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ScienceDirect
European Journal of Medicinal Chemistry 41 (2006) 1478–1493
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Short communication

Design, synthesis and activity against *Toxoplasma gondii*, *Plasmodium* spp., and *Mycobacterium tuberculosis* of new 6-fluoroquinolones

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Received 28 February 2006; received in revised form 28 June 2006; accepted 3 July 2006
Available online 25 September 2006

Abstract

This paper reports on the rational design of a series of new 6-fluoroquinolones by QSAR analysis against *Toxoplasma (T.) gondii*, their synthesis, their biological evaluation against *T. gondii* and *Plasmodium (P.)* spp., and their effect on *Mycobacterium (M.) tuberculosis* DNA gyrase and growth inhibition. Of the 12 computer-designed 8-ethyl(or methoxy)- and 5-ethyl-8-methoxy-6-fluoroquinolones predicted to be active against *T. gondii*, we succeeded in the synthesis of four 6-fluoro-8-methoxy-quinolones. The four 6-fluoro-8-methoxy-quinolones are active on *T. gondii* but only one is as active as predicted. One of these four compounds appears to be an antiparasitical drug of great potential with inhibitory activities comparable to or higher than that of trovafloxacin, gatifloxacin, and moxifloxacin. They also inhibit DNA supercoiling by *M. tuberculosis* gyrase with an efficiency comparable to that of the most active quinolones but are poor inhibitors of *M. tuberculosis* growth.

Keywords: Fluoroquinolone; 6-Fluoro-8-methoxy-quinolone; *Toxoplasma gondii*; *Plasmodium* spp.; *Mycobacterium tuberculosis*; Antiparasitical; Antibacterial; Malaria; Toxoplasmosis; QSAR

1. Introduction

Owing to the emergence and alarming spread of bacterial, parasitical and viral strains that are resistant against the drugs used at present in clinics, the discovery of new therapeutical targets and the development of new antibacterial, antiparasitical and antiviral drugs are urgently needed. According to the World Health Organization, one third of human population is infected by *Mycobacterium (M.) tuberculosis* and around two million people die from tuberculosis every year. The treatment of tuberculosis (isoniazid and rifampicin) [1] is long and observance is therefore a problem. Tuberculosis associated with AIDS (due to HIV infection) forms a fatal combination. Tuberculosis is currently responsible for 13% of the number of deaths due to HIV infection [2]. Toxoplasmosis, as well, is an opportunistic disease frequently associated with AIDS [3]. *Toxoplasma (T.) gondii* is the parasite responsible for toxoplasmosis [4]. Almost half of the human population is infected by this parasite, and even if most seropositive people do

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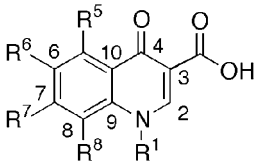
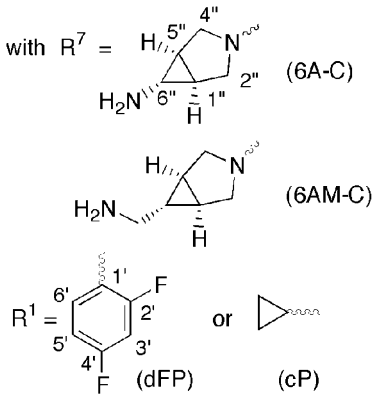
doi:10.1016/j.ejmech.2006.07.003

not develop any symptom of this disease, its transmission to immunocompromised patients such as AIDS patients is life threatening. The other problem concerns its transmission to the fetus, which can result in malformations or stillborns. Chemotherapeutic treatment against toxoplasmosis is limited by side effects or poor absorption of efficient drugs. *Plasmodium (P.)* spp., which are parasitic protozoans belonging to the same apicomplexan phylum as *T. gondii*, are also of great concern as they are responsible for another fatal disease, i.e. malaria. Indeed, it represents two million deaths per year and 90% are due to *Plasmodium falciparum* [5]. Numerous strains are resistant to the most frequently used antimalarial drugs, i.e. chloroquine and pyrimethamine/sulfadoxine [6,7].

In the search of new therapeutical targets and new anti-infective agents, fluoroquinolones (see generic structure in Table 1 with $R^6 = F$ and examples in Table 2) are particularly interesting because of their broad spectrum of activity against various bacteria, mycobacteria, and parasites [4,8–15]. Quinolones are bactericidal by interfering with two essential bacterial enzymes, DNA topoisomerases II (DNA gyrase) and IV, which are enzymes involved in DNA replication, decatenation, recombination and repair [11,16–19]. There are clues that quinolones act on similar targets of *T. gondii* and *P. falciparum*, i.e. the plastids which are circular DNA episomes located within the apicoplast organelle of both parasites, and through similar pathways, although no definitive evidence has been provided up to now [15]. Since the first antibacterial quinolone, i.e. nalidixic acid which was isolated in 1962 [20], more than ten thousands of quinolones have been patented, and the successive chemical modifications improved considerably their potency and spectrum of activity [for reviews, see Refs. [8–14]] and parasitical responses [15,21–24].

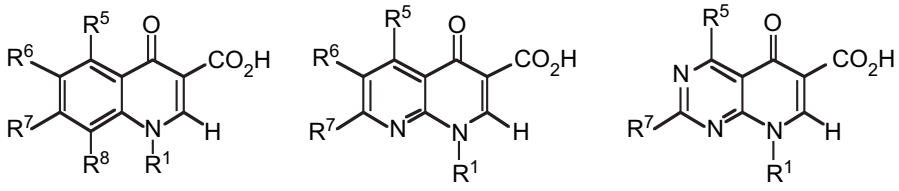
The most often used, relatively safe and well-tolerated 6-fluoroquinolones as antibacterials include norfloxacin (NFX), ofloxacin (OFX), ciprofloxacin (CPFX), levofloxacin (LVFX), moxifloxacin (MXFX), and gatifloxacin (GTFX). OFX was used as second-line agent against *M. tuberculosis* [25]. Furthermore, MXFX, which turns out to be as efficient as rifampicin and isoniazid [26], was recently suggested by the American Thoracic Society to be used against tuberculosis, as well as GTFX and LVFX. MXFX and GTFX with grepafloxacin (GPFX) and trovafloxacin (TVFX) account for among the most powerful antiparasitical fluoroquinolones known so far (for the chemical structure of MXFX, GTFX, GPFX and TVFX, see Table 2) [22,23]. However, GPFX and TVFX were taken off from the market more or less shortly after launch. If a huge number of (fluoro)quinolones differing by the nature of the R^7 substituent were elaborated enabling the establishment of structure/antibacterial [27], antituberculosis [28], or antiparasitical relationships [15,22,24], only a few R^5 or/and R^8 substituted 6-fluoroquinolones are known, probably due to the difficult access to precursors of these derivatives.

Table 1
Chemical structure of the targeted "virtual" 6-fluoroquinolones and their predicted anti-*Toxoplasma gondii* activity (the atom numbering is used for the description of the NMR data)

	Compound number		R^7	R^1	R^5	R^8	Predicted IC_{50} (μg/mL)
(a) generic structure 	**a** Series	**1**	6A-C	cP	H	MeO	0.4
		2	6AM-C	cP	H	MeO	0.5
		3	6A-C	dFP	H	MeO	0.3
		4	6AM-C	dFP	H	MeO	0.3
	b Series	**5**	6A-C	cP	Et	MeO	0.5
		6	6AM-C	cP	Et	MeO	0.6
		7	6A-C	dFP	Et	MeO	0.4
		8	6AM-C	dFP	Et	MeO	0.3
(b) targeted compounds 	**c** Series	**9**	6A-C	cP	H	Et	0.4
		10	6AM-C	cP	H	Et	0.5
		11	6A-C	dFP	H	Et	0.3
		12	6AM-C	dFP	H	Et	0.3

