*. Each MIC_{90} was determined three times in duplicate experiments.

Activity against other bacteria.

The activities of compounds **11**, **12**, **17** and **18** against other mycobacterial species and bacteria from other genera were then evaluated and are indicated in Table 2.

	M. smegmatis	*M. aurum*	*M. marinum*	*BCG*	*P. aeruginosa*
Ofloxacin	3.46	3.46	6.92	1.73	6.92
Amikacin	0.46	1.83	3.66	0.46	3.66
11	>100	>100	0.15	2.35	>100
12	>100	>100	0.61	2.5	>100
17	11.18	11.18	0.70	1.40	>100
18	21.35	10.67	1.3	2.67	>100
	A. baumannii	*E. aerogenes*	*E. cloacae*	*S. aureus*	*K. pneumonia*
Ofloxacin	1.73	0.22	6.92	1.73	n.d.
Amikacin	14.63	n.d.	n.d.	n.d.	n.d.
11	>100	309	>275	>275	>275
12	>100	314	>275	19.59	>275
17	>100	44.70	>275	22.35	>275
18	93.36	42.69	>275	10.67	>275

Table 2. Antibacterial activities [MIC_{90} (μM)] against different bacterial species. n.d.: not determined.

Toxicity assays.

Toxicity of compounds **11**, **12**, **17** and **18** was assayed on Vero cells line and human macrophages. The macrophages were incubated for six days. No major toxicity was observed in either cell types. Compound **11** is the least toxic with IC_{50} > 50 µg/mL. Results are shown in Figure 2 using rifampicin and moxifloxacin as a control.

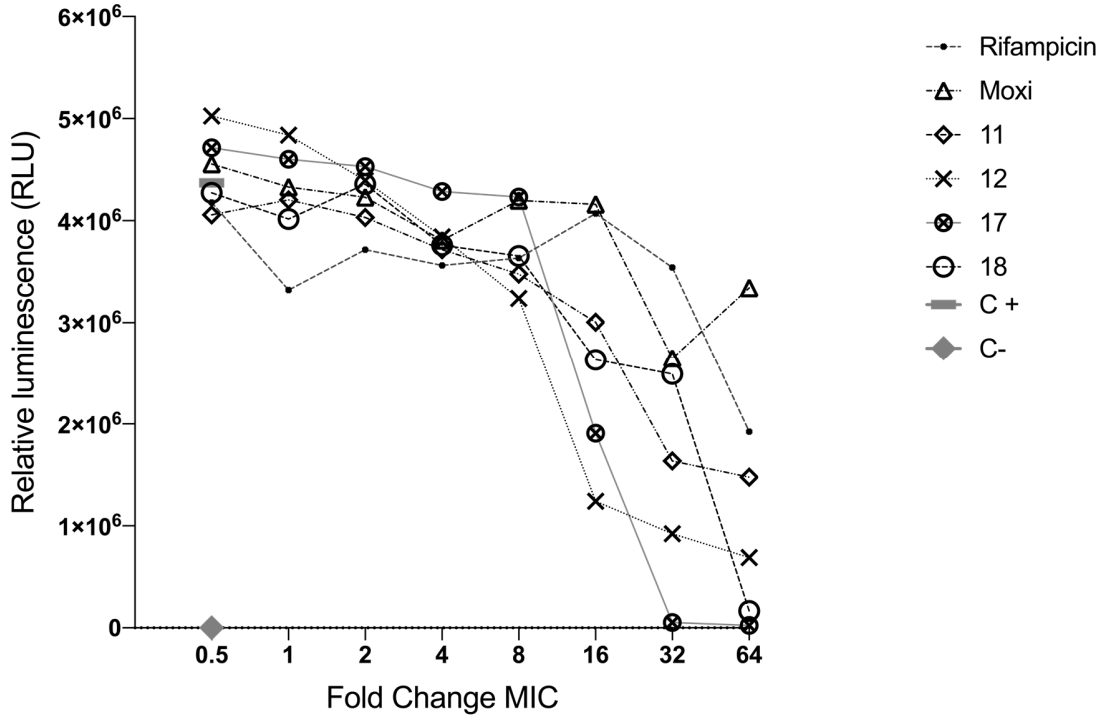


Figure 2. Cytotoxicity of compounds **11**, **12**, **17** and **18** in human macrophages after 6 days of incubation. Results are expressed in relative luminescence units.

Inhibitory effect on the growth of *M. tuberculosis* replicating in *in vitro* cultured macrophages.

The human macrophages were used for testing the activity of the molecules on the growth of *M. tuberculosis* replicating in *in vitro* cultured macrophages [15] In this case moxifloxacin was used as control.

		Moxifloxacin	**11**	**12**	**17**	**18**
	0 days	6 days	6 days	6 days	6 days	6 days
CONTROL +	5.57	6.43	5.79	5.68	5.72	5.53
1/2 MIC	5.57	4.02	4.22	4.42	3.78	4.19
MIC	5.57	3.77	3.63	3.59	3.65	3.69
2X MIC	5.57	3.93	3.45	3.1	2.77	3.21
4X MIC	5.57	2.54	3.06	3	3.01	3.24

Table 3. Activity of moxifloxacin and compounds **11**, **12**, **17** and **18** inside human macrophages at 0 and 6 days of incubation. Results are expressed in logUFC/mL. Cells infected without treatment were considered as the positive control.

***In vitro* activity of phenanthrolinic analogs of *M. tuberculosis* mutants.**

Compounds **11**, **12**, **17** and **18** were also tested against *M. tuberculosis* (H37Rv) mutants, resistant to FQ and against an extensively drug resistance clinical isolate (XDR-TB).

The FQ mutants carried either the mutations at position 88, 90, 91 or 94 of the *gyrA* gene or at position 500 or 540 of the *gyrB* gene. For some of the tested compounds no increase in MIC was observed in some of the mutants. Results are indicated in Table 4.

Compound	H37Rv	Mutants H37Rv						Clinical Isolate
		gyrA Ala-90-Val	*gyrA* Asp-94-Gly	*gyrA* Gly-88-Cys	*gyrA* Ser-91-Pro	*gyrA* Ala-90-Val *gyrB* Glu-540-Val	*gyrA* Asp-94-Gly *gyrB* Asp-500-Asn	XDR-TB
Ofloxacin	1.73	13.8	13.8	27.6	27.6	27.6	27.6	27.6
Moxifloxacin	0.77	3.08	3.08	6.16	6.16	6.16	6.16	6.16
Nalidixic acid	>50	>50	>50	>50	>50	>50	>50	n.d.
11	1.21	>50	1.21	>50	>50	>50	>50	1.21
12	4.99	>50	4.99	>50	>50	>50	>50	4.99
17	2.64	5.28	2.64	2.64	5.28	5.28	5.28	2.64
18	5.84	11.68	2.99	11.68	11.68	11.68	5.84	n.d.

Table 4. Antibacterial activities [MIC_{90} (μM)] of compounds **11**, **12**, **17**, **18**, ofloxacin, moxifloxacin and nalidixic acid against FQ resistant bacteria and XDR-TB, resistant to FQ, pyrazinamide, ethambutol and streptomycin ; n.d. not determined.

