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New 6-nitroquinolones: synthesis and antimicrobial activities

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Abstract

Pursuing our searches on quinolonecarboxylic acids we used a simple three-step one pot procedure to synthesize novel 1,7-disubstituted-6-nitroquinolones. The new derivatives were tested against *Mycobacterium tuberculosis* and *Mycobacterium avium* complex (MAC) as well as against both gram-positive and gram-negative bacteria. In vitro assays showed some derivatives were endowed with good inhibiting activities against tested mycobacteria. Some derivatives were also found more potent than ciprofloxacin and ofloxacin (used as reference drugs) against gram-positive bacteria.

Keywords: Nitroquinolones; Antimycobacterial agents; Antibacterial agents; Tuberculosis

1. Introduction

Tuberculosis (TB) is a growing international health concern, since it is the leading infectious cause of death in the world today [1,2]. Moreover, the resurgence of TB in industrialized countries and the worldwide increase in the prevalence of *Mycobacterium avium* complex (MAC) infections in immunocompromised hosts have prompted the quest for new antimycobacterial drugs [3-6].

In particular, the appearance of multidrug-resistant (MDR) strains of *M. tuberculosis*, which exhibit in vitro resistance to at least two major antituberculous drug (usually isoniazid and rifampin) and cause intractable TB, has greatly contributed to the increased incidence of TB [7-11].

Although *M. tuberculosis* (two of its strains) was the first bacterial species to have the entire genome sequenced, this was of little help in identifying targets for the development of novel antimycobacterial drugs, mainly due to the complexity of this genoma (with at least 100 genes involved, e.g., in the synthesis of mycobacterial cell wall) [12].

Therefore, because of the global health problems of TB, the increasing rate of MDR-TB and the high rate of a co-infection with HIV, the development of potent new antituberculous drugs without cross-resistance with known antimycobacterial agents is still urgently needed [13,14].

Many groups have recently reported [15-29] the synthesis and antimycobacterial activity of novel classes of compounds, including isonicotinic acid hydrazides, pyrazinoic acids, β-lactams, compounds containing a thiocarbonyl group (thioamides, thioureas), compounds containing an alkylthio group bound to an electrondeficient atom of carbon, oxazolidinones, fluoroquinolones, nitroimidazoles, and others.

Taking account that fluoroquinolones exhibit fairly good antimycobacterial activity [30] and having in mind the not fully expressed antitubercular potential of nitro pharmacophore [31], in a preliminary report we recently described the synthesis and evaluation of a few novel quinolones as potential antimycobacterial agents [18].

The compounds were evaluated against both gram-positive and gram-negative bacteria and against various mycobacteria. The results of these studies revealed good antibacterial activity of tested compounds against *Streptococcus* and *Staphylococcus* but, above all, some of these derivatives showed to be effective against *M. tuberculosis* and other mycobacteria.

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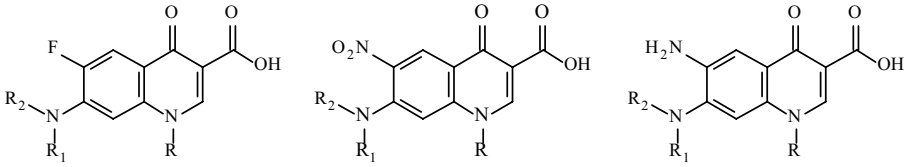


Fig. 1. Novel 6-Fluoro-, 6-nitro- and 6-aminoquinolones **1-3**. Simmetrically substituted quinolones (**1a-e, 2a-l, 3a,b**): R = R_1 = H, alkyl, cycloalkyl, 2,4-difluorophenyl; R_2 = H. Unsimmetrically substituted quinolones (**2m-r**): R = R_1 = *tert*-butyl, cyclopropyl; R_2 = H or R = *tert*-butyl, cyclopropyl; R_1-R_2 = morpholine, thiomorpholine.

In the present paper we wish to report the synthesis and the antimicrobial activities (both against mycobacteria and gram-positive and -negative bacteria) of a series of 6-nitroquinolone carboxylic acids together with a few 6-fluoro- and 6-amino-analogues (**1a-e, 2a-r**, and **3a,b**; Figure 1).

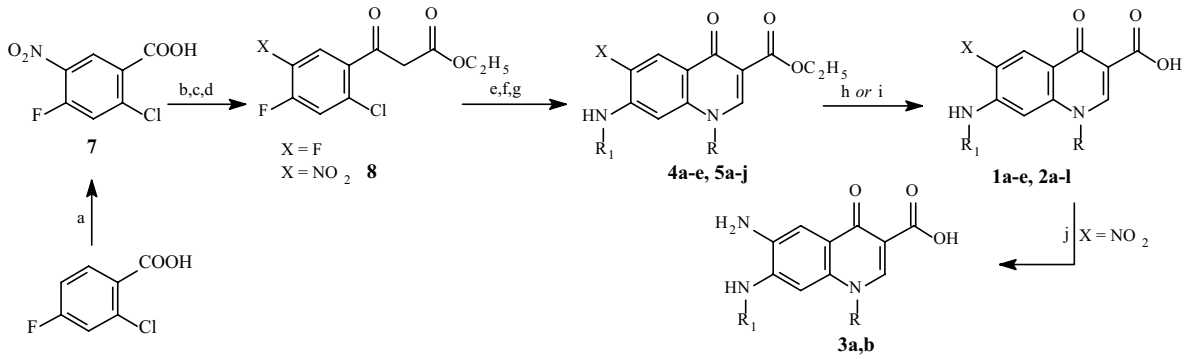
2. Chemistry

1,7-Disubstituted-6-fluoro-, 6-nitro- and 6-amino-4-oxo-1,4-dihydro-quinoline-3-carboxylic acids **1-3** were synthesized as outlined in Scheme 1 and in Scheme 2.

