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NITROQUINOLONES WITH BROAD-SPECTRUM ANTIMYCOBACTERIAL ACTIVITY *IN VITRO*

Marino Artico,[#*] Antonello Mai,[#] Gianluca Sbardella,[#] Silvio Massa,[+] Chiara Musiu,[≠] Stefania Lostia,[≠] Francesca Demontis,[≠] and Paolo La Colla[≠*]

[#]*Dipartimento di Studi Farmaceutici, Università di Roma "La Sapienza", p.le Aldo Moro 5, I-00185 Roma, Italy*
[+]*Dipartimento Farmaco Chimico Tecnologico, Università di Siena, Banchi di Sotto 55, I-53100 Siena, Italy*
[≠]*Dipartimento di Biologia Sperimentale, Sezione di Microbiologia, Università di Cagliari, Cittadella Universitaria, SS 554 - I-09042 Monserrato (Cagliari), Italy*

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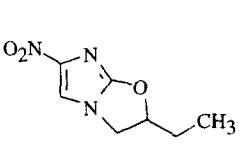
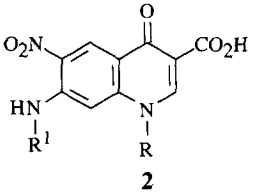
Abstract. During search on quinolonecarboxylic acids we used a facile, convenient two- or three-step procedure to synthesize new quinolone analogs, bearing at the C-7 position alkylamino substituents, and at the C-6 position a fluorine or alternatively a nitro group. The new derivatives were tested against both Gram-positive and Gram-negative bacteria and against a number of different mycobacteria. *In vitro* assays showed 1-*tert*-butyl-7-*tert*-butylamino-6-nitro-1,4-dihydro-4-quinolone-3-carboxylic acid to be a potent inhibitor of *Streptococcus* and *Staphylococcus* with potencies superior to those of ofloxacin and ciprofloxacin , used as reference drugs. Some 6-nitroquinolones were found to exert good inhibiting activities against *Mycobacterium tuberculosis* and various atypical mycobacteria, whereas the 6-fluoro counterparts showed poor or no activity against this bacterium.

Nitroheterocycles have been carefully studied as potential agents for the therapy of infectious diseases caused by pathogenic bacteria, fungi and protozoa.[1] Search on nitrofurans, nitroimidazoles and nitropyrroles has led to the discovery of potent drugs such as nitrofurantoin,[2] metronidazole[3] and pyrrolnitrin.[4] In particular, the nitrofuran and nitroimidazole groups have shown a remarkable broad spectrum of antimicrobial effects, antibacterial activity included.[5] Nevertheless, there are few reports concerning the antimycobacterial activity of members of these classes.

Some years ago, 2-ethyl-2,3-dihydro-6-nitroimidazo[2,1-b]oxazole (**1**)[6] has been claimed to possess potent *in vitro* and *in vivo* antimycobacterial activity, with a good therapeutic index. Unfortunately, this compound has been reported to be mutagenic in the Ames test and this finding has compromised further development.

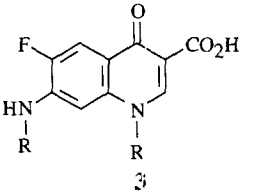


1



2

$R = R^1$ or $R \neq R^1$



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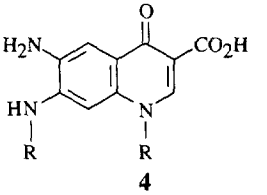

4

R,R^1 = cyclopropyl, *tert*-butyl

Nitroquinolones have been scarcely studied as antibacterial agents because of the poor activity shown by some members, such as 3-nitroquinolones,[7] against Gram-positive and Gram-negative bacteria. However,

* Corresponding authors: tel&fax +39 06 4462731/E-mail: artico@uniroma1.it and tel +39 070 670989, fax +39 070 670802/E-mail: placolla@unica.it