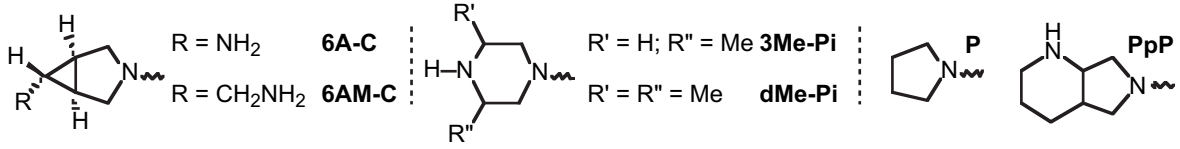
R^5/R^8 = H/MeO or Et/MeO or H/Et

Table 2
Structural features and in vitro anti-*T. gondii* activities (IC_{50}), antimalarial activities (IC_{50}) against blood stages of *P. falciparum* and hepatic stages of *P. yoelii yoelii*, and *M. tuberculosis* DNA gyrase (IC_{50}) and growth inhibition (MIC) of quinolones **1**-**4** in comparison with those of already known quinolones



Structure **A** (quinolone) Structure **B** (naphthyridone) Structure **C**

Quinolones	Base	Substituent					Inhibitory concentrations (μg/mL)				
		R^{1a}	R^5	R^6	R^{7b}	R^8	*T. gondii* RH (IC_{50})	*P. falciparum* NF54-R[c] (IC_{50})	*P. yoelii yoelii* 265By[d]* (IC_{50})	*M. tuberculosis* DNA gyrase (IC_{50})	*M. tuberculosis* MIC[e]
1	A	cP	H	F	6A-C	OMe	1.3	56	45*	3.5	64
2	A	cP	H	F	6AM-C	OMe	30	45	>100	6	64
Gatifloxacin (GTFX)	A	cP	H	F	3Me-Pi	OMe	4.1[f]	11[g]	>100[g]	3[h]	0.12[h]
Moxifloxacin (MXFX)	A	cP	H	F	PpP	OMe	5.1[f]	18[g]	>100[g]	4.5[h]	0.5[h]
Grepafloxacin (GPFX)	A	cP	Me	F	3Me-Pi	H	2.4[f]	3.1[g]	4.4[g]*	16[h]	1[h]
Sparfloxacin (SPFX)	A	cP	NH_2	F	dMe-Pi	F	40[f]	74[g]	53[g]	2[h]	0.25[h]
FQ4	B	cP	H	F	6A-C		0.6[i]	–	–	–	–
FQ9	B	cP	H	F	6AM-C		4.3[i]	–	–	–	–
3	A	dFP	H	F	6A-C	OMe	19	62	>100	20	256
4	A	dFP	H	F	6AM-C	OMe	22	24	>100	15	128
FQ6	A	dFP	H	F	6A-C	H	1.1[i]	–	–	–	–
Trovafloxacin (TVFX)	B	dFP	H	F	6A-C		0.4[f]	9.2[g]	31[g]*	15[h]	16[h]
FQ11	B	dFP	H	F	6AM-C		4.3[i]	–	–	–	–
Piromidic acid	C	Et	H		P		26[f]	14[g]	22[g]*	–	–



[a] dFP = 2,4-Difluorophenyl; cP = cyclopropyl.
[b] 6A-C = 6-Amino-3-azabicyclo[3.1.0]hexan-3-yl, 6AM-C = 6-(aminomethyl)-C, 3Me-Pi = 3-methyl-piperazin-1-yl, dMe-Pi = 3,4-dimethyl-Pi, P = pyrrolidin-1-yl; PpP = piperidinopyrrolidinyl.
[c] Against blood stages of chloroquine-resistant (NF54-R) strain.
[d] Against hepatic stages, symbol *indicates that in these cases only inhibition was also associated with an effect on schizont size.
[e] MIC: minimum inhibitory concentration.
[f] Data from Ref. [22].
[g] Data from Ref. [23].
[h] Data from Ref. [28].
[i] Data from Ref. [21].

In the search for new potent antiparasitical fluoroquinolones, a QSAR analysis by molecular connectivity of a series of quinolones active against *T. gondii* was performed [22,24]. This analysis led to the design of R^5- and/or R^8-substituted 6-fluoroquinolones which were predicted to display higher or at least comparable biological activities to those of already known fluoroquinolones. Among the virtually computer designed, potentially active derivatives, we selected those presented in Table 1 (compounds **1**–**12**). Their structures are all original combinations of the R^1, R^5, R^7 and/or R^8 substituents found in the most anti-*T. gondii* active quinolones MXFX, GTFX, GPFX, TVFX, FQ4, FQ6, FQ9 and FQ11 (see structures in Table 2). Moreover, the ethyl group in R^5 or in R^8 position has never been explored to our knowledge.

This paper is dedicated to (i) the rational design, by QSAR analysis against *T. gondii*, of the new series of fluoroquinolones shown in Table 1, (ii) the detailed synthesis of derivatives **1**–**4**, (iii) our unsuccessful attempts to prepare compounds **5**–**12**, and (iv) the results of in vitro biological tests, including antiparasitical activity against *T. gondii*, blood stages of *P. falciparum* and hepatic stages of *Plasmodium yoelii yoelii*, and antibacterial activity against *M. tuberculosis* (DNA gyrase and growth inhibition). Part of this work was briefly reported as a preliminary communication [24].

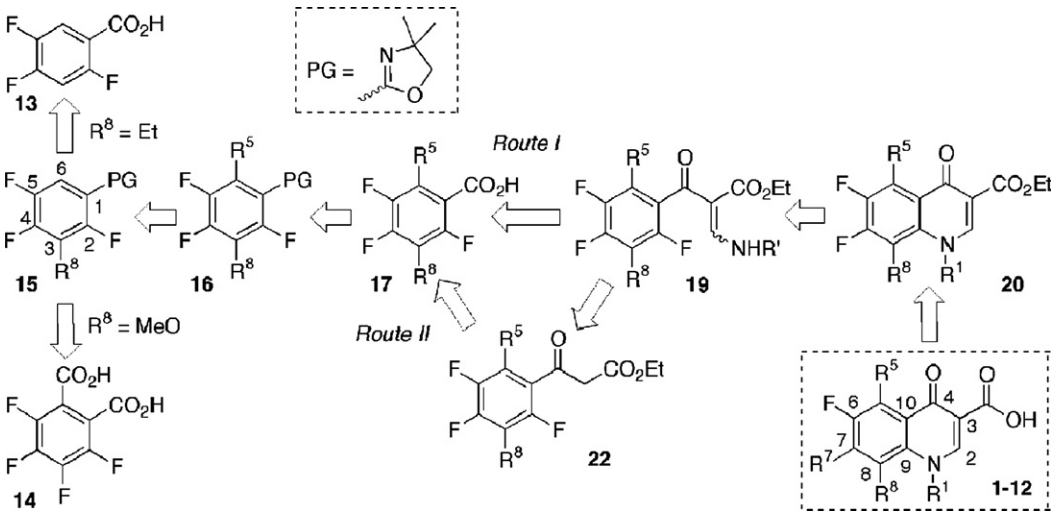
2. Chemistry

The retrosynthetic scheme to obtain the targeted R^5- and/or R^8-substituted 6-fluoroquinolones starting from commercially