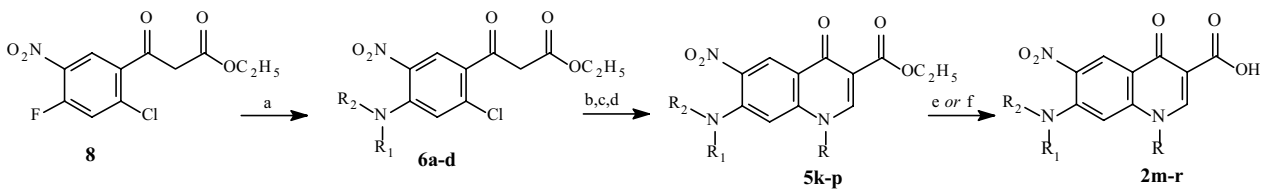
In particular, symmetrically substituted 6-fluoro- and 6-nitro-quinolones **1a-e** and **2a-l** were obtained starting from ethyl 3-(2-chloro-4,5-difluorophenyl)-3-oxopropanoate [32] or ethyl 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropanoate **8** [synthesized by us following the procedure illustrated in reference 32], respectively, by a three-step one-pot reaction with triethyl orthoformate in acetic anhydride, the proper amine (2.2 eq) in methyl sulphoxide and, at last, potassium carbonate to yield ethyl quinolonecarboxylates **4a-e** and **5a-j**, furtherly hydrolyzed (in acidic or basic conditions) to the corresponding quinolonecarboxylic acids **1a-e** and **2a-l** (Scheme 1). 6-Aminoquinolones **3a,b** were obtained by reduction of the corresponding 6-nitrosubstituted derivatives **2g,h** with stannous chloride in the presence of concentrated hydrochloric acid.

Unsymmetrically substituted 6-nitroquinolones **2m-r** were obtained starting from ethyl 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropanoate **8** by treatment with the proper amine (1.1 eq) in the presence of potassium carbonate, to yield 3-(2-chloro-5-nitro-4-substituted phenyl)-3-oxopropionic ethyl esters **6a-d** (Scheme 2). These intermediates were then converted into the ethyl quinolonecarboxylates **5k-p** by a three-step one-pot procedure similar to that described before. Ethyl esters were finally hydrolyzed (in acidic or basic conditions) to the desired quinolonecarboxylic acids **2m-r**.

When the 1-*tert*-butyl-7-*tert*-butylamino-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester **5e** was hydrolyzed in acidic conditions, unsubstituted 7-amino-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid **2a** was



Scheme 1. Synthesis of compounds **1a-e, 2a-l**, and **3a,b**. Reagents and conditions: a) HNO_3, H_2SO_4; b) $SOCl_2$, reflux; c) $KOOCCH_2COOEt$, $MgCl_2$, Et_3N, acetonitrile; d) H_2O, HCl; e) $HC(OEt)_3$, Ac_2O, reflux; f) RNH_2 (R = R_1), 2.2 eq, DMSO, r.t.; g) K_2CO_3, 90 °C; h) NaOH, H_2O, reflux; i) Acetic acid, H_2O, H_2SO_4, reflux; j) $SnCl_2$, HCl 37%, ethanol, reflux.



Scheme 2. Synthesis of compounds **2m-r**. Reagents: a) HNR_1R_2, K_2CO_3, CH_3CN, reflux; b) $HC(OEt)_3$, Ac_2O, reflux; c) RNH_2, DMSO, r.t.; d) K_2CO_3, 90 °C; e) NaOH, H_2O, reflux; f) Acetic acid, H_2O, H_2SO_4, reflux.

Table 1
In vitro antimycobacterial activity of compounds **1-3**

Compd	R	R_1	R_2	CC_{50}[a]	MIC_{50}[b]/MIC_{90}[c]	
				MT-4	M. tuberculosis *ATCC 27294 (wt)*	*M. avium* complex
1a[d]	cyclopropyl	cyclopropyl	H	>200	>200	>200
1b	cyclobutyl	cyclobutyl	H	>200	>200	>200
1c	cyclopentyl	cyclopentyl	H	>200	>200	>200
1d	cyclohexyl	cyclohexyl	H	>200	>200	>200
1e[d]	*tert*-butyl	*tert*-butyl	H	>200	126/>200	>200
2a	H	H	H	>200	>200	>200
2b	ethyl	ethyl	H	>200	>200	>200
2c	2-F-ethyl	2-F-ethyl	H	>200	77/>200	26/100
2d	ethenyl	2-F-ethyl	H	54	>200	>200
2e	*iso*-propyl	*iso*-propyl	H	80	2.0/22.6	>200
2f	*iso*-amyl	*iso*-amyl	H	>200	>200	>200
2g[d]	*tert*-butyl	*tert*-butyl	H	140	1.4/6	1.2/12
2h[d]	cyclopropyl	cyclopropyl	H	66.6	>200	>200
2i	cyclobutyl	cyclobutyl	H	>100	>200	75.5/>200
2j	cyclopentyl	cyclopentyl	H	>200	>200	81/>200
2k	cyclohexyl	cyclohexyl	H	>200	>200	>200
2l	2,4-F_2-phenyl	2,4-F_2-phenyl	H	118	>200	>200
2m[d]	*tert*-butyl	cyclopropyl	H	>200	31/>200	47/>200
2n	*tert*-butyl	morpholine		166	0.2/75	13.5/50
2o	*tert*-butyl	thiomorpholine		>200	1.6/100	9/46
2p[d]	cyclopropyl	*tert*-butyl	H	192	35/>200	54/>200
2q	cyclopropyl	morpholine		104	14.6/65	2.3/8.9
2r	cyclopropyl	thiomorpholine		78	8/100	1.1/5.8
3a[d]	cyclopropyl	cyclopropyl	H	>200	>200	129/>200
3b[d]	*tert*-butyl	*tert*-butyl	H	>200	168/>200	140/>200
CIP[e]				60	1/2.5	1/2
OFX[f]				>200	3/5	1.5/3