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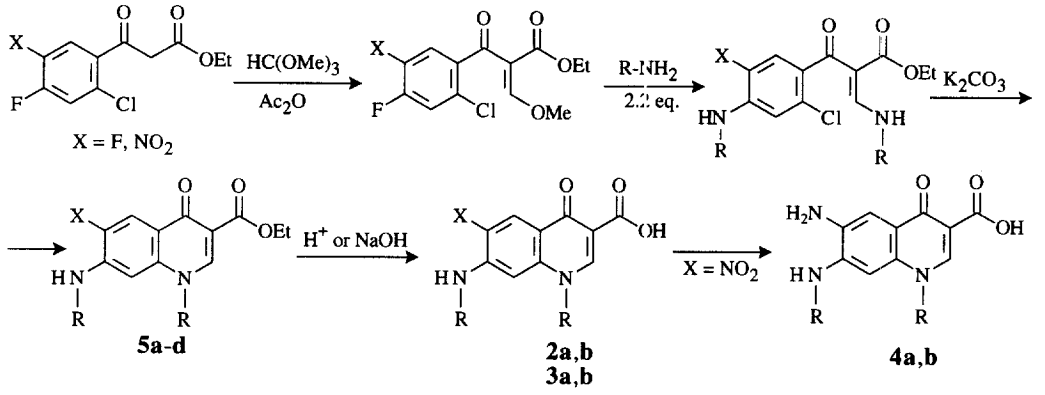
taking account that fluoroquinolonecarboxylic acids are good antibacterial[8] and antituberculosis[9] agents, and having in mind that the nitro group is a determinant for antimicrobial activity, we decided to design nitroquinolones as potential antimycobacterial agents. Therefore we have synthesized a series of 6-nitroquinolonecarboxylic acids bearing identical (compounds **2a,b**) or different (compounds **2c,d**) substituents at positions 1 and 7 of the quinolone moiety, and have compared **2a,b** with their 6-fluoro counterparts (compounds **3a,b**) with regard to both the antibacterial and the antimycobacterial activities. Some 6-aminoquinolones (**4a,b**) were also synthesized and tested as antimycobacterial agents due to the potent antibacterial activity claimed by Fravolini *et al.* for 6-amino analogues[10] of ciprofloxacin and related fluoroquinolones. Newly designed quinolonecarboxylic acids **2-4** were tested against *Mycobacterium tuberculosis* and other atypical mycobacteria, as well as against some Gram-positive and Gram-negative bacteria, using ciprofloxacin and ofloxacin as reference drugs.

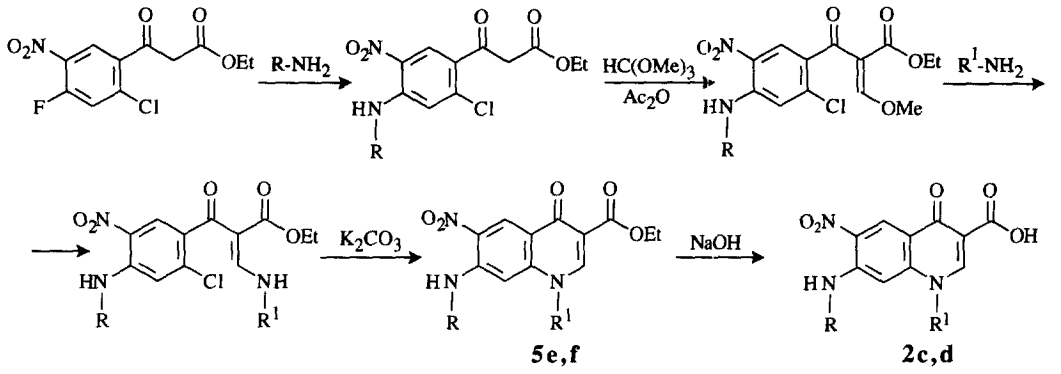
Scheme 1



2a R=cyclopropyl; X=NO_2. **2b** R=*tert*-butyl; X=NO_2. **3a** R=cyclopropyl. X=F. **3b** R=*tert*-butyl; X=F.
4a R=cyclopropyl; X=NH_2.**4b** R=*tert*-butyl; X=NH_2. **5a** R=cyclopropyl; X=NO_2. **5b** R=*tert*-butyl; X=NO_2.
5c R=cyclopropyl; X=F. **5d** R=*tert*-butyl; X=F.