Confocal fluorescent microscopy

The similarity of their structure to quinolones has suggested a similar mode of action i.e. the inhibition of the gyrase activity resulting in replication and division inhibition. Imaging methodology was chosen for investigating the mode of action of the phenanthrolinic analogs.[16,17] Indeed, due to a relative lack of water-solubility of the synthesized compounds, an in vitro gyrase inhibition assay could not be performed. In order to investigate morphological changes due to replication and division inhibition in bacterial cells after treatment by **17**, chosen among the most active compounds, we have used confocal fluorescent microscopy and combined staining with DAPI (fluorescent strain that binds DNA) and FM 4-64 (lipophilic dye that stains membrane). *E. coli* cells treated by **17** appear elongated and filamentous in comparison with untreated cells and have altered DNA morphology (Figure 3). *E. coli* treated by fluoroquinolone ofloxacin display also elongated morphology than untreated cells but however shorter than the cells in presence of **17**.[18] In order to overcome problems of clumping observed with BCG or *M. tuberculosis* cultures we have used *M. smegmatis* in imaging experiments. *M. smegmatis* cells treated by **17** or ofloxacin were slightly longer than untreated cells and appear more segmented as visualized by membrane staining with FM 4-64. These observations can also indicate an inhibition of cell division. These results are in accordance with a possible inhibition of gyrase.

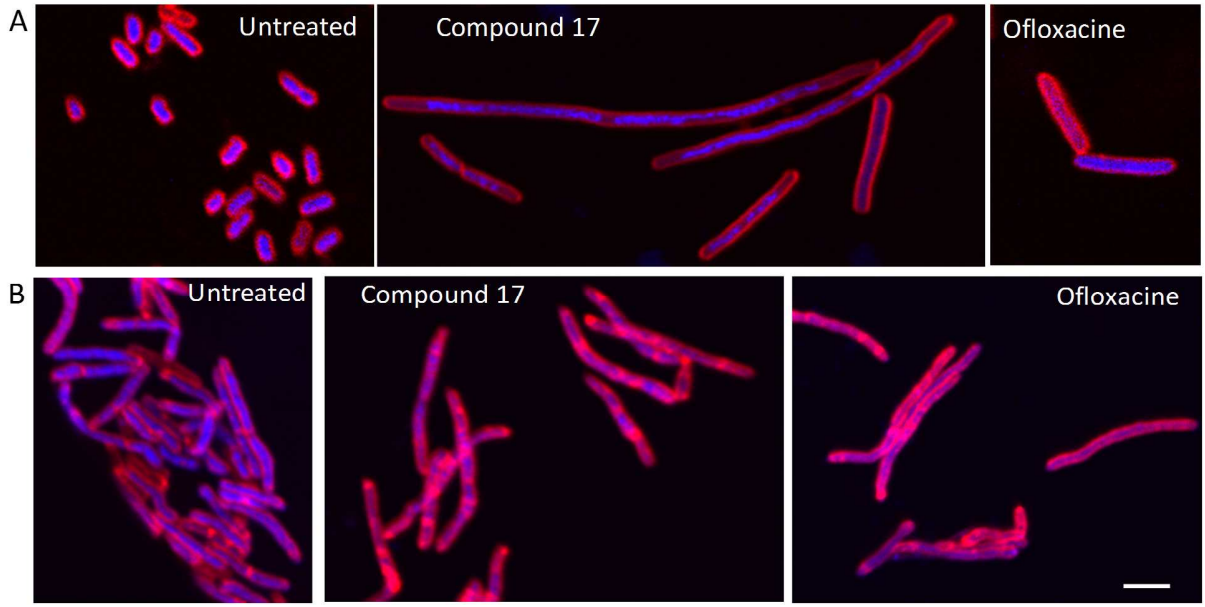


Figure 3. *E. coli* and *M. smegmatis* display altered morphology after treatment by **17**. The morphology of *E. coli* (A) and *M. smegmatis* (B) was visualized by confocal fluorescence microscopy after a combined staining with FM4-64 (membrane in red) and DAPI (DNA in blue). Bacterial cells were treated with 2.5 µg/mL of **17** or 2 µg/mL of ofloxacine for 1.5 hours for *E. coli* and for 6 hours for *M. smegmatis*. Elongation of bacterial cells in presence of **17** or ofloxacin is observed (Scale bar, 2 µm).

Genetic approaches

Mutants resistant to the compounds were selected by using different mycobacterial species. No mutant could be isolated either from *M. tuberculosis* or *M. smegmatis*. However, several mutants could be isolated from *M. marinum*. Their genomes were entirely sequenced and compared to the wild type strain used for the selection of the mutants. No mutations were observed in the gyrase genes or any enzymatic component of the replication process. Only

mutations likely affecting permeation or genes involved in peptidoglycan biosynthesis, *murF*, *murD, murA*, and other mutants in *mmpl4* gene were found.

Discussion

The first insight to come out of this study is that the phenanthrolinic series retains the antibacterial activity linked to the fluoroquinolone one, since among the twenty-six phenanthroline compounds tested, eight displayed an activity against *M. tuberculosis*, with MIC values < 10 µM. All these compounds possess a nitro group (**11**, **12**, **17**, **18**, **22**, **24**, **25**) at position 6, except **16** which is substituted on this position by a bromine atom. 6-Unsubstituted derivatives, the other 6-halogeno and 6-amino derivatives appear devoid of such an antibacterial activity. On the other hand, *N*-alkylated derivatives appear more active than the corresponding unsubstituted ones (ex **11** and **12** versus **10**) without significant differences between the *N*-methyl derivatives (**11**, **17**, **22**) and the corresponding *N*-ethyl ones (**12**, **18**, **24**). In a similar manner, the nature of the substituent in the 3-position appears as not discriminant for the activity and both acids (**17**, **18**), esters (**11**, **12**) and amides (**22**, **24**) are active in the low micromolar range.

Compounds **11**, **12**, **17** and **18** were chosen to be evaluated against other bacteria. No activity was observed against *Pseudomona aeruginosa, Enterobacter cloacae*, *Klebsiella* or *Acinetobacter baumanii*. Conversely, activity of the four compounds was observed on the *M. tuberculosis* complex and its ancestor *Mycobacterium marinum;* and when compounds **11** and **12** were tested, no activity was detected against *Mycobacterium smegmatis* and *Mycobacterium aurum*, selected non-tuberculous mycobacteria (NTMs). In this NTMs the activity of

compounds **17** and **18** presented MIC values >10 μM. These results indicate that these compounds have a narrow range of activity in tuberculous bacilli which is a desirable feature.

Compounds **11**, **12**, **17** and **18** are also effective against *M. tuberculosis* in *in vitro* conditions of replication inside macrophages without noticeable toxicity against the human macrophages cells. The antibacterial activity is in the same range of that observed for moxifloxacin. Moxifloxacin is an important second-line antibiotic frequently used in the treatment of MDR TB. The fact that the tested phenanthroline derivatives have *in vitro* activity in cultured macrophages similar to moxifloxacin is highly promising.

Concerning the *M. tuberculosis* mutants resistant to FQ, we can observe that compounds **11** and **12** showed significant activity only against mutants affected at position 94. Compounds **17** and **18**, however, showed activity against all types of mutation.

The phenanthrolinic analogs **11**, **12**, and **17** tested against XDR-TB clinical isolate mutant, remained active with a MIC identical to that observed on the parental H37Rv sensitive strain. In all cases, the phenanthrolinic analogs-susceptibility of the mutants was close to that of the parental H37Rv strain, indicating a more extensive spectrum of action than other currently used FQ.

The potential mechanism of action of the phenanthrolinic analogs, the morphologies of *E. coli* and *M. smegmatis* treated by the compound **17** and observed by confocal fluorescence microscopy, are consistent with the hypothesis that this compound targets DNA gyrase, thus leading to replication fork stalling.

The mutations, leading some *M. marinum* bacilli to become resistant to these compounds, did not concern the gyrase gene. This is a difference with the easy selection of *gyr* mutants resistant to FQ. However *gyrA* and *gyrB* are essential genes that could not support any mutations

leading to its inactivation. We may suggest that no permissive mutation can be isolated that confer resistance to our compounds without lethal phenotype. These findings show the usefulness of molecules blocking bacterial multiplication without provoking the selection of resistant mutants that will impair treatment using them during chemotherapy.