[a] Compound dose (μM) required to reduce the viability of MT-4 cells by 50%, as determined by the MTT method
[b] Minimum inhibitory concentration (μM) required to reduce the number of viable Mycobacteria by 50%, as determined by the MTT method
[c] Minimum inhibitory concentration (μM) required to reduce the number of viable Mycobacteria by 90%, as determined by the MTT method
[d] Rif. 18.
[e] CIP: ciprofloxacin; [f]OFX: ofloxacin

obtained. The reaction of ethyl 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropanoate with 2-fluoroethylamine according to the described procedure furnished the expected 1-(2-fluoroethyl)-7-(2-fluoroethylamino)-6-nitro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid ethyl ester **5a** together with the 7-(2-fluoroethylamino)-6-nitro-4-oxo-1-vinyl-1,4-dihydroquinoline-3-carboxylic acid ethyl ester **5b** resulting from the dehydrohalogenation of the fluoroethyl side chain at position 1 of quinolone ring. The hydrolysis of the two esters gave the corresponding derivatives **2c** and **2d**. The compound 1-ethyl-7-ethylamino-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid **2b** was directly obtained from 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropionic acid ethyl ester without isolating the ester intermediate since we use ethylamine hydrochloride in the presence of a double amount of potassium carbonate.

3. Biology

The quinolonecarboxylic acids **1-3** were evaluated *in vitro* against *M. tuberculosis* (ATCC 27294 strain), *M. avium* complex (MAC), and representative strains of gram-positive (group D *Streptococcus, Staphylococcus aureus*) and gram-negative (*Salmonella* spp., *Shigella* spp.) bacteria. Ciprofloxacin and ofloxacin were used as reference drugs.

4. Results and discussion

Results of the in vitro evaluation of antibacterial and antimycobacterial activity of tested compounds are reported in Tables 1,2.

When tested against mycobacteria, 1-*tert*-butyl-7-*tert*-butylamino derivative **2g** resulted the most active compound, exhibiting a potency (MIC_{50} = 0.5-1.5 μM) comparable, if not superior, to those of ciprofloxacin and ofloxacin (MIC_{50} = 1-1.2 μM and MIC_{50} = 1.5-3 μM, respectively). The introduction of a morpholine or a thiomorpholine group in the C_7 position of quinolone ring resulted in the very active compounds **2n,o** and **2q,r**, 1-*tert*-butyl derivatives **2n,o** being more active against *M. tuberculosis* whereas 1-cyclopropyl counterparts **2q,r** exhibited fairly good activity against

Table 2
In vitro antibacterial activity of compounds **1-3**

Compd	R	R_1	R_2	CC_{50}[a]	MIC[b]/MBC[c]			
				MT-4	group D *Streptococcus*	*S. aureus*	*Salmonella* spp.	*Shigella* spp.
1a[d]	cyclopropyl	cyclopropyl	H	>200	12/12	12/12	12/12	12/25
1b	cyclobutyl	cyclobutyl	H	>200	>200	>200	>200	>200
1c	cyclopentyl	cyclopentyl	H	>200	>200	>200	>200	>200
1d	cyclohexyl	cyclohexyl	H	>200	>200	>200	>200	>200
1e[d]	*tert*-butyl	*tert*-butyl	H	>200	22/22	7.4/22	7.4/7.4	7.4/7.4
2a	H	H	H	>200	>200	>200	>200	>200
2b	ethyl	ethyl	H	>200	>200	>200	>200	>200
2c	2-F-ethyl	2-F-ethyl	H	>200	6/6	6/6	1/1	1/3
2d	ethenyl	2-F-ethyl	H	54	50/50	25/25	1/3	2/3
2e	*iso*-propyl	*iso*-propyl	H	80	66/200	66/200	>200	>200
2f	*iso*-amyl	*iso*-amyl	H	>200	>200	>200	>200	>200
2g[d]	*tert*-butyl	*tert*-butyl	H	140	0.05/0.05	0.05/0.05	3/6	6/6
2h[d]	cyclopropyl	cyclopropyl	H	66.6	0.8/0.8	0.8/1.5	1.5/1.5	1.5/1.5
2i	cyclobutyl	cyclobutyl	H	>100	2.4/2.4	2.4/2.4	22/22	22/22
2j	cyclopentyl	cyclopentyl	H	>200	22/22	7.4/7.4	66/66	66/200
2k	cyclohexyl	cyclohexyl	H	>200	>200	>200	>200	>200
2l	2,4-F_2-phenyl	2,4-F_2-phenyl	H	118	22/200	22/>200	>200	>200
2m[d]	*tert*-butyl	cyclopropyl	H	>200	5/10	2.5/10	25/25	25/25
2n	*tert*-butyl	morpholine		166	22/22	22/22	22/22	22/66
2o	*tert*-butyl	thiomorpholine		>200	22/22	22/22	66/66	66/66
2p[d]	cyclopropyl	*tert*-butyl	H	192	0.6/2.5	0.6/1.2	25/25	25/25
2q	cyclopropyl	morpholine		104	0.8/0.8	0.8/2.4	0.8/0.8	0.8/0.8
2r	cyclopropyl	thiomorpholine		78	0.2/0.8	0.2/0.8	0.8/2.4	2.4/22
3a[d]	cyclopropyl	cyclopropyl	H	>200	50/50	25/25	>200	>200
3b[d]	*tert*-butyl	*tert*-butyl	H	>200	>125	>125	>125	>125
CIP[e]				60	0.4/0.4	0.4/0.4	0.01/0.01	0.01/0.01
OFX[f]				>200	1.5/1.5	0.8/0.8	0.4/0.4	0.4/0.4