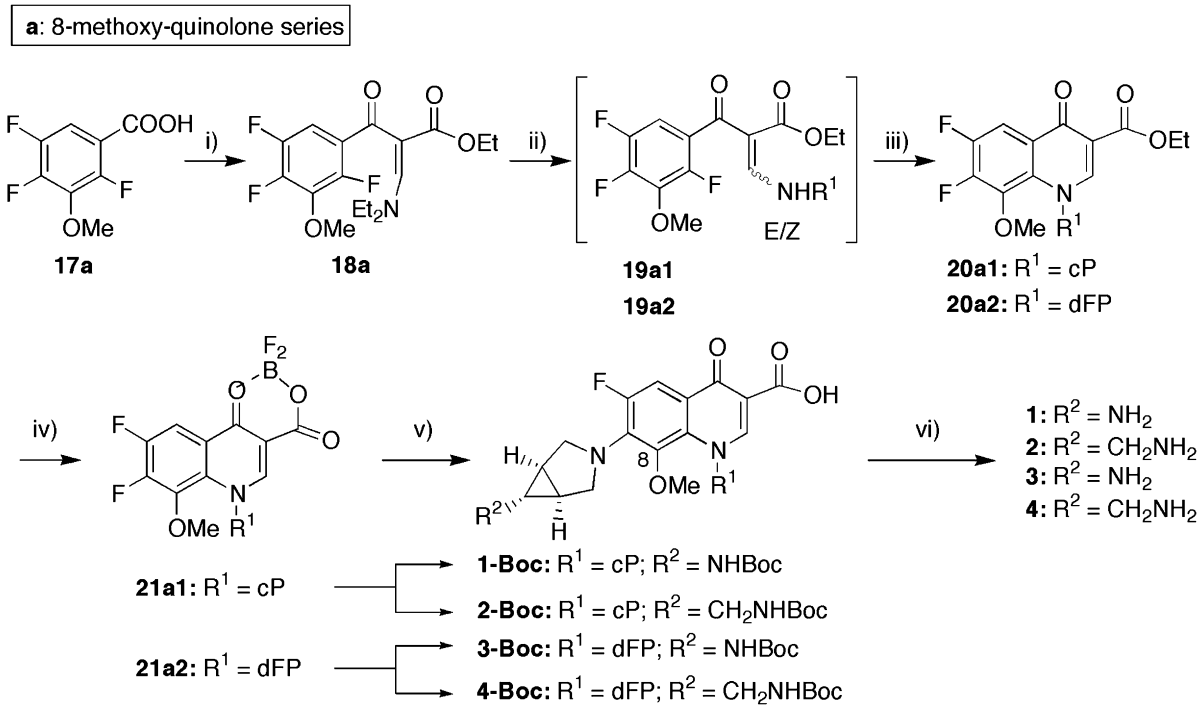
available derivatives **13** and **14** is shown in Scheme 1. The entry to quinolin-4-one nucleus relies on an intramolecular cyclization step in enaminone intermediates **19**. Such intermediates were used for the building-up of thousands of quinolone analogs known to date, though other peculiar alternatives have also been explored [19,29,30]. The approach to **19** from acid **17** is more classically performed through compounds of type **22** (route II) [19,29]. However, we used the alternative route I where the enaminone part was introduced in two steps from **17** using an (*N*-alkyl) acrylate derivative [30–33]. Moreover, this method is easier to implement (milder conditions) and more efficient than the one depicted in route II. The appropriate starting acids **13** and **14** were thus needed to get the key acids **17**, precursors of the target 6-fluoro-8-methoxy-quinolones (**a** and **b** series) and 6-fluoro-8-ethyl-quinolones (**c** series) listed in Table 1 (see Scheme 1). These acids **13** and **14** can be converted into **15** which contains (i) the ethyl or methoxy group in the 3 position (R^8 for the position numbering see Scheme 1), (ii) the fluor atom in the 5 position essential for the bioactivity of the target 6-fluoro-8-ethyl(or methoxy)-quinolones, (iii) two fluor atoms in the 2 and 4 positions, the former being necessary for the building of the heterocyclic nucleus, the latter for the regioselective introduction of the amino derivative moiety (e.g. R^7) through its nucleophilic displacement by suitable amines [19,29] and (iv) an aromatic C–H (position 6), which can be further used to introduce the R^5 substituent (i.e. the ethyl radical), thus generating the R^5 and R^8 substituted synthons **17**, precursor of the target 6-fluoro-5-ethyl-8-methoxy-quinolones (**b** series).

The detailed synthetic pathway to the 6-fluoroquinolone targets of series **a**, **b**, and **c** starting from synthons of type **17** is depicted in Schemes 2–4, respectively. The syntheses of the synthons **17** wherein R^5/R^8 is H/methoxy (i.e. **17a**), ethyl/methoxy (i.e. **17b**), and H/ethyl (i.e. **17c**) from **13** and **14** have been described elsewhere [34]. They were adapted from procedures that were elaborated in the literature for the preparation of R^5- and/or R^8-substituted **17**-type derivatives [34–36]. The 6-amino-3-aza-bicyclo[3.1.0]hexane **23** and its methyleneamino analog **24** (Fig. 1), which constitute the amino R^7 moiety of the target molecules, were synthesized from commercial *N*-benzylmaleimide in eight steps (about 10% overall yield) according to the published procedures [24,37,38].

Of the targeted R^5/R^8-6-fluoroquinolones **1–12** listed in Table 1, we succeeded only in the synthesis of the 6-fluoro-8-methoxy-quinolones **1–4** (**a** series). These compounds were prepared in seven steps from **17a** in 25–30% overall yield (Scheme 2). For the building of the key quinolone cycle **20a**, acid **17a** was first converted into its acid chloride derivative which was then reacted with ethyl 3-(diethylamino)-2(*E*)-propenoate [39], thus affording the *N*,*N*-diethyl enaminone intermediate **18a**. Transaminolysis of **18a** with cyclopropylamine or 2,4-difluorophenylamine followed by cyclization of the resulting cyclopropyl- or 2,4-difluorophenyl-enaminone intermediates **19a1** or **19a2** afforded **20a1** or **20a2**, respectively, in 65% yield. These two steps were performed in a one-pot process and ^{1}H NMR monitoring showed that compounds **19** consisted of *E*/*Z* mixture (data not shown). For cyclization, the mild K_2CO_3 base was preferred to the most frequently used NaH [36,40], which in our hands led to numerous by-products. Noticeably, this three-step two-pot process was more efficient than the two-step one-pot procedure [31,41,42] consisting of the condensation of the acid chloride derivative of **17** with ethyl 3-(cyclopropylamino)-2(*E*)-propenoate or ethyl 3-(2,4-difluorophenylamino)-2(*E*)-propenoate, followed by cyclization. Actually in these particular cases, the condensation step gave essentially the amide compounds of type **25** (example given in Scheme 5, data not shown), rather than the corresponding enaminone compound of type **19**, thus preventing all further cyclization steps. Next the introduction of the R^7 substituent onto **20a1** and **20a2** could only be achieved provided the C-7 position was activated, as



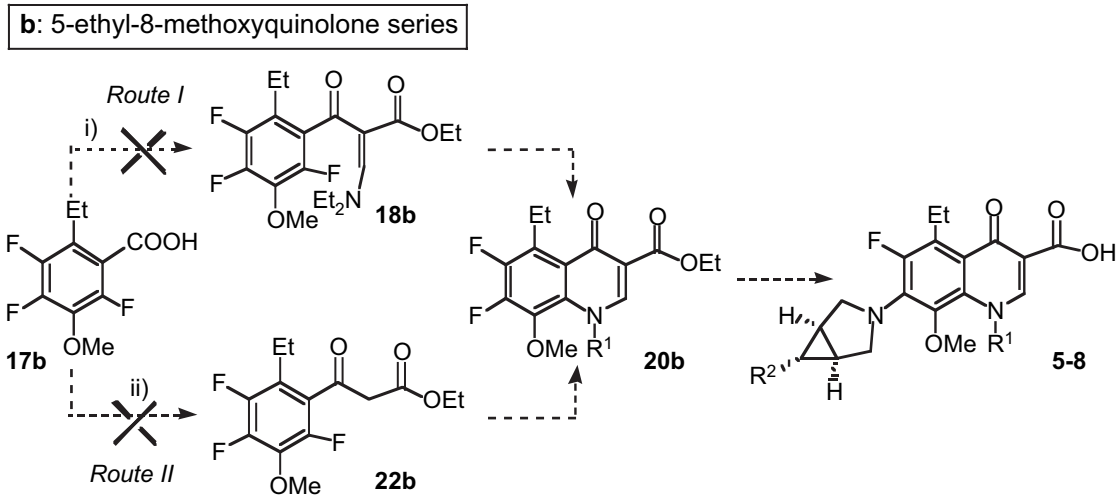
Scheme 1. Retrosynthetic pathway to the targeted quinolin-4-one derivatives **1–12**.