Conclusion

A new procedure for synthesizing novel phenanthrolinic analogs of quinolones was described. Several compounds showed activity against *M. tuberculosis*, including XDR isolates. Importantly, no mutant, impaired in the gyrase which is the major target of quinolones used in therapeutic treatment, was observed during *in vitro* selection experiments. Unlike FQ, that show activity against *M. tuberculosis*, the compounds described here do not carry a fluorine atom and a nitro group seems to be required for their activity. Even if, such a nitro group is sometimes considered as a toxicophore, recent works highlighted its importance in medicinal chemistry and especillay in antitubercular agents, with a specific activation through its bioreduction by *M. tuberculosis*.[19] These phenanthronilic analogs showed inhibitory effect on the growth of several mutants to fluoroquinolones, opening the way to new treatments for MDR isolates that are resistant to fluoroquinolones used in clinic. No significant toxicity is observed at concentrations used for the observation of inhibitory effect on in vitro or intracellular *M. tuberculosis* multiplication. The preclinical development of some of these derivatives is currently under progress. [20]

Methods.

Chemistry.

General material and methods:

All chemical reagents and solvents were purchased from commercial sources and used without further purification. Melting points were determined on a Kofler melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a BRUKER AVANCE III 400 MHz with chemical shifts expressed in parts per million (in chloroform-*d*, methanol-d_4 or DMSO-d_6) downfield from TMS as an internal standard and coupling in Hertz. IR spectra were recorded on a Perkin-Elmer BX FT-IR apparatus using KBr pellets. High resolution mass spectra (HRMS) were obtained by electrospray on a BrukermaXis. The purities of all tested compounds were analyzed by LC–MS, with the purity all being higher than 95%. Analyses were performed with a Waters Alliance 2695 as separating module (column XBridge C18 2.5 µM/4.6x50 mM) using the following gradients: A (95%)/B (5%) to A (5%)/B (95%) in 4.00 min. This ratio was hold during 1.50 min before return to initial conditions in 0.50 min. Initial conditions were then maintained for 2.00 min (A = H_2O, B = CH_3CN; each containing HCOOH: 0.1%). MS were obtained on a SQ detector by positive ESI.

Ethyl [(quinolin-8-ylamino)methylene]malonate (2). A mixture of 1 g of 8-aminoquinoline (1 equiv, 6.94 mmol) and 1.5 g of diethyl ethoxymethylenemalonate (1 equiv, 6.94 mmol) were refluxing for 2 h. After cooling to rt, 25 mL of EtOH were added. The new mixture was cooled to 0 °C. The precipitate was filtered and recristallized (EtOH) to give the desired product as a beige solid (83%). mp 113 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 12.37 (d, *J* = 14.4 Hz, 1H), 8.97 (dd, *J* = 4.2, 1.7 Hz, 1H), 8.80 (d, *J* = 14.4 Hz, 1H), 8.17 (dd, *J* = 8.3, 1.7 Hz, 1H), 7.55 (m, 3H), 7.49 (dd, *J* = 8.3, 4.2 Hz, 1H), 4.42 (q, *J* = 7.1 Hz, 2H), 4.30 (q, *J* = 7.2 Hz, 2H), 1.45 (t, *J* = 7.1 Hz, 3H), 1.37 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 178.6, 169.7, 155.5,

149.1, 143.9, 142.0, 141.3, 134.3, 131.0, 129.6, 128.7, 127.1, 117.3, 65.0 (2C), 19.5 (2C). IR (KBr) υ (cm^{-1}) 3336, 2973, 2897, 1645, 1683, 1585, 1259. LC-MS (ESI) t_R = 5.040 min; *m/z* $[M+H]^+$ 315.48. HRMS/ESI: m/z calcd for $C_{17}H_{19}N_2O_4$ $[M+H]^+$ 315.0968, found 315.0965.

Ethyl 1,4-dihydro-4-oxo-1,10-phenanthroline-3-carboxylate (3). 1.8 g of ethyl [(quinolin-8-ylamino)methylene]malonate **2** (1 equiv, 6.71 mmol) was dissolved in 40 mL of diphenyl ether. The solution was refluxing during 45 min under stirring. After cooling to rt, EP was added. The precipitate was filtered and recrystallized (DMF) to obtain the product as a brown solid (71%). mp 242 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 12.85 (s, 1H), 9.08 (dd, *J* = 4.3, 1.6 Hz, 1H), 8.55 (d, *J* = 8.0 Hz, 2H), 8.21 (d, *J* = 8.8 Hz, 1H), 7.88 (d, *J* = 8.9 Hz, 1H), 7.83 (dd, *J* = 8.3, 4.3 Hz, 1H), 4.26 (q, *J* = 7.1 Hz, 2H), 1.30 (t, *J* = 7.1 Hz, 3H).^{13}C NMR (100.53 MHz, DMSO-d_6) δ 173.6, 165.0, 150.5, 144.4, 139.1, 137.2, 136.6, 129.6, 126.5, 124.8, 124.0, 122.9, 113.1, 60.3, 14.8. IR (KBr) υ (cm^{-1}) 3219, 2897, 1706, 1631, 1510, 1082, 728. LC-MS (ESI) t_R = 3.457 min; *m/z* $[M+H]^+$ 269.47. HRMS/ESI: m/z calcd for $C_{15}H_{13}N_2O_3$ $[M+H]^+$ 269.0578, found 283.0576.

General procedure for *N*-alkylation of ethyl 1,4-dihydro-4-oxo-1,10-phenanthroline-3-carboxylate (3). To a suspension of ethyl 1,4-dihydro-4-oxo-1,10-phenanthroline-3-carboxylate **3** (1 equiv) in DMF (7 mL/mmol) was added K_2CO_3 (1 equiv). The solution was stirring at rt for 30 min and the alkyl iodide (1.5 equiv) was added dropwise. The reaction mixture was warmed for 10 h between 70 and 100 °C. After cooling to rt, the mixture was evaporated under reduced pressure. The residue was extended with water and extracted with DCM (4 x). The combined organic layers were washed with brine, dried over $MgSO_4$ and then evaporated under reduced pressure. The final compound was obtained after recrystallization (water/DMF 50/50).

Ethyl 1-methyl 1,4-dihydro 4-oxo 1,10-phenanthroline 3-carboxylate (4). Prepared from 1 g of compound **3** (3.73 mmol). White solid (74%). mp 181 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 9.07 (dd, *J* = 4.2, 1.8 Hz, 1H), 8.69 (s, 1H), 8.54 (dd, *J* = 8.3, 1.8 Hz, 1H), 8.38 (d, *J* = 8.7 Hz, 1H), 7.95 (d, *J* = 8.7 Hz, 1H), 7.79 (dd, *J* = 8.3, 4.2 Hz, 1H), 4.64 (s, 3H), 4.29 (q, *J* = 7.1 Hz, 2H), 1.34 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 172.5, 164.9, 152.4, 148.8, 141.4, 137.8, 137.3, 131.3, 129.5, 125.6, 123.7, 123.5, 112.7, 60.4, 49.9, 14.8. IR (KBr) υ (cm^{-1}) 3020, 2924, 1708, 1693, 1517, 1364, 1096, 824, 718. LC-MS (ESI) t_R = 3.808 min; *m/z* $[M+H]^+$ 283.53. HRMS/ESI: m/z calcd for $C_{16}H_{15}N_2O_3$ $[M+H]^+$ 283.1081, found 283.1083.