[b]Minimum inhibitory concentration (μM). [c]Minimum bactericidal concentration (μM). [d]Rif. 18. [e]CIP: ciprofloxacin; [f]OFX: ofloxacin
[a] Compound dose (μM) required to reduce the viability of MT-4 cells by 50%, as determined by the MTT method.

M. avium complex. Particularly, 1-*tert*-butyl-7-morpholin-4-yl derivative **2n** (MIC_{50} = 0.2 μM) resulted 7-fold more active than derivative **2g** (MIC_{50} = 1.5 μM), 5-fold more active than ciprofloxacin (MIC_{50} = 1 μM) and 15-fold more active than ofloxacin (MIC_{50} = 3 μM) against *M. tuberculosis*, while 1-cyclopropyl-7-thiomorpholin-4-yl derivative **2r** was slightly less potent than ciprofloxacin and as potent as ofloxacin against *M. avium* complex. The other test compounds resulted essentially inactive.

Contrary to ciprofloxacin and ofloxacin, test compounds were more active against gram-positive than gram-negative bacteria [33-35] except for 7-morpholin-4-yl derivatives **2n** and **2q**, which showed to be active both against gram-positive and -negative bacteria. Derivative **2g** resulted up to 30-fold more potent than reference drugs against gram-positive bacteria, whereas 1-cyclopropyl derivatives **2h** and **2p-r** exhibited a lower but still comparable activity respect to ciprofloxacin and ofloxacin.

In conclusion, the results of our studies confirm the importance of *tert*-butyl group as antimycobacterial pharmacophore, as previously reported [33-35]. Interestingly, even if conferring to test derivatives lower efficacy than the *tert*-butyl moiety, the 1-cyclopropyl group increased the potency of molecules against *M. avium* complex. It is also to be noticed that, when the nitro group is replaced by fluorine or amino group, the antimicrobial activity of quinolones diminishes or vanishes, thus suggesting the presence of strong electronwithdrawing groups at quinolone C6 position as essential for biological activity.

5. Experimental

5.1. Chemistry

Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. Infrared (IR) spectra (KBr) were recorded on a Perkin-Elmer 310 instrument. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 200 MHz on a Bruker AC 200 spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). All compounds were routinely checked by thin-layer chromatography (TLC) and ^{1}H NMR. TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standard methods.

Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within ±0.40% of the theoretical values. All chemicals were purchased from Aldrich Chimica, Milan (Italy), or Lancaster Synthesis GmbH, Milan (Italy), and were of the highest purity.

*5.1.1. Synthesis of 2-chloro-4-fluoro-5-nitrobenzoic acid **7***

To a stirred solution of 2-chloro-4-fluorobenzoic acid (28.64 mmol) in sulphuric acid 96 % (40 ml), nitric acid 65% (42.96 mmol) was added and the resulting mixture was stirred at room temperature for 1 h. The mixture was then diluted with water and extracted with ethyl acetate (3 x 50 ml) and the combined organic extracts were washed with brine (3 x 40 ml), dried (Na_2SO_4), filtered and evaporated under reduced pressure to yield a TLC pure solid. (yield: 96 %); ^{1}H NMR ($CDCl_3$) δ 7.44-7.49 (d, 1H, H_3 – Ar), 8.66 (br. s, 1H, OH, exchanged with D_2O), 8.76-8.80 (d, 1H, H_6 – Ar).

*5.1.2. Synthesis of 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropionic acid ethyl ester **8***

A solution of 2-chloro-4-fluoro-5-nitrobenzoic acid (28.42 mmol) in thionyl chloride (10 ml) was refluxed for 2 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. Toluene was added (3 x 10 ml) and the mixture concentrated again to yield the corresponding acyl chloride as a TLC pure residual oil. Potassium ethyl malonate (59.13 mmol) was placed in a flask under a nitrogen blanket. Acetonitrile (60 ml) was added and the mixture stirred and cooled to 10-15 °C. To this mixture was added triethylamine (92.65 mmol) followed by magnesium chloride (65.36 mmol) and stirring continued at 20-25 °C for 2.5 h. The resulting slurry was re-cooled to 0 ° and a solution of the acyl chloride in dry acetonitrile (10 ml) was added dropwise over 25 min followed by the addition of more triethylamine (9.27 mmol). The mixture allowed to stir overnight at room temperature and the next cooled to 0° C before adding 13 % aq HCl (15 ml) cautiously while keeping the temperature below 25 °C. The two phases were separated and the organic layer was evaporated under reduced pressure to remove acetonitrile while the aqueous layer was back-extracted with ethyl acetate (3 x 40 ml). The combined organic extracts were washed with $NaHCO_3$ saturated solution (2 x 40 ml) followed by brine (2 x 40 ml) and then filtered, dried (Na_2SO_4) and concentrated under reduced pressure to give the required product as a TLC pure solid. (yield: 97 %); ^{1}H NMR ($CDCl_3$) δ 1.28-1.35 (t, 3H, CH_2CH_3), 4.01 (s, 2H, $COCH_2CO$), 4.22-4.32 (q, 2H, CH_2CH_3), 7.38-7.43 (d, 1H, H_3 – Ar), 8.31-8.41 (m, 1H, H_6 – Ar).