In general, the synthesis of quinolonecarboxylic acids requires a multi-step procedure, being the introduction of the piperazine moiety and the quinoline ring annelation with proper amines two separate events. In order to obtain by a simple procedure quinolonecarboxylic acids deprived of the 7-piperazine group, we devised a chemical pathway which allowed us to introduce simultaneously the proper substituent at positions 1 and 7 of the quinolone ring. In the attempt to modify the structure of ofloxacin and ciprofloxacin without losing their main chemicophysical features, we synthesized a series of new derivatives containing F, NO_2 or NH_2 at position 6, a secondary amine group at position 7 instead of the piperazine and aliphatic substituents at the 4-pyridone nitrogen. As potential pharmacophores we chose the cyclopropyl and *t*-butyl groups, which were introduced by reacting ethyl 3-(2-chloro-4-fluoro-5-nitro(or fluoro)phenyl)-2-methoxymethylene-3-oxopropanoate with cyclopropylamine or *tert*-butylamine (Scheme 1) or treating 3-(2-chloro-4-*t*-butyl (or cyclopropyl)amino-5-nitrophenyl)-2-methoxymethylene-3-oxopropanoate with cyclopropylamine (or *t*-butylamine) (Scheme 2). The importance of *t*-butyl group as antimycobacterial pharmacophore has been previously reported.[10-12] Chemical and physical data of newly synthesized quinolones are reported in Table 1; ^{1}H-NMR data were consistent with the proposed structures.

Scheme 2



2c R=cyclopropyl; R^1=*tert*-butyl. **2d** R=*tert*-butyl; R^1=cyclopropyl. **5e** R=cyclopropyl; R^1=*tert*-butyl. **5f** R=*tert*-butyl; R^1=cyclopropyl.

The quinolonecarboxylic acids **2-4** were evaluated *in vitro* against atypical mycobacteria strains (*Mycobacterium fortuitum*, *Mycobacterium smegmatis* and *Mycobacterium avium* complex (MAC)), *Mycobacterium tuberculosis* strains (ATCC 27294 and clinical isolate 1104), and representative strains of Gram-positive and Gram-negative bacteria (*Streptococcus* group D , *Staphylococcus aureus*, *Salmonella* sp., *Shigella* sp.). Ciprofloxacin and ofloxacin were used as reference drugs.

Table 1. Chemical and Physical Data of Quinolone Derivatives **2-5**

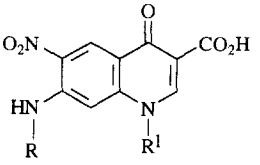
compd	R	R^1	m.p. (°C)	yield (%)	crystallized from	formula[a]
2a	cyclopropyl	-	>280	88	DMF	$C_{16}H_{15}N_3O_5$
2b	*t*-butyl	-	>280	75	DMF	$C_{18}H_{23}N_3O_5$
2c	cyclopropyl	*t*-butyl	>280	82	acetonitrile	$C_{17}H_{19}N_3O_5$
2d	*t*-butyl	cyclopropyl	>280	85	acetonitrile	$C_{17}H_{19}N_3O_5$
3a	cyclopropyl	-	>280	49	-	$C_{16}H_{17}N_3O_3$
3b	*t*-butyl	-	>280	52	-	$C_{18}H_{25}N_3O_3$
4a	cyclopropyl	-	>280	90	-	$C_{16}H_{15}FN_2O_3$
4b	*t*-butyl	-	177-178	89	benzene	$C_{18}H_{23}FN_2O_3$
5a	cyclopropyl	-	>280	92	ethanol	$C_{18}H_{19}N_3O_5$
5b	*t*-butyl	-	>280	93	benzene/cyclohexane	$C_{20}H_{27}N_3O_5$
5c	cyclopropyl	-	218-219	85	acetonitrile	$C_{18}H_{19}FN_2O_3$
5d	*t*-butyl	-	208-210	89	benzene/cyclohexane	$C_{20}H_{27}FN_2O_3$
5e	cyclopropyl	*t*-butyl	>280	83	acetonitrile	$C_{19}H_{23}N_3O_5$
5f	*t*-butyl	cyclopropyl	>280	86	acetonitrile	$C_{19}H_{23}N_3O_5$

[a] Microanalytical results were within ± 0.4% of theoretical values.