Ethyl 1-ethyl-1,4-dihydro-4-oxo-1,10-phenanthroline-3-carboxylate (5). Prepared from 1 g of compound **3** (3.73 mmol). White solid (60%). mp 169 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 9.10 (dd, *J* = 4.2, 1.8 Hz, 1H), 8.73 (s, 1H), 8.58 (dd, *J* = 8.3, 1.9 Hz, 1H), 8.43 (d, *J* = 8.6 Hz, 1H), 7.99 (d, *J* = 8.7 Hz, 1H), 7.82 (dd, *J* = 8.2, 4.2 Hz, 1H), 5.23 (q, *J* = 6.9 Hz, 2H), 4.30 (q, *J* = 7.1 Hz, 2H), 1.47 (t, *J* = 6.9 Hz, 3H), 1.34 (t, *J* = 7.1 Hz, 3H).^{13}C NMR (100.53 MHz, DMSO-d_6) δ 171.8, 164.3, 152.8, 149.9, 142.0, 141.0, 140,3, 133.1, 127.1, 125.6, 123.3, 122.8, 114.9, 60.9, 55.2, 16.8, 14.7. IR (KBr) υ (cm^{-1}) 3012, 2821, 1700, 1608, 1501, 1473, 1317, 807, 734. LC-MS (ESI) t_R = 3.81 min; *m/z* $[M+H]^+$ 297.43. HRMS/ESI: m/z calcd for $C_{17}H_{17}N_2O_3$ $[M+H]^+$ 297.2169, found 297.2169.

General procedure for halogenation of 1,10-phenanthrolinone. To a suspension of *N*-alkylphenantrolinone (1 equiv) in CH_3CN (25 mL/mmol) was added NBS or NCS (1.1 equiv) by portion. The resulting mixture was stirred at 40 °C for 48 h. After cooling to rt, the mixture was concentrated *in vacuo*, dissolved in water and then extracted with DCM (4 x). The combined

organic layers were washed with brine, dried over $MgSO_4$ and then evaporated under reduced pressure. The compound was then purified by recrystallization (CH_3CN).

Ethyl 6-chloro-1-methyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (6). Prepared from 1 g of compound **4** (3.54 mmol). White solid (52%). mp 181 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 8.99 (dd, *J* = 4.2, 1.8 Hz, 1H), 8.61 (dd, *J* = 8.5, 1.8 Hz, 1H), 8.56 (s, 1H), 8.47 (s, 1H), 7.67 (dd, *J* = 8.5, 4.2 Hz, 1H), 4.63 (s, 3H), 4.43 (q, *J* = 7.1 Hz, 2H), 1.44 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 172.2, 165.2, 152.0, 148.1, 142.1, 136.5, 133.3, 129.8, 128.7, 128.4, 123.8, 123.3, 112.9, 61.1, 50.3, 14.4. IR (KBr) υ (cm^{-1}) 2971, 1705, 1693, 1507, 1458, 1320, 1089, 708. LC-MS (ESI) t_R = 4.24 min; *m/z* $[M+H]^+$ 317.39/319.38. HRMS/ESI: m/z calcd for $C_{16}H_{14}ClN_2O_3$ $[M+H]^+$ 317.1728, found 317.1726.

Ethyl 6-bromo-1-methyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (7). Prepared from 1 g of compound **4** (3.54 mmol). White solid (49%). mp 191 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 8.91 (dd, *J* = 4.2, 1.7 Hz, 1H), 8.81 (s, 1H), 8.60 (dd, *J* = 8.5, 1.8 Hz, 1H), 8.46 (s, 1H), 7.62 (dd, *J* = 8.5, 4.1 Hz, 1H), 4.59 (s, 3H), 4.37 (q, *J* = 7.1 Hz, 2H), 1.37 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 172.2, 165.3, 152.1, 148.1, 142.2, 137.2, 136.2, 130.3, 130.0, 128.3, 127.9, 123.7, 119.0, 61.2, 50.3, 14.4. IR (KBr) υ (cm^{-1}) 2989, 1703, 1691, 1501, 1473, 1063, 727. LC-MS (ESI) t_R = 4.410 min; *m/z* $[M+H]^+$ 362.45/364.35. HRMS/ESI: m/z calcd for $C_{16}H_{14}BrN_2O_3$ $[M+H]^+$ 362.2198, found 362.2196.

Ethyl 6-chloro-1-ethyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (8). Prepared from 1 g of compound **5** (3.37 mmol). White solid (56%). mp 192 °C. ^{1}H NMR (399.75 MHz,

$CDCl_3$-*d*) δ 9.04 (dd, *J* = 4.1, 1.7 Hz, 1H), 8.72 (m, 2H), 8.56 (s, 1H), 7.72 (dd, *J* = 8.5, 4.1 Hz, 1H), 5.28 (q, *J* = 6.9 Hz, 2H), 4.45 (q, *J* = 7.1 Hz, 2H), 1.57 (t, *J* = 6.9 Hz, 3H), 1.45 (t, *J* = 7.1 Hz, 3H).^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 172.4, 165.5, 151.2, 148.3, 141.6, 135.8, 133.7, 130.4, 128.8, 128.6, 124.3, 123.4, 113.5, 61.2, 55.5, 16.7, 14.5. IR (KBr) υ (cm^{-1}) 2969, 1754, 1632, 1592, 1453, 1313, 1087, 819, 763. LC-MS (ESI) t_R = 4.60 min; *m/z* $[M+H]^+$ 331.41/333.40. HRMS/ESI: m/z calcd for $C_{17}H_{16}ClN_2O_3$ $[M+H]^+$ 331.0732, found 331.0739.

Ethyl 6-bromo-1-ethyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (9). Prepared from 1 g of compound **5** (3.37 mmol). White solid (48%). mp 194 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 9.01 (dd, *J* = 4.2, 1.8 Hz, 1H), 8.95 (s, 1H), 8.72 (dd, *J* = 8.5, 1.8 Hz, 1H), 8.57 (s, 1H), 7.71 (dd, *J* = 8.5, 4.1 Hz, 1H), 5.29 (q, *J* = 7.0 Hz, 2H), 4.45 (q, *J* = 7.1 Hz, 2H), 1.57 (t, *J* = 7.0 Hz, 3H), 1.45 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$) δ 172.4, 165.5, 151.2, 148.3, 141.6, 135.8, 133.7, 130.4, 128.8, 128.6, 124.3, 123.4, 113.5, 61.2, 55.5, 16.7, 14.5. IR (KBr) υ (cm^{-1}) 2991, 1716, 1646, 1496, 1368, 1174,1049, 839, 745. LC-MS (ESI) t_R = 4.64 min; *m/z* $[M+H]^+$ 376.37/377.37/378.37. HRMS/ESI: m/z calcd for $C_{17}H_{16}BrN_2O_3$ $[M+H]^+$ 376.0262, found 376.0263.

General procedure for nitration. To a suspension of phenanthrolinone (1 equiv) in Ac_2O (7 mL/mmol) was added dropwise a solution of HNO_3 65 % (4.38 equiv) in the same amount of Ac_2O as previously. The mixture was stirred at rt for 3 h and then diluted in EtOAc. The precipitate was filtered and recrystallized (DMF/EtOAc 50/50).