*5.1.3. General procedure for the preparation of 3-(4-(cyclo) alkylamino-2-chloro-5-nitrophenyl)-3-oxopropionic acid ethyl esters **6a-d***

*Example: Synthesis of 3-(4-*tert*-Butylamino-2-chloro-5-nitrophenyl)-3-oxopropionic acid ethyl ester **6b**.*

A mixture of 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropionic acid ethyl ester (1.73 mmol), dry acetonitrile (30 ml), anhydrous potassium carbonate (1.73 mmol) and *tert*-butylamine (1.89 mmol) was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate (150 ml), washed with brine and dried (Na_2SO_4). After filtration and evaporation of the solvent in vacuum, the residue was purified by chromatography on silica gel column (*n*-hexane/ethyl acetate/methanol 12/3/1) to give the TLC pure desired ester. (yield: 96%); ^{1}H NMR ($CDCl_3$) δ 1.24-1.31 (t, 3H, CH_2CH_3), 1.54 (s, 9H, *t*-butyl), 4.00 (s, 2H, $COCH_2CO$), 4.10-4.27 (q, 2H, CH_2CH_3), 7.13 (s, 1H, H_3 – Ar), 8.70 (br. s, 1H, NH, exchanged with D_2O), 8.83 (s, 1H, H_6 – Ar).

Compounds **6a-d** were obtained as TLC pure solids. Chemical and physical data are reported in Table 4.

*5.1.4. General procedure for the preparation of unsymmetrically disubstituted 1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid ethyl esters **5k-p***

*Example: Synthesis of 7-*tert*-butylamino-1-cyclopropyl-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid ethyl ester **5n***

To a stirred solution of 3-(4-*tert*-butylamino-2-chloro-5-nitrophenyl)-3-oxopropionic acid ethyl ester **6b** (1.92 mmol) in acetic anhydride (5 ml) triethyl orthoformate (2.98 mmol) was added and the resulting mixture was refluxed for 1 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. Toluene was added (3 x 10 ml) and the mixture concentrated again. The residual oil was dissolved in DMSO (3 ml) and a solution of cyclopropylamine (2.12 mmol) in DMSO was added while maintaining the temperature between 15-20 °C. The resulting solution was stirred at room temperature for 1 h. Then anhydrous K_2CO_3 (2.12 mmol) was added and the mixture was heated to 90 °C overnight. After cooling to room temperature, the mixture was diluted with water and filtered to collect a crude precipitate which was used in the following hydrolysis step without further purification (yield: 86 %); ^{1}H NMR (DMSO-d_6) δ 1.21-1.29 (m, 4H, CH_2-cyclopropyl), 1.46-1.52 (t, 3H, CH_2CH_3), 1.52 (t, 9H, *t*-butylamino, overlapped signal), 3.62 (m, 1H, CH-cyclopropyl), 4.14-4.24 (q, 2H, CH_2CH_3), 7.32 (s, 1H, H_8-Ar), 8.12 (br. s, 1H, NH, exchanged with D_2O), 8.36 (s, 1H, H_5-Ar), 8.81 (s, 1H, H_2 – Ar). IR (KBr): 3340 (NH), 1710 and 1630 (CO).

Compounds **5k-p** were obtained as TLC pure solids and were used without any further purification. Chemical and physical data are reported in Table 4.