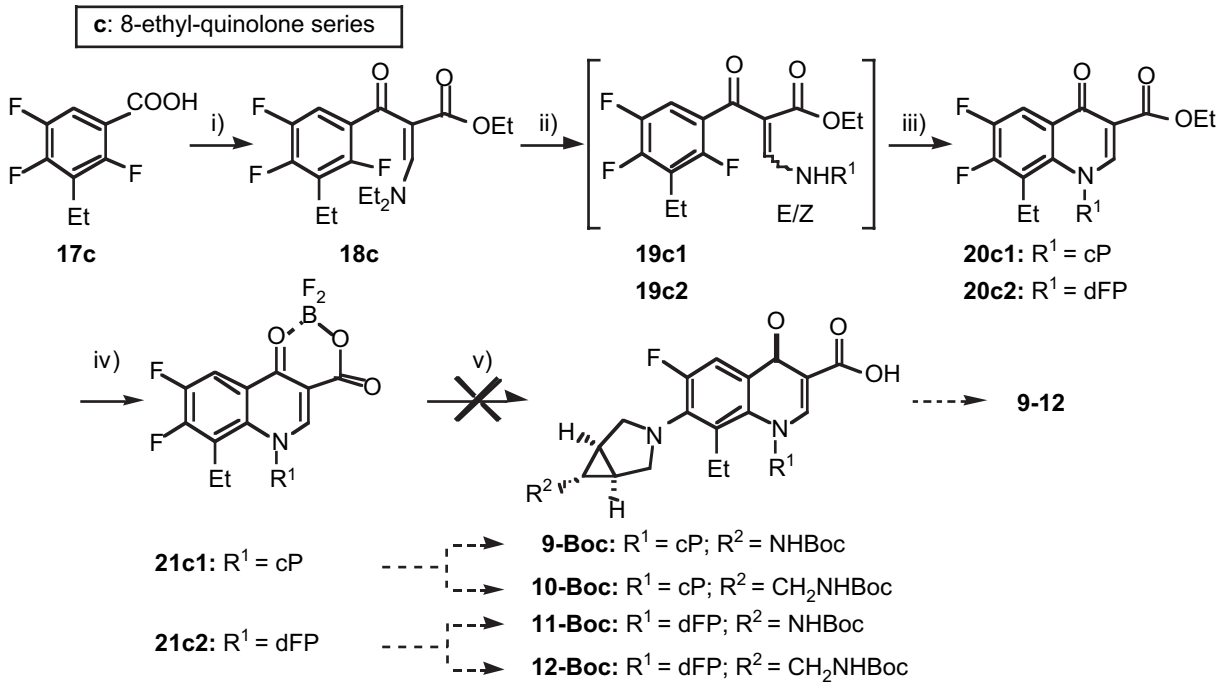
Scheme 2. Synthetic pathways to the quinolin-4-one derivatives **1**–**4** (**a** series), compounds **1**–**4**: (i) oxalyl chloride, then $Et_2NCH{=}CHCO_2Et$, NEt_3, toluene, 90 °C, 5 h; (ii) R^1NH_2, 1:2 EtOH/Et_2O rt, 3 h; (iii) K_2CO_3, DMF, 100 °C, 5 h; (iv) $BF_3{\cdot}Et_2O$, THF, reflux; (v) amine **23** or **24**, CH_3CN, seven days, reflux; (vi) 1:1 CH_2Cl_2/TFA; cP = cyclopropyl; dFP = 2,4-difluorophenyl.

reported in the literature for similar analogs [36,43–45]. Indeed, no reaction occurred between **20a1** and **20a2** and the heterobicyclic amines **23** or **24** (Fig. 1). Compounds **20a1** and **20a2** were thus activated as their boron difluoride derivatives **21a1** and **21a2**, respectively, by reaction with boron trifluoride etherate in refluxing THF. The successive but nevertheless time-consuming (seven days) nucleophilic displacement of the C-7 fluorine atom in **21a1** (resp. **21a2**) with 2.5–3 equiv of amines **23** or **24** followed by *N*-Boc deprotection using an excess of 1:1 TFA/CH_2Cl_2 gave the desired fluoroquinolones **1** or **2** (resp. **3** or **4**), as their TFA salts in 40–44% overall yield from **20a1** and **20a2**.

Concerning the 5-ethyl-8-methoxy quinolones **5**–**8** (**b** series, Scheme 3), all our attempts to obtain the ethyl R^5-substituted enaminone derivative **18b** or β-ketoester **22b** from **17b**, which constitutes the entry to these quinolones,



Scheme 3. Synthetic pathways explored to the quinolin-4-one derivatives **5**–**8** (**b** series): (i) oxalyl chloride, then $Et_2NCH{=}CHCO_2Et$, NEt_3, toluene, 90 °C, 5 h; (ii) $HO_2CCH_2CO_2Et$, *n*-BuLi (2 equiv).



Scheme 4. Synthetic pathways explored to the quinolin-4-one derivatives **9**–**12** (**c** series). See Scheme 2 for (i)–(v). cP = cyclopropyl; dFP = 2,4-difluorophenyl.

failed. Indeed, the acid chloride of the ethyl-methoxy disubstituted acid **17b** led, under the same conditions as used for the synthesis of quinolones **20a** (and also **20c**, see below), to a complex mixture from which we could not isolate the expected **18b** (Scheme 3, route I), thus prohibiting the building of the key quinolone cycle **20b**, and, consequently, the access to the targeted quinolones **5**–**8** of series **b**. The more classical route II shown in Scheme 3, which involves the intermediary preparation of **22b**, by reacting the previous acid chloride with monoethylmalonate was also unsuccessful.

Concerning the synthesis of the 8-ethyl-quinolones **9**–**12** (**c** series, Scheme 4), we were fully successful in building the key quinolones **20c1** and **20c2** from **17c** using the same strategy as that described above for the synthesis of their **21a1** and **21a2** analogs. However, and in sharp contrast with the **a** series, the aromatic substitution of the C-7 fluorine in compounds **20c1** and **20c2** (Scheme 2) whether in the presence of a Lewis acid such as LiCl or $Al(OTf)_3$, or activated as their BF_2-derivatives **21c1** and **21c2** could not be performed with amines **23** or **24**. Indeed, reacting **20c2** or **21c2** with **23** or **24** led to the decarboxylation of **20c2** or **21c2** giving **26c1** or **26c2** (Fig. 2), while derivative **21c1**, under the same conditions, was inert. It should be further noticed that the aromatic substitution of the C-7 fluorine in **21c1** was observed when **21c1** was reacted with piperazine. Thus, 25% yield of the 7-piperazine compound **27** (Fig. 2), i.e. the analog of **9-Boc** and **10-Boc** which were expected from the reaction between **21c1** and amines **23** or **24**, were obtained (data not shown), indicating that the nucleophilicity of the entering amine is of importance.

The chemical structures of fluoroquinolones **1**–**4** (as their TFA salts) and of all intermediates shown in Schemes 2 and 4 were unambiguously attested by ^1H, ^{13}C, ^{19}F NMR, ^1H–^{13}C DEPT, HSQC, ESI-high resolution mass spectrometry, and by comparison with the ^1H, ^{13}C and ^{19}F NMR data reported for similar analogs [46]. The cyclization, and consequently the quinolone structures of **20a1**, **20a2**, **20c1**, and **20c2**, were established by the presence of two fluor resonances in their ^{19}F NMR spectra (as compared to three for **18**) and the deshielding of the H-2 proton (~0.5–1 ppm) with respect to the chemical shift of the vinylic one in the ^1H NMR spectra of **18**. The introduction of the azabicyclic moieties into **1**–**4**

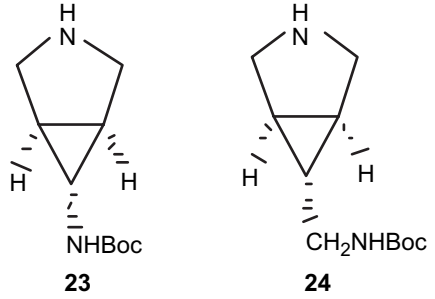
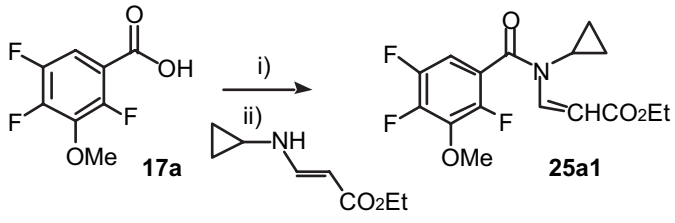


Fig. 1. Structure of Boc-protected 6-amino-3-aza-bicyclo[3.1.0]hexane **23** and its 6-aminomethyl analog **24**.



Scheme 5. Formation of amide **25a1**: (i) oxalyl chloride, CH_2Cl_2 rt, 24 h; (ii) toluene, 90 °C, 5 h.