Ethyl 6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (10). Prepared from 1 g of compound **3** (3.73 mmol). Yellow solid (40%). mp > 260 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 14.53 (s, 1H), 8.91 (d, *J* = 6.9 Hz, 1H), 8.76 (s, 1H), 8.50 (d, *J* = 1.9 Hz, 1H), 8.48 (s, 1H), 8.29 (d, *J* = 9.3 Hz, 1H), 4.26 (q, *J* = 7.1 Hz, 2H), 1.31 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 171.8, 164.6, 144.6, 142.5, 138.7, 134.0, 132.5, 128.6, 127.3, 125.5, 121.7, 118.5, 114.2, 60.5, 14.7. IR (KBr) υ (cm^{-1}) 3121, 2902, 1706, 1693, 1521, 1468, 1326, 1012, 817, 738. LC-MS (ESI) t_R = 3.70 min; *m/z* $[M+H]^+$ 314.49. HRMS/ESI: m/z calcd for $C_{15}H_{12}N_3O_5$ $[M+H]^+$ 314.2173, found 314.2169.

Ethyl 1-methyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (11). Prepared from 1 g of compound **4** (3.54 mmol). Yellow solid (58%). mp: > 260 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 9.29 (s, 1H), 9.07 – 8.98 (m, 2H), 8.50 (s, 1H), 7.73 (dd, *J* = 8.7, 4.2 Hz, 1H), 4.62 (s, 3H), 4.38 (q, *J* = 7.1 Hz, 2H), 1.38 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 172.3, 164.8, 153.2, 148.4, 142.1, 141.7, 141.2, 132.6, 127.7, 124.6, 123.7, 123.5, 114.6, 61.5, 50.4, 14.4. IR (KBr) υ (cm^{-1}) 3249, 2924, 1692, 1652, 1524, 1479, 1298, 1111, 701. LC-MS (ESI) t_R = 4.109 min; *m/z* $[M+H]^+$ 328.42. HRMS/ESI: m/z calcd for $C_{16}H_{14}N_3O_5$ $[M+H]^+$ 328.0933, found 328.0935.

Ethyl 1-ethyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (12). Prepared from 1 g of compound **5** (3.37 mmol). Yellow solid (52%). mp 239 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 9.18 (dd, *J* = 4.1, 1.7 Hz, 1H), 9.02 (dd, *J* = 8.8, 1.7 Hz, 1H), 8.97 (s, 1H), 8.75 (s, 1H), 7.99 (dd, *J* = 8.8, 4.2 Hz, 1H), 5.23 (q, *J* = 6.9 Hz, 2H), 4.30 (q, *J* = 7.1 Hz, 2H), 1.47 (t, *J* = 6.9 Hz, 3H), 1.34 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 172.8, 165.3, 153.8,

150.9, 143.0, 142.0, 141.2, 134.1, 128.1, 126.6, 124.3, 123.8, 115.9, 61.9, 56.2, 16.9, 14.9. IR (KBr) υ (cm^{-1}) 3170, 2964, 1712, 1683, 1502, 1468, 798. LC-MS (ESI) t_R = 3.99 min; *m/z* $[M+H]^+$ 342.32. HRMS/ESI: m/z calcd for $C_{17}H_{16}N_3O_5$ $[M+H]^+$ 342.1783, found 342.1781.

General procedure for hydrolyse of ester. A suspension of the ester derivative in HCl 3N was refluxed for 5 h. After cooling to rt, the precipitate was filtered and washed several times with water.

4-Oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (13). Prepared from 1 g of compound **3** (4.11 mmol). White solid (91%). mp > 260 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 15.61 (s, 1H), 13.92 (s, 1H), 9.19 (dd, *J* = 4.3, 1.9 Hz, 1H), 8.80 (s, 1H), 8.67 (dd, *J* = 8.3, 1.9 Hz, 1H), 8.33 (d, *J* = 8.9 Hz, 1H), 8.08 (d, *J* = 8.9 Hz, 1H), 7.95 (dd, *J* = 8.3, 4.4 Hz, 1H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 172.5, 164.9, 152.4, 148.8, 141.4, 137.8, 137.3, 131.3, 129.5, 125.6, 123.7, 123.5, 112.7. IR (KBr) υ (cm^{-1}) 2981, 1709, 1618, 1468, 1302, 1271, 810, 719. LC-MS (ESI) t_R = 4.25 min; *m/z* $[M+H]^+$ 241.50. HRMS/ESI: m/z calcd for $C_{13}H_9N_2O_3$ $[M+H]^+$ 241.0575, found 241.0573.

1-Methyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (14). Prepared from 1 g of compound **4** (3.54 mmol). White solid (90%). mp > 260 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 15.53 (s, 1H), 9.17 (dd, *J* = 4.2, 1.8 Hz, 1H), 9.10 (s, 1H), 8.66 (dd, *J* = 8.3, 1.8 Hz, 1H), 8.47 (d, *J* = 8.7 Hz, 1H), 8.15 (d, *J* = 8.7 Hz, 1H), 7.90 (dd, *J* = 8.3, 4.2 Hz, 1H), 4.83 (s, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 172.5, 164.9, 152.4, 148.8, 141.4, 137.8, 137.3, 131.3, 129.5, 125.6, 123.7, 123.5, 112.7, 49.9 IR (KBr) υ (cm^{-1}) 2919, 1790, 1598, 1421,1325, 1081,

815, 758. LC-MS (ESI) t_R = 3.80 min; *m/z* $[M+H]^+$ 255.44. HRMS/ESI: m/z calcd for $C_{14}H_{11}N_2O_3$ $[M+H]^+$ 255.0994, found 255.0993.

1-Ethyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (15). Prepared from 1 g of compound **5** (3.37 mmol). White solid (93%). mp > 260 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 15.17 (s, 1H), 9.01 (dd, *J* = 4.2, 1.9 Hz, 1H), 8.76 (s, 1H), 8.51 (d, *J* = 8.8 Hz, 1H), 8.28 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.81 (d, *J* = 8.8 Hz, 1H), 7.63 (dd, *J* = 8.3, 4.2 Hz, 1H), 5.39 (q, *J* = 7.0 Hz, 2H), 1.56 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 177.5, 166.9, 150.2, 148.7, 140.7, 137.6, 137.0, 131.7, 127.0, 126.5, 123.6, 123.2, 111.2, 56.6, 16.9. IR (KBr) υ (cm^{-1}) 2935, 1768, 1603, 1531, 1468, 1309, 1082, 809, 728. LC-MS (ESI) t_R = 3.85 min; *m/z* $[M+H]^+$ 269.36. HRMS/ESI: m/z calcd for $C_{15}H_{13}N_2O_3$ $[M+H]^+$ 269.2198, found 269.2195.

6-Bromo-1-ethyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (16). Prepared from 1 g of compound **9** (2.67 mmol). White solid (95%). mp > 260 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 14.91 (s, 1H), 9.10 (dd, *J* = 4.2, 1.8 Hz, 1H), 8.90 (s, 1H), 8.85 (s, 1H), 8.80 (dd, *J* = 8.5, 1.8 Hz, 1H), 7.81 (dd, *J* = 8.5, 4.2 Hz, 1H), 5.43 (q, *J* = 7.0 Hz, 2H), 1.64 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*)) δ 176.4, 166.4, 150.5, 149.0, 141.3, 137.2, 136.8, 130.6, 128.1, 126.8, 124.5, 120.6, 111.6, 56.8, 16.8. IR (KBr) υ (cm^{-1}) 3431, 3054, 2935, 1730, 1614, 1526, 1455,1260, 1093, 833, 732. LC-MS (ESI) t_R = 4.29 min; *m/z* $[M+H]^+$ 348.29 /349.45. HRMS/ESI: m/z calcd for $C_{15}H_{12}BrN_2O_3$ $[M+H]^+$ 348.2124, found 348.2122.