Table 3
Chemical and physical data of compounds **1-3**

Compd	R	R_1	R_2	Yield (%)	Mp (°C)	Crystallization solvent	Formula[a]
1a[b]	cyclopropyl	cyclopropyl	H	49	>280	acetonitrile	$C_{16}H_{15}FN_2O_3$
1b	cyclobutyl	cyclobutyl	H	55	>280	acetonitrile	$C_{18}H_{19}FN_2O_3$
1c	cyclopentyl	cyclopentyl	H	58	259-260	DMF	$C_{20}H_{23}FN_2O_3$
1d	cyclohexyl	cyclohexyl	H	60	287-289	acetonitrile	$C_{22}H_{27}FN_2O_3$
1e[b]	*tert*-butyl	*tert*-butyl	H	52	177-178	benzene	$C_{18}H_{23}FN_2O_3$
2a	H	H	H	78	>280	DMF	$C_{10}H_7N_3O_5$
2b[c]	ethyl	ethyl	H	77	>280	acetonitrile	$C_{14}H_{15}N_3O_5$
2c	2-F-ethyl	2-F-ethyl	H	82	>280	acetonitrile	$C_{14}H_{13}F_2N_3O_5$
2d	ethenyl	2-F-ethyl	H	83	261-263	acetonitrile	$C_{14}H_{12}FN_3O_5$
2e	*iso*-propyl	*iso*-propyl	H	79	>280	acetonitrile	$C_{16}H_{19}N_3O_5$
2f	*iso*-amyl	*iso*-amyl	H	76	>280	acetonitrile	$C_{20}H_{27}N_3O_5$
2g[b]	*tert*-butyl	*tert*-butyl	H	75	>280	DMF	$C_{18}H_{23}N_3O_5$
2h[b]	cyclopropyl	cyclopropyl	H	88	>280	DMF	$C_{16}H_{15}N_3O_5$
2i	cyclobutyl	cyclobutyl	H	77	258-260	acetonitrile	$C_{18}H_{19}N_3O_5$
2j	cyclopentyl	cyclopentyl	H	79	232-234	acetonitrile	$C_{20}H_{23}N_3O_5$
2k	cyclohexyl	cyclohexyl	H	80	280-281	ethanol 95°	$C_{22}H_{27}N_3O_5$
2l	2,4-F_2-phenyl	2,4-F_2-phenyl	H	80	>280	ethanol 95°	$C_{22}H_{11}F_4N_3O_5$
2m[b]	*tert*-butyl	cyclopropyl	H	82	>280	acetonitrile	$C_{17}H_{19}N_3O_5$
2n	*tert*-butyl	morpholine		68	>280	benzene/acetonitrile	$C_{18}H_{21}N_3O_6$
2o	*tert*-butyl	thiomorpholine		75	>280	acetonitrile	$C_{18}H_{21}N_3O_5S$
2p[b]	cyclopropyl	*tert*-butyl	H	85	>280	acetonitrile	$C_{17}H_{19}N_3O_5$
2q	cyclopropyl	morpholine		72	198-200	benzene/acetonitrile	$C_{17}H_{17}N_3O_6$
2r	cyclopropyl	thiomorpholine		64	238-239	acetonitrile	$C_{17}H_{17}N_3O_5S$
3a[b]	cyclopropyl	cyclopropyl	H	90	>280	acetonitrile	$C_{16}H_{17}N_3O_3$
3b[b]	*tert*-butyl	*tert*-butyl	H	89	>280	acetonitrile	$C_{18}H_{25}N_3O_3$

[a] Analytical results for C, H, N were within ± 0.4% of the calculated values.
[b] Rif. 18.
[c] Directly obtained from the 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropionic acid ethyl ester .

*5.1.5. General procedure for the preparation of symmetrically disubstituted 1,4-dihydro-6-fluoro- and -6-nitro-4-oxoquinoline-3-carboxylic acid ethyl esters **4a-e** and **5a-j***

*Example: Synthesis of 1-*tert*-butyl-7-*tert*-butylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid ethyl ester **5e***

To a stirred solution of 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropionic acid ethyl ester (3.41 mmol) in acetic anhydride (8 ml) triethyl orthoformate (2.98 mmol) was added and the resulting mixture was refluxed for 1 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. Toluene was added (3 x 10 ml) and the mixture concentrated again. The residual oil was dissolved in DMSO (5 ml) and a solution of *tert*-butylamine (7.50 mmol) in DMSO was added while maintaining the temperature between 15-20 °C. The resulting solution was stirred at room temperature for 1 h. Then anhydrous K_2CO_3 (7.50 mmol) was added and the mixture was heated to 90 °C overnight. After cooling to room temperature, the mixture was diluted with water and filtered to collect a crude precipitate which was used in the following hydrolysis step without further purification (yield: 93 %); ^{1}H NMR (DMSO-d_6) δ 1.22-1.29 (t, 3H, CH_2CH_3), 1.51 (s, 9H,C_7-*t*-butylamino), 1.80 (s, 9H, N_1-*t*-butyl), 4.14-4.25 (q, 2H, CH_2CH_3), 7.09 (s, 1H, H_8-Ar), 8.11 (br. s, 1H, NH, exchanged with D_2O), 8.69 (s, 1H, H_5-Ar), 8.89 (s, 1H, H_2 – Ar).

Compounds **5a** and **5b** were obtained as different products by reacting 2-fluoroethylamine according to this procedure.

Compounds **4a-e** and **5a-j** were obtained as TLC pure solids and were used without any further purification. Chemical and physical data are reported in Table 4.

*5.1.6. General procedure for the preparation of 1,4-dihydro-1,7-disubstituted-6-fluoro- and -6-nitro-4-oxoquinoline-3-carboxylic acids **1a-d, 2a,c,d**, and **2h-l***

*Example: Synthesis of 1-cyclopropyl-7-cyclopropylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid **2h***

To a solution of 1-cyclopropyl-7-cyclopropylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid ethyl ester **5f** (10.27 mmol) in acetic acid (50 ml) and water (40 ml), concentrated sulphuric acid (7 ml) was added and the resulting mixture was refluxed for 1.5 h. After cooling, the reaction mixture was diluted with water and extracted with ethyl acetate (3 x 50 ml) and the combined organic extracts were washed with brine (3 x 40 ml) and dried (Na_2SO_4). After filtration the solvent was evaporated under reduced pressure to yield a TLC pure solid residue, which was recrystallized from DMF (yield: 88%). ^{1}H NMR (CF_3COOD) δ: 0.90-0.94 (m, 2H, CH_2 C_7-cyclopropylamino), 1.23-1.26 (m, 2H, CH_2 N_1-cyclopropyl), 1.46-1.49 (m, 2H, CH_2 C_7-cyclopropylamino), 1.70-1.73 (m, 2H, CH_2 N_1-cyclopropyl),