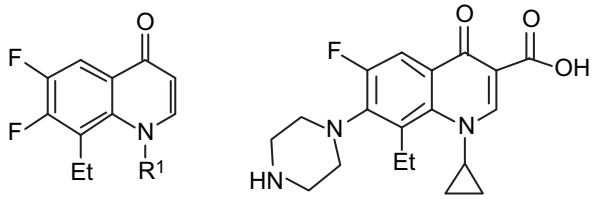


Fig. 2. Structure of decarboxylated by-product derivatives **26c1** and **26c2**, and 7-piperazinyl-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, **27**; cP = cyclopropyl; dFP = 2,4-difluorophenyl.

was attested by the presence of their characteristic ^{1}H and ^{13}C patterns. Its location in R^7 was confirmed by ^{1}H NMR which showed the presence of a doublet for H-5 at 7.4–7.7 ppm with $J \sim 13.0$–13.5 Hz, characteristic of a $^{3}J_{H-F}$ coupling. Concerning the degree of purity of the target fluoroquinolones **1**–**4**, the samples used for the determination of their antiparasitical and antibacterial activities (see Section 3) were obtained by recrystallization from appropriate solvents, and analyzed by two distinct HPLC systems which showed the absence of any trace of impurities.

3. Pharmacology

The antiparasitical activities of quinolones **1**–**4** were evaluated in vitro against *T. gondii* (RH strain), blood stages of *P. falciparum* (chloroquine-resistant strain NF54-R), and hepatic stages of *P. yoelii yoelii* (265BY strain) according to the published procedures [22,23]. The quinolones **1**–**4** were also assessed in vitro for their ability to inhibit *M. tuberculosis* DNA gyrase activity (IC_{50}) and the growth of *M. tuberculosis* (MIC). Table 2 lists all the data from these biological evaluations together with those of selected (fluoro)quinolones and naphthyridones taken from literature for comparison and determined strictly under the same conditions.

4. Results and discussion

4.1. Design rationale

Numerous structure–activity relationships concerning quinolones have been examined to rationalize the various biological activities of quinolones as well as to design new powerful compounds [19,47–50]. In our search for new potent antiparasitical fluoroquinolones, we used the previously described QSAR analysis by molecular connectivity that was performed on quinolones active against *T. gondii* [22] and validated on TVFX analogs [21] for which a good agreement was observed between the predicted and experimental IC_{50} values [22,51,52]. This analysis by molecular topology and virtual computational techniques led to the design of virtual structures which were expected to display higher or at least comparable anti-*T. gondii* activities to those of already known fluoroquinolones. Among these virtually active structures, we selected the structures shown in Table 1 for the feasibility of their synthesis and for their predicted high anti-*T. gondii* activities within the 0.3–0.6 μg/mL range. For their design, we used, as topological indices (TIs), the *E*-state indices, which are specific for each atom and reflect the electronic and topological atomic features taking into account the interaction with the rest of the molecule [53]. These TIs were related to the anti-*T. gondii* activity of known quinolones, thus providing some insights into the substitutions leading to higher anti-*T. gondii* activity. Computational screening was then used to select new quinolones with improved efficacy. Virtual structures were designed around the quinolone chemotype by using a home-made software that allows the introduction of virtual radicals on the most active quinolones (i.e. TVFX, GPFX, GTFX and MXFX with IC_{50} values below 5 μg/mL) and their systematic combination, thus leading to a "virtual combinatorial library" of compounds. Their TIs were calculated, and linear discriminant analysis (LDA) and multilinear regression (MLR) equations were used to determine their activity/inactivity and IC_{50} values, respectively.

The originality of the selected 12 6-fluoroquinolones listed in Table 1 lies in the combination of R^1, R^5, R^7 and R^8 substituents which are found in the most potent anti-*T. gondii* TVFX, FQ4 and FQ9 naphthyridones [21], and MXFX, GTFX, GPFX quinolones (see structures in Table 2). Indeed, these fluoroquinolones contain on a quinolone skeleton (i) as R^1, the cyclopropyl moiety of GTFX, MXFX, and GPFX, or the 2,4-difluorophenyl one of TVFX, FQ6 and FQ11, (ii) as R^7, the azabicyclohexyl (6A-C or 6AM-C) substituent of TVFX, FQ6 and FQ11, (iii) as R^8, the methoxy group of MXFX and GTFX, and (iv) as R^5 and/or R^8, the ethyl radical which has up to now not been investigated. Although R^5 or/and R^8-substituted 6-fluoroquinolones have been the focus of intensive search, R^5 or R^8 substitutions are restricted to F, Cl, Br, NH_2, Me, MeO, EtO, MeS, OH, F_2CHO, or CF_3O [29,35,36,40,43,54,55]. R^5- and R^8-substituted 6-fluoroquinolones are also scarce and R^5/R^8 substitutions are limited to Me/F, Me/Cl, Me/Me, Me/OMe, Cl/Me, NH_2/Me, NHMe/Me, NMe_2/Me, NH_2/OMe, NH_2/OEt, NH_2/F, NH_2/Cl [35,40,43,56–58].

It is further expected that the original R^1, R^5, R^7 and/or R^8 combinations provide not only active antiparasitical/antibacterial drugs but also new drugs displaying reduced side effects as compared with TVFX and GPFX which were withdrawn from the market owing to hepatotoxicity, phototoxicity and/or CNS reactions [59]. Lower toxic side effects are more particularly expected for the derivatives containing a methoxy as R^8. Indeed, this substituent in MXFX and GTFX contributed favorably to the reduction of phototoxicity [19], the selection of less resistant strains [60], and to their activity on resistant strains [61,62].

4.2. Anti-T. gondii activity

The four fluoroquinolones **1**–**4**, which were predicted to display an anti-*T. gondii* activity, were indeed found to be active. However, only compound **1** had an activity (1.3 μg/mL) close to that predicted (0.4 μg/mL), while the other three derivatives were less active than predicted. It should be noted that the anti-*T. gondii* activity of **1** is in the range found for

the most potent quinolones, e.g. TVFX, GPFX, FQ4, and FQ6 (see Table 2). The data from these evaluations show the usefulness and limits of the predictive QSAR models based on molecular topology and multilinear regression analysis which led to the identification of the basic chemical structures of quinolones responsible for anti-toxoplasmosis activity and to the design of quinolones **1**–**4**.

4.3. Anti-*P. falciparum* and *P. yoelii yoelii* activities

The four quinolones **1**–**4** display a moderate activity against blood stages of the chloroquine-resistant strain (NF54-R) of *P. falciparum* (IC_{50} range 24–62 μg/mL). Their activity is much lower than that of the most active quinolones, i.e. GPFX and TVFX. Moreover, of these four quinolones, only **1** is active (45 μg/mL) against the hepatic stages of *P. yoelii yoelii*, and interestingly shows an alteration of *P. yoelii yoelii* schizonts. Among the 30 quinolones tested in the literature against chloroquine-sensitive and resistant blood stages of *P. falciparum*, and hepatic stages of *P. yoelii yoelii*, only fluoroquinolone **1** and three other quinolones, i.e. GPFX, TVFX, and piromidic acid, were associated with a marked morphology alteration, number and size reduction of the *P. yoelii yoelii* schizonts [23].

The analysis of these data indicates that the predictive QSAR models established for *T. gondii* cannot be reliably extended for the design of highly active antimalarial drugs. Although all the four quinolones that were active against *T. gondii* were also found to be active against the chloroquine-resistant blood stages of *P. falciparum*, only one of them displayed inhibitory effects against the hepatic stages of *P. yoelii yoelii*.

4.4. SAR studies

Concerning SAR at position 8 (C–OMe, C–H or N), the analysis of the experimental IC_{50} values of **1**–**4** and of structurally-related quinolones published in literature indicates that the anti-*T. gondii* and anti-*P. falciparum* activities are increasing, in a homogenous series, when replacing at position 8 C–OMe by C–H or by N. Indeed, in the 1-difluorophenyl-substituted quinolone series (**3**, **4**, FQ6, TVFX and FQ11), the replacement of C-OMe in **3** (resp. **4**) by C–H as in FQ6 or by N as in TVFX (resp. FQ11) induces a substantial decrease of the IC_{50} values [**3** (resp. **4**) > FQ6 > TVFX (resp. FQ11) for anti-*T. gondii*; **3** (resp. **4**) > TVFX (resp. FQ11) for anti-*P. falciparum*]. A similar trend but to a much lesser extent is also found in the cyclopropyl series (IC_{50} of **1** > FQ4; **2** > FQ9). That at position 8 a C–OMe is likely less suited for antiparasitical activity than C–H or N seems to be supported by the fact that among the six fluoroquinolones possessing a methoxy group as R^8 (**1**–**4**, GTFX, and MXFX), only one (i.e. **1**) was found to be active, though moderately, against *P. yoelii yoelii*.