1-Methyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (17). Prepared from 1 g of compound **11** (3.06 mmol). Yellow solide (94%). mp 248 °C. ^{1}H NMR (399.75

MHz, DMSO-d_6) δ 14.75 (s, 1H), 9.28 (dd, *J* = 4.1, 1.7 Hz, 1H), 9.19 (s, 1H), 9.11 (s, 1H), 9.07 (dd, *J* = 8.8, 1.7 Hz, 1H), 8.09 (dd, *J* = 8.8, 4.1 Hz, 1H), 4.79 (s, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 176.7, 165.4, 154.4, 150.1, 143.2, 142.1, 141.2, 133.0, 132.8, 126.0, 124.6, 123.7, 111.5, 51.2. IR (KBr) υ (cm^{-1}) 3428, 3123, 2921, 1726, 1624, 1501, 1408, 1296, 1087, 828, 730 LC-MS (ESI) t_R = 3.75 min; *m/z* $[M+H]^+$ 299.37. HRMS/ESI: m/z calcd for $C_{14}H_{10}N_3O_5$ $[M+H]^+$ 299.2613, found 299.2614.

1-Ethyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (18). Prepared from 1 g of compound **12** (2.93 mmol). Yellow solid (91%). mp 205 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 14.70 (s, 1H), 9.29 (dd, *J* = 4.2, 1.7 Hz, 1H), 9.19 (s, 1H), 9.10 (d, *J* = 1.5 Hz, 1H), 9.07 (dd, *J* = 8.7, 1.7 Hz, 1H), 8.10 (dd, *J* = 8.8, 4.2 Hz, 1H), 5.47 (q, *J* = 6.9 Hz, 2H), 1.54 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 176.8, 165.1, 153.2, 150.3, 143.4, 141.3, 141.1, 133.1, 126.0, 125.3, 123.7, 121.2, 112.4, 56.4, 16.7. IR (KBr) υ (cm^{-1}) 3358, 2921, 1798, 1644, 1536, 1424, 1325, 1287, 1042, 738. LC-MS (ESI) t_R = 3.58 min; *m/z* $[M+H]^+$ 314.29. HRMS/ESI: m/z calcd for $C_{15}H_{12}N_3O_5$ $[M+H]^+$ 314.2535, found 314.2532.

General procedure for reduction of nitro group. To a suspension of nitro compound (1 equiv) in CH_3CO_2H (6 mL/mmol) was added iron (11.71 equiv) by portion. The reaction mixture was warmed at 80 °C for 2 h. After cooling to rt, EtOH (12 mL/mmol) was added. The mixture was then filtered through Celite. The filtrate was concentrated *in vacuo* and the residue was triturated in water (12 mL/mmol). After cooling to 0 °C and filtration, the amino compound was obtained.

Ethyl 6-amino-1-methyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (19). Prepared from 1 g of compound **17** (3.05 mmol). Yellow solid (60%). mp 234 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 9.00 (dd, *J* = 4.1, 1.7 Hz, 1H), 8.71 (dd, *J* = 8.5, 1.8 Hz, 1H), 8.50 (s, 1H), 7.71 (dd, *J* = 8.5, 4.1 Hz, 1H), 7.54 (s, 1H), 6.27 (s, 2H), 4.56 (s, 3H), 4.24 (q, *J* = 7.1 Hz, 2H), 1.31 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 172.2, 165.3, 150.0, 148.2, 143.1, 142.3, 135.8, 131.9, 131.7, 128.7, 121.8, 109.7, 101.7, 60.0, 49.8, 14.8. IR (KBr) υ (cm^{-1}) 3398, 3330, 2921; 1673, 1597, 1487, 1287, 1099, 858, 744. LC-MS (ESI) t_R = 3.49 min; *m/z* $[M+H]^+$ 298.54. HRMS/ESI: m/z calcd for $C_{16}H_{16}N_3O_3$ $[M+H]^+$ 298.2584, found 298.2581.

Ethyl 6-amino-1-ethyl-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (20). Prepared from 1 g of compound **12** (2.92 mmol). Yellow solid (70%). mp > 260 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 9.07 (dd, *J* = 4.1, 1.7 Hz, 1H), 8.72 (dd, *J* = 8.5, 1.8 Hz, 1H), 8.53 (s, 1H), 7.77 (dd, *J* = 8.5, 4.1 Hz, 1H), 7.56 (s, 1H), 6.28 (s, 2H), 5.27 (q, *J* = 6.8 Hz, 2H), 4.25 (q, *J* = 7.0 Hz, 2H), 1.37 (t, *J* = 6.6 Hz, 3H), 1.31 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 172.1, 165.3, 150.0, 148.2, 143.1, 142.3, 135.8, 131.9, 131.7, 128.7, 121.8, 109.7, 101.7, 60.0, 56.5, 16.7, 14.8. IR (KBr) υ (cm^{-1}) 3314, 3153,1682, 1591, 1519, 1499, 1395, 1201, 1095, 846, 735. LC-MS (ESI) t_R = 3.65 min; *m/z* $[M+H]^+$ 312.50. HRMS/ESI: m/z calcd for $C_{17}H_{18}N_3O_3$ $[M+H]^+$ 312.2587, found 312.2587.

1-Ethyl-4-oxo-*N*-phenyl-1,4-dihydro-1,10-phenanthroline-3-carboxamide (21). 615 □L of aniline (2 equiv, 6.74 mmol) were added to a suspension of 1 g of compound **5** (3.37 mmol) in 15 mL of diphenyl ether. The reaction mixture was refluxed for 45 min. After cooling to rt, some EP was added, the resulting solid is obtained by filtration and then purified on flash

chromatography (SiO_2 ; eluent : DCM) to obtain the product as a white solid (48%). mp 256 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 12.33 (s, 1H), 8.94 (dd, *J* = 4.2, 1.9 Hz, 1H), 8.85 (s, 1H), 8.57 (d, *J* = 8.7 Hz, 1H), 8.21 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.78 – 7.71 (m, 3H), 7.54 (dd, *J* = 8.2, 4.1 Hz, 1H), 7.33 – 7.27 (m, 2H), 7.04 (t, *J* = 7.3 Hz, 1H), 5.36 (q, *J* = 7.0 Hz, 2H), 1.54 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 175.7, 162.7, 149.8, 148.2, 140.9, 138.8, 137.1, 136.7, 131.3, 129.1, 128.9 (2C), 125.4, 123.8, 123.8, 123.1, 120.4 (2C), 114.4, 56.0, 16.9. IR (KBr) υ (cm^{-1}) 3136, 2884, 1667, 1504, 1340, 1201, 845, 789. LC-MS (ESI) t_R = 3.49 min; *m/z* $[M+H]^+$ 344.46. HRMS/ESI: m/z calcd for $C_{21}H_{18}N_3O_2$ $[M+H]^+$ 344.9388, found 344.9385.

General procedure for the preparation of amides and hydrazides 22-26. Oxalyl chloride (1.5 equiv) and DMF (2 drops) were added to a suspension of the acid in dry THF (30 mL/mmol). The reaction mixture was stirred at rt for 3 h and then evaporated to give the acyl chloride. This latter is dissolved in dry CH_3CN (30 mL/mmol) and then a solution of amine or hydrazine (1.2 equiv) in the same amount of dry CH_3CN as previously was added dropwise. The reactional mixture was stirred at rt for 2 h and filtered. The resulting precipitate was purified by some washes with EP or recristallization ou or by column chromatography.