Table 4
Chemical and physical data of compounds **4-8**

Compd	R	R_1	R_2	Yield (%)	Mp (°C)	Crystallization solvent	Formula[a]
4a[b]	cyclopropyl	cyclopropyl	H	85	218-219	acetonitrile	$C_{18}H_{19}FN_2O_3$
4b	cyclobutyl	cyclobutyl	H	86	248-249	acetonitrile	$C_{20}H_{23}FN_2O_3$
4c	cyclopentyl	cyclopentyl	H	87	207-208	acetonitrile	$C_{22}H_{27}FN_2O_3$
4d	cyclohexyl	cyclohexyl	H	90	244-245	acetonitrile	$C_{24}H_{31}FN_2O_3$
4e[b]	*tert*-butyl	*tert*-butyl	H	89	208-210	cyclohexane-benzene	$C_{20}H_{27}FN_2O_3$
5a	2-F-ethyl	2-F-ethyl	H	50[c]	245-246	acetonitrile	$C_{16}H_{17}F_2N_3O_5$
5b	ethenyl	2-F-ethyl	H	45[c]	218-220	ethyl acetate	$C_{16}H_{16}FN_3O_5$
5c	*iso*-propyl	*iso*-propyl	H	88	205-207	benzene	$C_{18}H_{23}N_3O_5$
5d	*iso*-amyl	*iso*-amyl	H	89	191-192	acetonitrile	$C_{20}H_{27}N_3O_5$
5e[b]	*tert*-butyl	*tert*-butyl	H	93	>280	cyclohexane-benzene	$C_{20}H_{27}N_3O_5$
5f[b]	cyclopropyl	cyclopropyl	H	92	>280	DMF	$C_{18}H_{19}N_3O_5$
5g	cyclobutyl	cyclobutyl	H	90	266-267	acetonitrile	$C_{20}H_{23}N_3O_5$
5h	cyclopentyl	cyclopentyl	H	92	247-248	cyclohexane-benzene	$C_{22}H_{27}N_3O_5$
5i	cyclohexyl	cyclohexyl	H	93	191-192	acetonitrile	$C_{24}H_{31}N_3O_5$
5j	2,4-F_2-phenyl	2,4-F_2-phenyl	H	90	244-245	acetonitrile	$C_{24}H_{15}F_4N_3O_5$
5k[b]	*tert*-butyl	cyclopropyl	H	83	>280	acetonitrile	$C_{19}H_{23}N_3O_5$
5l	*tert*-butyl	morpholine		82	>280	benzene	$C_{20}H_{25}N_3O_6$
5m	*tert*-butyl	thiomorpholine		74	>280	acetonitrile	$C_{20}H_{25}N_3O_5S$
5n[b]	cyclopropyl	*tert*-butyl	H	86	>280	acetonitrile	$C_{19}H_{23}N_3O_5$
5o	cyclopropyl	morpholine		78	248-250 dec	acetonitrile	$C_{19}H_{21}N_3O_6$
5p	cyclopropyl	thiomorpholine		76	241-243 dec	benzene	$C_{19}H_{21}N_3O_5S$
6a		cyclopropyl	H	95	72-73	*n*-hexane	$C_{14}H_{15}ClN_2O_5$
6b		*tert*-butyl	H	96	95-96	*n*-hexane	$C_{15}H_{19}ClN_2O_5$
6c		morpholine		88	172-174	benzene	$C_{15}H_{17}ClN_2O_6$
6d		thiomorpholine		86	160-162	benzene	$C_{15}H_{17}ClN_2O_5S$
7				96	166-168	benzene	$C_7H_3ClFNO_4$
8				97	63-64	cyclohexane-diethyl ether	$C_{11}H_9ClFNO_5$

[a] Analytical results for C, H, N were within ± 0.4% of the calculated values.
[b] Rif. 18.
[c] Derivatives **5a** and **5b** were obtained as different products from the same reaction.

2.85-2.93 (m, 1H, CH C_7-cyclopropylamino), 3.97-4.06 (m, 1H, CH N_1-cyclopropyl), 8.23 (s, 1H, C_8-H), 9.34 (s, 1H, C_5-H), 9.52 (s, 1H, C_2–H). IR (KBr): 3350 (NH), 2900 (OH), 1705 (COOH), 1615 (CO), 1460-1370 (NO_2) cm^{-1}.

Compound **2a** was obtained by this procedure starting from 1-*tert*-butyl-7-*tert*-butylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid ethyl ester **5e**. Compounds **1a-d, 2a,c,d**, and **2h-l** were obtained as TLC pure solids and chemical and physical data are reported in Table 3.

*5.1.7. General procedure for the preparation of 1,4-dihydro-1,7-disubstituted-6-fluoro- and -6-nitro-4-oxoquinoline-3-carboxylic acids **1e, 2e-g**, and **2m-r***

*Example: Synthesis of 1-*tert*-butyl-7-*tert*-butylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid **2g***

To a solution of NaOH (11.29 mmol) in water (30 ml) 1-*tert*-butyl-7-*tert*-butylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid ethyl ester **5e** (2.26 mmol) was added and the resulting mixture was refluxed for 3 h. After cooling, the reaction mixture was diluted with water and acidified with HCl 2N. After stirring for about 10 min, the resulting slurry was filtered to collect a precipitate which was recrystallized from DMF to yield the title compound as TLC pure solid. (yield: 75 %) ^{1}H NMR (CF_3COOD) δ: 1.78 (s, 9H, N_1-*t*-butyl), 2.22 (s, 9H, C_7-*t*-butylamino), 8.10 (s, 1H, H_8-Ar), 8.11 (br. s, 1H, NH, exchanged with D_2O), 9.67 (s, 1H, H_5-Ar), 9.71 (s, 1H, H_2 – Ar). IR (KBr): 3200 (NH), 2900 (OH), 1690 (COOH), 1635 (CO), 1450-1360 (NO_2) cm^{-1}.