Concerning R^7 SAR, our data with those of literature indicate that the contribution of R^7 to antiparasitical activity is also contrasting. In the 1-cyclopropyl-quinolone series, it decreases along (i) 6A-C (as in **1**) ∼ 3-MePi (as in GTFX) ∼ PpP (as in MXFX) > 6AM-C (as in **2**), for anti-*T. gondii*, (ii), it varies, however, along 3-MePi (as in GTFX) ∼ PpP (as in MXFX) > 6AM-C (as in **2**) ∼ 6A-C (as in **1**) for anti-*P. falciparum*, and (iii), 6A-C (as in **1**) > 6AM-C (as in **2**), 3-MePi (as in GTFX), or PpP (as in MXFX) for anti-*P. yoelii yoleii*. In the 2,4-difluorophenyl-quinolone series, the contribution of 6A-C to anti-*T. gondii* activity is almost comparable to that of 6AM-C (IC_{50} **3** ∼ **4**) while in the 2,4-difluorophenyl-naphtyridone series, replacement of 6A-C by 6AM-C induces a substantial decrease of activity (IC_{50} TVFX < FQ11).

Concerning R^1 SAR, replacement of cyclopropyl for 2,4-difluorophenyl is also contrasting, in line with literature, and depends on R^7 and on the nature of the antiparasitical activity: when R^7 is 6A-C, this replacement decreases anti-*T. gondii* and anti-*P. yoleii yoleii* activities (IC_{50} **1** < **3**) but is rather of low incidence on anti-*P. falciparum* activity (IC_{50} **1** ∼ **3**); when R^7 is 6AM-C, it increases anti-*P. falciparum* activity (IC_{50} **2** > **4**) and is rather of low incidence on anti-*T. gondii* activity (IC_{50} **2** ∼ **4**).

4.5. Antibacterial activity against *M. tuberculosis* and SAR study

The quinolones **1**–**4** were found to inhibit DNA gyrase and growth of *M. tuberculosis* (see data Table 2). If they display a DNA gyrase activity comparable to that of commercially available fluoroquinolones, two of them demonstrated an activity close to the most active compounds, e.g. MXFX, SPFX and GTFX. Our data confirm that anti-*M. tuberculosis* activity is higher for the cyclopropyl fluoroquinolone series (i.e. **1**, **2**) than for the 2,4-difluorophenyl series (i.e. **3**, **4**), in line with literature (GTFX, MXFX vs TVFX) [28]. Interestingly, it appears also that the nature of substituent R^7 (6A-C, 6AM-C, 3Me-Pi, PpP) has almost no impact on DNA gyrase inhibition (IC_{50} **1** ∼ **2** ∼ GTFX, MXFX).

MICs of all the four compounds were very high (>64 μg/mL) and they account for among the lowest active quinolones for inhibiting the growth of *M. tuberculosis*. Moreover, no correlation was found for quinolones **1**–**4** between DNA gyrase and growth inhibition of *M. tuberculosis*, in contrast to the other quinolones listed in Table 2. This could be due to either a poor bacterial uptake of quinolones **1**–**4**, as a result of low diffusion across the cell wall or efficient efflux. Instability or degradation of these compounds during MIC determination experiment, which requires incubation at 37 °C for 21–30 days, seems unlikely as they were found to be very stable under even more drastic conditions. However, we may observe that MIC is indeed increasing along 3Me-Pi (as in GTFX) < PpP (as in MXFX) < 6A-C (as in **1**/resp. **3**) = 6AM-C (as in **2**/resp. **4**), indicating that the high MIC of **1**–**4** and consequently their poor bacterial uptake could mainly be related to the R^7 6A-C and 6MA-C azabicyclic rings. These results further indicate that MIC measurements are essential to determine whether a quinolone is a promising antituberculosis agent or not.

5. Conclusion

The synthesis of the four new 8-methoxy-substituted fluoroquinolones **1**–**4** was performed with 15–18% overall yield

in seven steps from commercially available acid materials. Unfortunately, the synthetic strategy applied for **1**–**4** was unsuccessful for the preparation of the corresponding 8-ethyl-substituted fluoroquinolones **9**–**12**, the introduction of the substituent R^7 through an aromatic nucleophilic substitution of the C-7 fluorine atom in synthons **20c1**,**c2** or **21c1**,**c2** being the most difficult step to achieve. It was also unsuccessful for the 5-ethyl-8-methoxy analogs **5**–**8**, the main difficulty being here the condensation step leading to the key enaminone **18b** or malonate **22**, from the 6-ethyl-3-methoxy synthon **17b**.

The biological results show the usefulness and limits of our predictive QSAR models for *T. gondii*. Indeed, the four computer-designed fluoroquinolones were active on *T. gondii* but only one of these derivatives, i.e. **1**, was as active as predicted, showing that the models remain of interest to direct the synthesis of active anti-*T. gondii* quinolones. Concerning anti-*Plasmodium* spp. activity, the four compounds are active against blood stages of *P. falciparum* though at high concentration and one of them, i.e. **1**, is also inhibitory for hepatic stages associated with an effect on schizont size reduction. However, further studies are necessary to define more adapted and specific QSAR models for the design of new antimalarial drugs. Moreover, fluoroquinolones **1**–**4** inhibit DNA supercoiling by *M. tuberculosis* gyrase, the IC_{50} of **1** and **2** being below 6 μg/mL. This promising antituberculosis activity was unfortunately invalidated by their poor inhibition of *M. tuberculosis* growth as shown by their high MIC values. These results confirm the SAR deduced from previous study [28]. Nevertheless, it would be worth to explore the pharmacological properties and safety profile of **1** which is active against *T. gondii*, as well as against both erythrocytic and hepatic stages of *P. falciparum*, and against hepatic *P. yoelii yoelii* schizonts, thus appearing to be an antiparasitical drug with great potential.

6. Materials and methods

6.1. Design of quinolones

The mathematical QSAR models used for the design of the fluoroquinolones listed in Table 1 and for the prediction of their anti-*T. gondii* activity are detailed elsewhere [22,51,52]. Briefly, these models were based on the numerical description of a set of "training" compounds by topological indices (TIs) and the application of the statistical LDA technique. The obtained QSAR-LDA equations were applied to 24 known quinolones and the results indicate theoretical activity against *T. gondii* to be in good agreement with the experimentally in vitro IC_{50} data. These latter data were used for developing a new QSAR model by multilinear regression (MLR). The equation thus obtained accurately matched experimental and calculated IC_{50} values ($r^2 = 0.87$), and a very good predictive capacity of the model was confirmed by the cross-validation test ($r^2_{cv} = 0.74$). From both the experiments and the mathematical model, four fluoroquinolones emerged as being more active than the other compounds, as their IC_{50} values were below 10 mg/L. Trovafloxacin was the most active drug, with experimental and calculated IC_{50} values below 0.5 mg/L, followed by grepafloxacin, gatifloxacin and moxifloxacin. Furthermore, 11 trovafloxacin analogs experimentally tested [21] were submitted to the QSAR model, and good agreement was observed between the predicted and experimental IC_{50} values.

We then used as TIs, the *E*-state indices, which are specific for each atom and reflect the electronic and topological atomic features taking into account the interaction with the rest of the molecule [53]. These TIs were related to the anti-*T. gondii* activity of 24 known quinolones, thus providing some insights about the substituents leading to higher anti-*Toxoplasma* activity. Computational screening was finally used to select new quinolones with improved efficacy. Virtual structures were designed by the omission or substitution of some radicals on the most active quinolones tested (trovafloxacin, grepafloxacin, gatifloxacin and moxifloxacin, with IC_{50} values below 5 mg/L). Their TIs were calculated, and LDA and MLR equations were used to determine their activity/inactivity and IC_{50} values, respectively. The LDA and MLR models were then used to identify the pharmacophoric structures responsible for anti-*Toxoplasma* activity of quinolones [22].