1-Methyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxamide (22). Prepared from 1 g of compound **17** (3.34mmol). Yellow solid (38 %) after wash with EP. mp > 260 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 9.22 (dd, *J* = 4.1, 1.7 Hz, 1H), 9.12 (m, 2H), 9.06 (dd, *J* = 8.7, 1.7 Hz, 1H), 8.94 (s, 1H), 8.03 (dd, *J* = 8.8, 4.2 Hz, 1H), 7.78 (d, *J* = 4.2 Hz, 1H), 4.69 (s, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 174.6, 164.8, 153.2, 149.4, 142.4, 141.9, 132.6, 131.4, 126.1, 125.5, 123.4, 122.3, 115.7, 50.0. IR (KBr) υ (cm^{-1}) 3363, 3119, 2972, 1686, 1575, 1505,

1350, 1092, 817, 738. LC-MS (ESI) t_R = 3.96 min; *m/z* $[M+H]^+$ 299.42. HRMS/ESI: m/z calcd for $C_{14}H_{11}N_4O_2$ $[M+H]^+$ 299.9551, found 299.9549.

1-Methyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carbohydrazide (23). Prepared from 1 g of compound **17** (3.34 mmol). Yellow solid (54%) after wash with EP. mp 252 – 254 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 10.43 (s, 1H), 9.18 (dd, *J* = 4.1, 1.7 Hz, 1H), 9.10 (s, 1H), 9.06 (dd, *J* = 8.7, 1.7 Hz, 1H), 8.85 (s, 1H), 7.99 (dd, *J* = 8.8, 4.2 Hz, 1H), 4.72 (s, 3H), 4.61 (s, 2H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 174.7, 163.7, 149.6, 149.1, 140.8, 137.5, 137.0, 131.4, 128.8, 125.7, 123.8, 123.5, 113.8, 55.1. IR (KBr) υ (cm^{-1}) 3495, 3290, 2917, 1695; 1517, 1458, 1329, 1094, 819, 781. LC-MS (ESI) t_R = 3.42 min; *m/z* $[M+H]^+$ 314.40. HRMS/ESI: m/z calcd for $C_{14}H_{12}N_5O_4$ $[M+H]^+$ 314.0800, found 314.0795.

1-Ethyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxamide (24). Prepared from 1 g of compound **18** (3.19 mmol). Yellow solid (45%) after wash with EP. mp > 260 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 9.23 (dd, *J* = 4.1, 1.7 Hz, 1H), 9.16 (s, 1H), 9.07 (dd, *J* = 8.8, 1.6 Hz, 1H), 9.03 (s, 1H), 8.93 (s, 1H), 8.03 (dd, *J* = 8.8, 4.2 Hz, 1H), 7.49 (s, 1H), 5.38 (q, *J* = 6.9 Hz, 2H), 1.53 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 174.6, 164.7, 152.1, 149.7, 141.2, 141.1, 132.8, 131.4, 126.5, 125.6, 123.2, 122.5, 116.2, 55.4, 16.6. IR (KBr) υ (cm^{-1}) 3314, 3153, 2942, 1682, 1591, 1519, 1499, 1334, 815, 795. LC-MS (ESI) t_R = 4.04 min; *m/z* $[M+H]^+$ 313.38. HRMS/ESI: m/z calcd for $C_{15}H_{13}N_4O_4$ $[M+H]^+$ 313.0855, found 313.0853.

1-Ethyl-6-nitro-4-oxo-1,4-dihydro-1,10-phenanthroline-3-carbohydrazide (25). Prepared from 1 g of compound **18** (3.19 mmol). Yellow solid (52%) after wash with EP. mp 248 °C. ^{1}H

NMR (399.75 MHz, DMSO-d_6) δ 10.43 (s, 1H), 9.19 (dd, *J* = 4.2, 1.7 Hz, 1H), 9.01 (dd, *J* = 8.8, 1.6 Hz, 1H), 8.98 (s, 1H), 8.87 (s, 1H), 8.00 (dd, *J* = 8.7, 4.1 Hz, 1H), 5.32 (q, *J* = 6.8 Hz, 2H), 4.72 (s, 2H), 1.48 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 176.7, 165.1, 153.3, 150.3, 143.2, 141.3, 141.0, 131.1, 126.1, 125.1, 123.6, 121.2, 112.3, 56.4, 16.8. IR (KBr) υ (cm^{-1}) 3274, 3115, 2824, 1667, 1620, 1461, 1193, 1002, 759. LC-MS (ESI) t_R = 3.58 min; *m/z* $[M+H]^+$ 328.42. HRMS/ESI: m/z calcd for $C_{15}H_{14}N_5O_4$ $[M+H]^+$ 328.2229, found 328.2227.

1-Ethyl-6-nitro-4-oxo-*N*-phenyl-1,4-dihydro-1,10-phenanthroline-3-carboxamide (26). Prepared from 1 g of compound **18** (3.19 mmol). Yellow solid (54%) after purification on flash chromatography (SiO_2 ; eluent : DCM/EtOAc/TEA (8.25/0.5/0.25)). mp > 260 °C. ^{1}H NMR (399.75 MHz, DMSO-d_6) δ 11.96 (s, 1H), 9.24 (dd, *J* = 4.2, 1.8 Hz, 1H), 9.17 (s, 1H), 9.06 (dd, *J* = 8.8, 1.8 Hz, 1H), 9.03 (s, 1H), 8.03 (dd, *J* = 8.7, 4.1 Hz, 1H), 7.72 (d, *J* = 7.9 Hz, 2H), 7.39 (t, *J* = 7.9 Hz, 2H), 7.13 (t, *J* = 7.3 Hz, 1H), 5.43 (q, *J* = 6.9 Hz, 2H), 1.58 (td, *J* = 6.9, 1.4 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 174.8, 161.7, 152.2, 149.9, 142.6, 141.1, 140.9, 138.9, 132.9, 129.4 (2C), 126.2, 125.7, 124.3, 123.4, 122.2, 120.3 (2C), 115.4, 55.9, 16.8. IR (KBr) υ (cm^{-1}) 3323, 3019, 2927, 1676, 1603, 1421, 1378, 1278, 1150, 795. LC-MS (ESI) t_R = 3.48 min; *m/z* $[M+H]^+$ 389,46. HRMS/ESI: m/z calcd for $C_{21}H_{17}N_4O_4$ $[M+H]^+$ 389.1834, found 389.1831.

Ethyl 10-hydroxy-4-oxo-1H-1,10-phenanthroline-3-carboxylate (27). To a solution of 0.5 g of compound **3** (1 equiv; 1.86 mmol) in 25 mL of DCM at 0 °C were added 0.48 g of *m*-CPBA (1.5 equiv) by portion. The reaction mixture was stirred under room temperature for 15 h and then neutralized with a saturated K_2CO_3 solution. The precipitate was filtered, washed with water and then recristallized with DMF to obtain the desired product (67%). mp 204 °C. ^{1}H NMR (399.75

MHz, DMSO-d_6) δ 15.10 (s, 1H), 8.79 (m, 2H), 8.33 (d, *J* = 8.8 Hz, 1H), 8.20 (d, *J* = 1.1 Hz, 1H), 7.94 (d, *J* = 8.9 Hz, 1H), 7.77 (dd, *J* = 8.4, 6.2 Hz, 1H), 4.26 (q, *J* = 7.1 Hz, 2H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, DMSO-d_6) δ 172.3, 165.0, 143.9, 139.0, 134.2, 133.7, 130.5, 128.6, 127.3, 125.5, 125.0, 124.3, 113.5, 60.3, 14.8. IR (KBr) υ (cm^{-1}) 3334, 2975, 1701, 1609, 1508, 1365, 1239, 1165, 842, 733. LC-MS (ESI) t_R = 2.84 min; *m/z* $[M+H]^+$ 285,46. HRMS/ESI: m/z calcd for $C_{15}H_{13}N_2O_4$ $[M+H]^+$ 285.9935, found 285.9931.