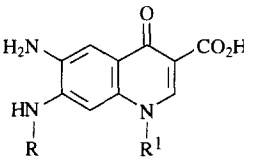
Because of an ongoing screening program carried out to identify new antiretrovirus compounds,[13] test derivatives were also evaluated for anti-HIV-1 activity in MT-4 cells. None of them, however, was capable of protecting the cells from the cytopathic effect induced by the virus (data not shown). Cytotoxicity against MT-4 cells, carried out in parallel with the anti-HIV-1 activity, was evaluated to determine whether the

compounds were endowed with selective antiviral/antimicrobial activity. When tested against mycobacteria (Table 2) the most active compound was **2b**, a di-*t*-butyl derivative which showed a potency (MIC_{50} = 0.5-1.5 μM) comparable, if not superior, to those of ciprofloxacin and ofloxacin (MIC_{50} = 1-2 μM and MIC_{50} = 1.5-3 μM, respectively). In decreasing order of activity **2d** and **2c** (MIC_{50} range 2->125 μM), and then **2a** (MIC_{50} range 3.2->200 μM) follow. It is also noteworthy that **2b** is less cytotoxic than ciprofloxacin. The other test compounds resulted essentially inactive.

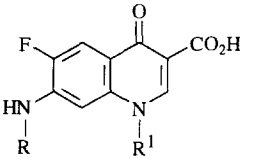
Table 2. Antimycobacterial Activity of Quinolonecarboxylic Acids



2a-d



3a,b



4a,b

compd	R	R^1	MT-4 cells	*M. fortuitum* clinical isolate		*M. smegmatis* ATCC 19420		MAC ATCC 49601		*M. tuberculosis*			
										ATCC 27294		clin. isol. 1104	
			CC_{50}[a]	MIC_{50}[b]	MIC_{90}[c]	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
2a	cyclopropyl	cyclopropyl	40	3.6	100	3.2	50	>200		≥200	>200	72	>200
2b	*t*-butyl	*t*-butyl	140			0.5	1.5	1.2	12	1.4	6	1.5	6
2c	cyclopropyl	*t*-butyl	≥200	>125		2	7	4	>200	31	>200	47	>200
2d	*t*-butyl	cyclopropyl	192	>125		2	5	5	>200	35	>200	16	>200
3a	cyclopropyl	cyclopropyl	>200	>200		156	>200	129	>200	>200		>200	
3b	*t*-butyl	*t*-butyl	>200	>125		>125		140	>200	168	>200	>200	>200
4a	cyclopropyl	cyclopropyl	>200	>125	>200	150	>200	>200	>200	>200		>200	
4b	*t*-butyl	*t*-butyl	>200	36	129	34.5	>200	>200		126	≥200	>200	
CIP[d]			60	1.5	4	8.8	1	2	3	1.5	3	1	1.5
OFL[e]			>200	2	5	2	4	1.5	3	3	3	2	3

[a] Cytotoxic concentration: compound dose (μM) required to reduce the viability of mock-infected MT-4 cells by 50%. [b] Minimum inhibitory concentration (μM) required to reduce the number of viable of *Mycobacterium* by 50%. [c] Minimum inhibitory concentration (μM) required to reduce the number of viable of *Mycobacterium* by 90%. [d] CIP: ciprofloxacin. [e] OFL: ofloxacin.