6.2. Chemistry

6.2.1. General methods, reagents and starting materials

Reactions were conducted under an anhydrous nitrogen atmosphere using freshly distilled and dry solvents. Anhydrous solvents were prepared by standard methods. Column chromatography purifications were carried out on Silica Gel 60 (E. Merck, 70–230 mesh). The purity of all new compounds was checked by thin-layer chromatography (TLC) and NMR. All reactions were monitored by TLC analyses on precoated Silica Gel F254 plates (E. Merck) with detection by UV and by $KMnO_4$ (0.5% in 1 N aq NaOH solution, w/v) or charring with ninhydrin (0.3% in MeOH containing 3 vol% of acetic acid, w/v) or with 50% methanol–sulfuric acid solution. The purity of the final products (>99.5%) was checked by HPLC analyses (flow of 1 mL min^{-1}) using a Waters 996 photodiode array detector apparatus (PDA, UV detector from 195 to 290 nm) using a Lichrospher 100 RP-18 (5 μm)-packed column (250 × 4 mm) and two solvent systems, i.e. solvent A [isocratic H_2O/CH_3CN (65:35) 0.1% TFA] or solvent B [gradient H_2O/CH_3CN (from 80:20 to 0:100) 0.1% TFA over 30 min]. Melting points, determined with an Electrothermal model 3100 apparatus, are uncorrected. The ^1H, ^{13}C, and (^1H decoupled) ^{19}F NMR spectra were recorded with a Bruker AC 200 spectrometer at 200, 50.3, and 188.3 MHz, respectively. Chemical shifts (δ) were expressed in parts per million relative to the signal indirectly (i) to $CHCl_3$ (δ 7.27) for ^1H and (ii) to $CDCl_3$ (δ 77.16) for ^{13}C, and directly (iii) to $CFCl_3$ (internal standard) (δ 0.0) for ^{19}F. Coupling constants are expressed in hertz, and multiplicities are referred to as s (singlet), br s (broad singlet), d (doublet), t (triplet), and m (multiplet). Concerning the description of the NMR spectra, the atom numbering is indicated in Table 1. Electron-spray ionization mass spectra in positive mode [ESI(+) MS] were recorded on a Finnigan MAT TSQ 7000 apparatus equipped with an atmospheric pressure ionization source. The high resolution mass spectrometry (HRMS) analyses were

performed by the "Service Commun de Spectrométrie de Masse" at the Institut de Chimie des Substances Naturelles, Gif sur Yvette, France.

N-Benzylmaleimide, boron trifluoride diethyl etherate, triethylamine, trifluoroacetic acid (TFA) and oxalyl chloride were purchased from Aldrich. Ethyl propiolate, cyclopropylamine and 2,4-difluoraniline were obtained from Fluka.

2,4,5-Trifluoro-3-methoxybenzoic acid **17a** was prepared from 3,4,5,6-tetrafluorophthalic acid (Lancaster) as described in literature [36,63]. The 3-ethyl-2,4,5-trifluorobenzoic acid **17b** was synthesized from 2,4,5-trifluorobenzoic acid (Lancaster) according to literature [34].

The synthesis of (1α,5α,6α)-6-*tert*-butoxycarbonylamino-3-azabicyclo[3.1.0]hexane **23** and (1α,5α,6α)-6-(*tert*-butoxycarbonyl)aminomethyl-3-azabicyclo[3.1.0]hexane **24** was performed from *N*-benzylmaleimide in eight steps (about 10% overall yield) according to published procedures [37,38].

Compound 23: Mp = 110−111 °C; R_f = 0.35 (90:10:1 $CHCl_3$/MeOH/NH_4OH, UV, ninhydrin); ^{1}H NMR ($CDCl_3$) δ 1.42 [s, 10H, NH and $C(CH_3)_3$], 1.54 (s, 2H, H_1 and H_5), 2.26 (br s, 1H, H_6), 2.88 and 3.10 (AB system, 4H, H_2 and H_4, $^2J_{H-H}$ = 11.6 Hz), 4.69 (s, 1H, NHBoc); ^{13}C NMR ($CDCl_3$) δ 26.4 (C_1 and C_5), 28.5 [$C(CH_3)_3$], 30.4 (C_6), 48.8 (C_2 and C_4), 79.7 [$C(CH_3)_3$], 156.4 [NC(O)].

Compound 24: R_f = 0.30 (1:1 AcOEt/MeOH, UV, ninhydrin); ESI-MS (positive mode): $(M+H)^+$ = 213.3 (calcd for $C_{11}H_{20}N_2O_2$ 212.15); ^{1}H NMR ($CDCl_3$) δ 0.70 (tt, 1H, H_6, $^3J_{H-H}$ = 6.9 Hz, $^3J_{H-H}$ = 3.4 Hz), 1.21 (m, 2H, H_1 and H_5), 1.34 [s, 9H, $C(CH_3)_3$], 1.88 (br s, 1H, NH), 2.78−2.96 (m, 6H, H_2, H_4 and CH_2NHBoc), 4.92 (br s, 1H, NHBoc); ^{13}C NMR ($CDCl_3$) δ 20.4 (C_6), 21.1 (C_1 and C_5), 28.2 [$C(CH_3)_3$], 41.4 (CH_2NHBoc), 46.7 (C_2 and C_4), 79.0 [$C(CH_3)_3$], 155.9 [NHC(O)].

The synthesis of ethyl 3-(diethylamino)-2(*E*)-propenoate was carried out by heating an equimolar amount of diethylamine and ethyl propiolate in acetonitrile as described in Ref. [39]. After solvent evaporation, the Michael adduct was used without further purification [^{1}H NMR ($CDCl_3$) δ 4.55 (d, 1H, *H*C=CHN, $^3J_{H-H}$ = 13.1 Hz), and 7.42 (d, 1H, HC=C*H*N, $^3J_{H-H}$ = 13.1 Hz)].

6.2.2. Synthesis of α-enamino-β-ketoesters

6.2.2.1. Ethyl α(E)-[(diethylamino)methylene]-2,4,5-trifluoro-3-methoxy-β-oxo-benzenepropanoate, ***18a***. A CH_2Cl_2 solution (30 mL) of **17a** (1.36 g, 6.60 mmol), oxalyl chloride (0.80 mL, 9.17 mmol) and five drops of DMF was stirred for 24 h at room temperature. The reaction mixture was then subjected to concentrated evaporation under reduced pressure, solubilized in toluene (15 mL) and added dropwise to a toluene solution (15 mL) of triethylamine (3 mL, 16.5 mmol) and ethyl 3-(diethylamino)-2*E*-propenoate (1.29 g, 7.54 mmol). After 5 h of stirring at 90 °C, the cooled reaction mixture was washed with water. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude residue was purified by flash chromatography on silica gel (95:5 to 60:40 hexane/AcOEt) to give **18a** (1.88 g, 5.22 mmol, 79%) as a colourless oil. R_f = 0.25 (7:3 hexane/AcOEt, UV); ^{1}H NMR ($CDCl_3$) δ 0.77 (t, 3H, OCH_2CH_3, $^3J_{H-H}$ = 7.1 Hz), 0.84 (br s, 3H, NCH_2CH_3), 1.08 (br s, 3H, NCH_2CH_3), 3.25 (br s, 4H, NCH_2CH_3), 3.76 (q, 2H, OCH_2CH_3, $^3J_{H-H}$ = 7.1 Hz), 3.78 (s, 3H, OCH_3), 6.87 (ddd, 1H, H_{Ar}, $^3J_{H-F}$ = 10.1 Hz, $^4J_{H-F}$ = 8.5 Hz, $^4J_{H-F}$ = 6.0 Hz), 7.53 (s, 1H, C=CHN); ^{19}F NMR ($CDCl_3$) δ −135.1 (dd, F_2, $^5J_{F-F}$ = 13.8 Hz, $^4J_{F-F}$ = 7.6 Hz), −141.5 (dd, F_5, $^3J_{F-F}$ = 20.6 Hz, $^5J_{F-F}$ = 13.7 Hz), −149.2 (br d, F_4, $^3J_{F-F}$ = 16.5 Hz); ^{13}C NMR ($CDCl_3$) δ 10.8, 14.2 (br s, NCH_2CH_3), 13.4 (OCH_2CH_3), 45.0, 53.8 (br s, NCH_2CH_3), 59.4 (OCH_2CH_3), 61.5 (t, OCH_3, $^4J_{C-F}$ = 3.5 Hz), 101.6 (*C*=CHN), 109.5 (dd, C_6, $^2J_{C-F}$ = 20.1 Hz, $^3J_{C-F}$ = 3.3 Hz), 126.2 (ddd, C_1, $^2J_{C-F}$ = 14.8 Hz, $^3J_{C-F}$ = 5.6 Hz, $^4J_{C-F}$ = 3.9 Hz), 137.1 (ddd, C_3, $^2J_{C-F}$ = 16.6 Hz, $^2J_{C-F}$ = 11.1 Hz, $^3J_{C-F}$ = 2.1 Hz), 145.1 (ddd, C_4, $^1J_{C-F}$ = 253.2 Hz, $^2J_{C-F}$ = 15.2 Hz, $^3J_{C-F}$ = 5.3 Hz), 146.6 (ddd, C_5, $^1J_{C-F}$ = 246.1 Hz, $^2J_{C-F}$ = 11.5 Hz, $^4J_{C-F}$ = 3.3 Hz), 149.1 (dt, C_2, $^1J_{C-F}$ = 248.8 Hz, $^3J_{C-F}$ = $^4J_{C-F}$ = 3.3 Hz), 154.4 (C=*C*HN), 167.3 [C(O)O], 184.5 [C(O)C].