Ethyl 7-nitro-10-hydroxy-4-oxo-1H-1,10-phenanthroline-3-carboxylate (28). To a solution of 0.304 g *N*-oxide compound **27** (1 equiv; 1.05 mmol) in 1.08 mL of concentrated H_2SO_4 was added dropwise 496 □L of HNO_3 65 % (6.7 equiv; 7.05 mmol). The resulting mixture was warmed at 80°C for 4 h. After cooling, ice was added and the obtained precipitate was filtered, washed with a solution of $NaHCO_3$ 5%. The product was purified by recristallization (DMF/AcOEt) (34%). mp 196 °C. ^{1}H NMR (399.75 MHz, $CDCl_3$-*d*) δ 14.77 (s, 1H), 8.86 (d, *J* = 9.4 Hz, 1H), 8.69 (d, *J* = 6.7 Hz, 1H), 8.60 (d, *J* = 6.9 Hz, 1H), 8.49 (d, *J* = 9.4 Hz, 1H), 8.26 (d, *J* = 6.9 Hz, 1H), 4.44 (q, *J* = 7.1 Hz, 2H), 1.44 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100.53 MHz, $CDCl_3$-*d*) δ 171.8, 164.6, 144.6, 142.5, 138.7, 134.0, 132.5, 128.6, 127.3, 125.6, 121.7, 118.5, 114.2, 60.5, 14.7. IR (KBr) υ (cm^{-1}) 3224, 3100, 2985, 1685, 1589, 1405, 1249, 1185, 1032, 723. LC-MS (ESI) t_R = 3.44 min; *m/z* $[M+H]^+$ 330.50. HRMS/ESI: m/z calcd for $C_{15}H_{12}N_3O_6$ $[M+H]^+$ 330.1139, found 330.1134.

Bacterial Strains and Growth Conditions

M. smegmatis,[21] *M. aurum*,[22] *M. marinum*,[23] were grown in Middlebrook 7H9 broth (Difco) supplemented with ADC.

M. tuberculosis strains H37Rv [24] and XDR-TB was grown in Middlebrook 7H9 broth (Difco) supplemented with ADC and 0,05% Tween 80, except when referred, or in 7H11 Middlebrook broth (Difco) supplemented with OADC and 0,5% glycerol for CFU analysis.

Spontaneous ofloxacin resistant mutants of *M. tuberculosis* (H37Rv) were selected on 7H11 plates supplemented with OADC and 20X MIC of antibiotic at 37°C after four weeks of incubation. The *gyrA* and *gyrB* genes were sequenced. Mutations known to confer fluoroquinolone resistance were found.

Enterobacter aerogenes, *Enterobacter cloacae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii* were grown in Mueller Hinton Broth. These isolates consisted of clinical isolates from the Bichat-Claude Bernard Hospital in Paris as well as XDR-TB.

Resazurin Microtiter Assay (REMA)

The assays were carried out as previously described [13, 14] with some modifications. The microdilution tests were performed in 96-well plates. Two-fold dilutions of each drug were prepared in the test wells in complete 7H9 broth, the final drug concentrations were as follows: rifampicin, ofloxacin and amikacin were prepared in a stock solution with a final concentration ranging from 10 to 0.0048 µg/mL. Exponential phase culture is diluted to a final OD_{600} of 0.005 in 100µL of the 7H9 medium without tween, were subsequently treated with different compounds concentrations. Control wells were prepared with culture medium only and bacterial suspension only. The plates were sealed and incubated for 7 days at 37°C. After the incubation

time, 30 μL of 0,01 % resazurin solution were added per well, coloring them blue. Plates were incubated at 37°C for additional 24h. After incubation, plates were red for color change from blue to pink in live bacteria-containing wells. MIC was defined as the lowest drug concentration that prevented resazurin colour change from blue to pink. Each MIC was determined three times in duplicate experiments. Viable bacteria from control wells and from tests wells were plated onto 7H11 agar medium in order to determine Minimal bactericidal concentration (MBC).

Toxicity Assays

Vero monkey cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mm glutamine and Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture. When the cell monolayer became confluent, Vero cells were removed from the flask and seeded at 25000 cells per well in a tissue-culture treated 96-well plate. The mixture was incubated for 24 hours after the compound was added while keeping the final DMSO concentration at 1%. The plates were incubated 24 and 48 hours before ATP content determination. The CellTiter-Glo kit from Promega was used to measure ATP content of the cell represented by a luminescent signal as an indicator of viability. IC_{50} was defined as the concentration of compound that caused a 50% decrease in the ATP signal compared to the DMSO control.

Macrophage Assays

The assays were carried out as described by Tailleux et al., 2003 [15] with some modifications. Briefly, monocytes were isolated from healthy donors by CD14 positive selection using CD14 microbeads (Miltenyi Biotec). For the assay, cells were differentiated in RPMI-1640 medium

containing 20 ng/mL GM-CSF (R&D Systems) at 10% O_2, 5% CO_2. After 1 week, cells were plated on 24 well plates and infected with wild-type *M. tuberculosis* H37Rv at multiplicity of infection (MOI) of 0.5. After 24 h of infection, test compound was added. Infected human macrophages containing drug or not were then incubated for 6 days. Macrophages were visually inspected for viability and CellTiter-Glo assay was used as control. If drug-treated macrophages looked healthy, they were lysed with 0.1% Triton-X for enumeration of intracellular bacteria by plating on Middlebrook 7H11 agar plates containing OADC supplement.

Fluorescence microscopy

Escherichia coli ATCC 25922 were cultivated in LB broth and *Mycobacterium smegmatis* in 7H9 broth. 2 mL of exponential-phase cell cultures was treated with antibiotic ofloxacine (2 µg/mL final) or compound **17** (2.5 µg/mL final) and grown at 37°C during 1.5 hour in the case of *E. coli*, 6 hours in the case of *M. smegmatis*. Then, treated culture were harvested, pelleted by centrifugation, washed with PBS, and fixed with 4% PFA in PBS during 10 min. After washing in PBS, the pellet was resuspended in PBS containing 2 µg/mL DAPI and 1 µg/mL FM 4-64 (invitrogen) and incubated at room temperature for 20 minutes in order to stain DNA and cell membrane. After a final washing in PBS, 5 µL stained culture was placed between glass slide and coverslip for microscopy. Fluorescence signals were detected by using Olympus FV1200 confocal microscope equipped with an oil immersion 60x objective. Images were analyzed using Olympus Fluoview software.

Resistance study

For single step resistance *Mycobaterium marinum* at 10^{10} c.f.u. were plate onto 7H10 containing 4x, 8x, 12x and 16 x MIC of **11**, **12**, **17** and **18**. After five days of incubation at 32 °C, no resistant colonies were detected except for g (8x and 12xMIC). For *M. tuberculosis*, cells were culture in 7H9 medium and plated at 10^8 cells per mL on 10 plates and incubated for 3 weeks a 37°C for colony counts. No colonies were detected.

For resistance development by sequential passages *M. marinum* cells at exponential phase were diluted to an A600nm (OD_{600}) of 0.01 in 1 mL of 7H9 supplemented with ADC containing of **11**, **12**, **17** and **18**. Cells were incubated at 32°C with agitation, and passaged at four days intervals in the presence of each compound. The MIC was determined by broth microdilution. Experiments were performed with biological replicates.

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Author Contributions

SC and MC contributed equally to the study. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

Abbreviations: Tuberculosis, TB; fluoroquinolones, FQ; Multi-drug resistant, MDR; rifampicin, RMP; isoniazid INH; extensively drug resistant, XDR; multi-drug resistance, MDR; kanamycin, KM; amikacin, AMK; capreomycin, CPM.

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Graphical abstract