Compounds **1e, 2e-g** and **2m-r** were obtained as TLC pure solids and chemical and physical data are reported in Table 3.

*5.1.8. Synthesis of 1,4-dihydro-1-ethyl-7-ethylamino-6-nitro-4-oxoquinoline-3-carboxylic acid **2b***

To a stirred solution of 3-(2-chloro-4-fluoro-5-nitrophenyl)-3-oxopropionic acid ethyl ester (3.41 mmol) in acetic anhydride (8 ml) triethyl orthoformate (2.98 mmol) was added and the resulting mixture was refluxed for 1 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. Toluene was added (3 x 10 ml) and the mixture concentrated again. The residual oil was dissolved in DMSO (5 ml) and ethylamine hydrochloride (7.50 mmol) and anhydrous K_2CO_3 (7.50 mmol) was added while maintaining the temperature between 15-20 °C. The resulting solution was stirred at room temperature for 1 h. More anhydrous K_2CO_3 (7.50 mmol) was added and the mixture was heated to 90 °C overnight. After cooling to room temperature, the mixture was diluted with water, neutralized

and filtered to collect a TLC pure solid, which was recrystallized from acetonitrile (yield: 77%).

*5.1.9. General procedure for the preparation of 6-amino-1,4-dihydro-1,7-disubstituted-4-oxoquinoline-3-carboxylic acids **3a,b***

*Example: Synthesis of 6-amino-1-*tert*-butyl-7-*tert*-butylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid **3b***

To a stirred solution of 1-*tert*-butyl-7-*tert*-butylamino-1,4-dihydro-6-nitro-4-oxoquinoline-3-carboxylic acid **2g** (1.88 mmol) in ethanol 95° (4 ml) a solution of $SnCl_2 \cdot 2H_2O$ (6.58 mmol) in 37% hydrochloric acid (2 ml) was cautiously added and the resulting mixture was refluxed for 30 min. After cooling, the reaction mixture was diluted with water, made neutral (pH ≈ 7) with KOH 2N and extracted with ethyl acetate (3 x 50 ml). The combined organic extracts were washed with brine (3 x 40 ml) and dried over anhydrous Na_2SO_4. After filtration and evaporation of the solvent under reduced pressure a residue was obtained which was triturated with ethanol/diethyl ether to give the desired compound as a TLC pure solid (yield: 89 %). ^{1}H NMR (DMSO-d_6) δ: 1.48 (s, 9H, N_1-*t*-butyl), 1.92 (s, 9H, C_7-*t*-butylamino), 5.68-5.78 (m, 2H, NH_2, exchanged with D_2O), 7.17 (s, 1H, NH, exchanged with D_2O), 7.28 (s, 1H, H_8-Ar), 7.76 (s, 1H, H_5-Ar), 8.92 (s, 1H, H_2 – Ar). IR (KBr): 3300 (NH), 2900 (OH), 1690 (COOH), 1600 (CO) cm^{-1}.

Compounds **3a-b** were obtained as TLC pure solids and chemical and physical data are reported in Table 3.

5.2. Microbiology

5.2.1. Compounds

Test compounds were dissolved in DMSO at an initial concentration of 200 mM and then were serially diluted in culture medium.

5.2.2. Cells

Cell line were from American Type Culture Collection (ATCC); bacterial and fungal strains were either clinical isolates (obtained from Clinica Dermosifilopatica, University of Cagliari) or collection strains from ATCC. Cell cultures were checked periodically for the absence of mycoplasma contamination with a Myco Tect Kit (Gibco).

5.2.3. Antibacterial Assays

Group D *Streptococcus, S. aureus, Shighella* spp. and *Salmonella* spp. were recent clinical isolates. Tests were carried out in nutrient broth, pH 7.2, with an inoculum of 10^3 cells/tube. MICs were determined after 18 h incubation at 37 °C in the presence of serial dilutions of the test compounds. The minimal bactericidal concentration (MBC) was determined by subcultivating in Triptosio agar samples from cultures with no apparent growth.

5.2.4. Antimybacterial Assays

M. tuberculosis 27294 and *M. avium* complex (MAC) were ATCC strains. MICs were assessed in microtiter plates by adding 20 ml aliquots of a culture suspension [whose turbidity was equal to that of a no. 1 McFarland standard containing 10^8 colony forming units (CFU)/ml] to 80 ml of Middlebrook 7H9 medium containing 0.5% glycerol and 10% albumin-dextrose-catalase (ADC) and various concentrations of test compounds. Plates were then incubated for 9 days at 37 °C. At the end of incubation, the number of viable mycobacteria was determined by the MTT method, as already reported [36].

Microbial and cell growth at each drug concentration were expressed as percentage of untreated controls and concentrations resulting in 50% (CC_{50}, MIC_{50}) or 90% (MIC_{90}) growth inhibition was determined by linear regression analysis.

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