Contrary to ciprofloxacin and ofloxacin, test compounds were more active against Gram-positive than Gram-negative bacteria.[10-12] Derivative **2b** was (up to 30-fold) more potent and significantly more selective than ciprofloxacin and ofloxacin against Gram-positive bacteria, whereas **2d** and **2a** were as active as the two reference drugs but less selective than ofloxacin. All the other compounds were 3- to 125-fold less active than ciprofloxacin and ofloxacin (Table 3). The most active compound against the Gram-negative bacteria tested was **2a** which was 150 and 4 times less potent than ciprofloxacin and ofloxacin, respectively.

SAR studies show that, in the 6-nitroquinolonecarboxylic acid series, compounds bearing identical substituents at positions 1 and 7 (compounds **2b** and **2a**) are more potent than those carrying different substituents (compounds **2c** and **2d**). Interestingly, the *tert*-butyl group confer higher potency than the cyclopropyl group. It is also to be noticed that, when the nitro group is replaced by fluorine or amino group, the antimicrobial activity diminishes or vanishes, respectively. This suggests that the presence of electron-withdrawing groups is essential for biological activity.

For the biological assays test compounds were dissolved in DMSO at an initial concentration of 200 mM and then were serially diluted in culture medium. Cell lines were from American Type Culture Collection (ATCC). Bacterial and fungal strains were either collection strains from ATCC or clinical isolates (obtained from Clinica Dermosifilopatica, University of Cagliari). H9/IIIB, MT-4 and C8166 cells [grown in RPMI 1640 containing 10% foetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin] were used for anti-HIV-1 assays. Cell cultures were checked periodically for the absence of mycoplasma contamination with a Myco Tect Kit (Gibco). Human immunodeficiency virus type-1 (HIV-1, III_B strain) was obtained from supernatants of persistently infected H9/III_B cells. HIV-1 stock solutions had a titre of 5 x 10^7 cell culture infectious dose fifty ($CCID_{50}$)/mL. Activity against the HIV-1 multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells.[14-16]

Table 3. Antibacterial Activity of Quinolonecarboxylic Acids

compd	CC_{50}[a] MT-4	MIC[b] / MBC[c]			
		Streptococcus D	*Staphylococcus aureus*	*Salmonella*	*Shigella*
2a	40	0.8 / 0.8	0.8 / 1.5	1.5 / 1.5	1.5 / 1.5
2b	140	0.05 / 0.05	0.05 / 0.05	3 / 6	6 / 6
2c	>200	5 / 10	2.5 / 10	25 / 25	25 / 25
2d	192	0.6 / 2.5	0.6 / 1.2	25 / 25	25 / 25
3a	>200	50 / 50	25 / 25	>200	>200
3b	>200	>125	>125	>125	>125
4a	>200	12 /12	12 /12	12 / 12	12 / 25
4b	>200	12 / 12	12 / 12	12 / 12	12 / 25
Ciprofloxacin	60	0.4 / 0.8	0.4 / 0.4	0.01 / 0.01	0.01 / 0.01
Ofloxacin	>200	1.5 / 1.5	0.8 / 0.8	0.4 / 0.4	0.4 / 0.4

[a]Compound concentration (μM) required to reduce the viability of MT-4 cells by 50%. [b]Minimum inhibitory concentration (μM). [c]Minimum bactericidal concentration (μM).

Assays for antibacterial activity were carried out in nutrient broth, pH 7.2, with an inoculum of 10^3 bacterial cells/tube. *Streptococcus* group D, *Staphylococcus aureus*, *Salmonella* and *Shigella* spp., were recent clinical isolates. Minimum inhibitory concentrations (MICs) were determined after incubation at 37°C for 18 hours in the presence of serial dilutions of test compounds.

Mycobacterium smegmatis, *Mycobacterium avium* complex (MAC) and *Mycobacterium tuberculosis* 27294 were ATCC strains, whereas *Mycobacterium fortuitum* and *Mycobacterium tuberculosis* 1104 were clinical isolates. 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 μL 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 1 day (*M. smegmatis* and *M. fortuitum*) or 9 days (MAC and *M. tuberculosis*) at 37°C. At the end of incubation, the number of viable mycobacteria was determined by the MTT method, as already reported.[13]

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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