6.2.2.2. Ethyl α(E)-[(diethylamino)methylene]-3-ethyl-2,4,5-trifluoro-β-oxo-benzenepropanoate, ***18c***. The procedure as described for **6**, when applied to **17c** (524 mg, 2.58 mmol), 0.35 mL (4.0 mmol) of oxalyl chloride, 1.4 mL (10 mmol) of Et_3N and 790 mg (4.61 mmol) of ethyl 3-(diethylamino)-2*E*-propenoate gave, after silica gel chromatography (90:10 to 75:25 hexane/AcOEt), 750 mg (2.10 mmol, 80%) of **18c** as a colourless oil. R_f = 0.25 (7:3 hexane/AcOEt, UV); ^{1}H NMR ($CDCl_3$) δ 0.84 (t, 3H, OCH_2CH_3, $^3J_{H-H}$ = 7.1 Hz), 0.90 (br s, 3H, NCH_2CH_3), 1.08 (t, 3H, CH_3(Ar), $^3J_{H-H}$ = 7.6 Hz), 1.10 (br s, 3H, NCH_2CH_3), 2.59 (q, 2H, CH_2(Ar), $^3J_{H-H}$ = 7.5 Hz), 3.36 (br s, 4H, $2NCH_2CH_3$), 3.86 (q, 2H, OCH_2CH_3, $^3J_{H-H}$ = 7.1 Hz), 7.16 (td, 1H, H(Ar), $^3J_{H-F}$ = $^4J_{H-F}$ = 9.2 Hz, $^4J_{H-F}$ = 6.6 Hz), 7.64 (s, 1H, C=CHN); ^{19}F NMR ($CDCl_3$) δ −122.8 (dd, 1F, F_2, $^5J_{F-F}$ = 15.8 Hz, $^4J_{F-F}$ = 6.9 Hz), −137.1 (br s, 1F, F_4), −143.4 (dd, F_5, $^3J_{F-F}$ = 21.3 Hz, $^5J_{F-F}$ = 15.8 Hz); ^{13}C NMR ($CDCl_3$) δ 11.2, 14.5 (2br s, $2NCH_2CH_3$), 13.7 (2s, CH_3(Ar) and OCH_2CH_3), 16.1 [CH_2(Ar)], 45.3, 54.1 (2br s, $2NCH_2CH_3$), 59.7 (OCH_2CH_3), 102.1 (*C*=CHN), 114.3 (dd, C_6, $^2J_{C-F}$ = 19.9 Hz, $^3J_{C-F}$ = 3.1 Hz), 121.1 (dd, C_3, $^2J_{C-F}$ = 23.8 Hz, $^2J_{C-F}$ = 17.2 Hz), 126.3 (dt, C_1, $^2J_{C-F}$ = 17.2 Hz, $^3J_{C-F}$ = $^4J_{C-F}$ = 4.4 Hz), 146.5 (ddd, C_5, $^1J_{C-F}$ = 245.1 Hz, $^2J_{C-F}$ = 13.5 Hz, $^4J_{C-F}$ = 3.3 Hz), 149.8 (ddd, C_4, $^1J_{C-F}$ = 251.0 Hz, $^2J_{C-F}$ = 14.3 Hz, $^3J_{C-F}$ = 9.2 Hz), 153.8 (ddd, C_2, $^1J_{C-F}$ = 247.7 Hz, $^3J_{C-F}$ = 7.0 Hz, $^4J_{C-F}$ = 2.4 Hz), 154.5 (C=*C*HN), 167.9 [C(O)O], 185.7 [C(O)C].

6.2.3. Synthesis of ethyl 6,7-difluoro-8-substituted quinoline-carboxylates (cyclization step)

6.2.3.1. Ethyl 1-cyclopropyl-6,7-difluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinoline-carboxylate, ***20a1***. Compound **18a** (850 mg, 2.37 mmol) in 1:2 EtOH/Et_2O (20 mL) was added to cyclopropylamine (0.38 mL, 5.48 mmol). After 3 h of stirring at room temperature, the reaction mixture was evaporated under reduced pressure. The oily residue containing

19a1 (^{1}H NMR monitoring) was dissolved in DMF (20 mL) and K_2CO_3 (1.32 g, 9.57 mmol) was then added. After 5 h of stirring at 100 °C, cold water (3 mL) was added. The yellow precipitate was filtered and dried affording **20a1** (627 mg, 1.94 mmol, 82%). $R_f = 0.60$ (97:3 $CHCl_3$/MeOH, UV); mp = 183–184 °C; ^{1}H NMR ($CDCl_3$) δ 1.04 and 1.19 [2m, 4H, CH_2(cPr)], 1.37 (t, 3H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz), 3.97 [tt, 1H, CH(cPr), $^3J_{H-H} = 7.5$ Hz, $^3J_{H-H} = 3.7$ Hz], 4.07 (d, 3H, OCH_3, $^5J_{H-F} = 1.9$ Hz), 4.35 (q, 2H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz), 7.97 (dd, 1H, H_5, $^3J_{H-F} = 10.0$ Hz, $^4J_{H-F} = 8.8$ Hz), 8.56 (s, 1H, H_2); ^{19}F NMR ($CDCl_3$) δ −136.9 and −145.1 (2d, 2F, F_6 and F_7, $^3J_{F-F} = 21.3$ Hz); ^{13}C NMR ($CDCl_3$) δ 9.2 [CH_2(cPr)], 14.5 (OCH_2CH_3), 39.8 [CH(cPr)], 61.1 (OCH_2CH_3), 62.9 (d, OCH_3, $^4J_{C-F} = 7.7$ Hz), 108.7 (dd, C_5, $^2J_{C-F} = 18.7$ Hz, $^3J_{C-F} = 1.1$ Hz), 110.1 (C_3), 126.1 (dd, C_{10}, $^3J_{C-F} = 5.9$ Hz, $^4J_{C-F} = 1.8$ Hz), 131.6 (dd, C_9, $^3J_{C-F} = 3.7$ Hz, $^4J_{C-F} = 2.2$ Hz), 140.4 (d, C_8, $^2J_{C-F} = 12.1$ Hz), 148.2 (dd, C_7, $^1J_{C-F} = 253.7$ Hz, $^2J_{C-F} = 15.6$ Hz), 149.2 (dd, C_6, $^1J_{C-F} = 251.4$ Hz, $^2J_{C-F} = 12.4$ Hz), 150.7 (C_2), 165.2 [C(O)O], 172.3 (C_4).

6.2.3.2. Ethyl 1-(2,4-difluorophenyl)-6,7-difluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinoline-carboxylate, ***20a2***. The procedure described for **20a1** when applied first to **18a** (672 mg, 1.87 mmol) and 2,4-difluoroaniline (0.40 mL, 3.97 mmol) for 24 h at room temperature, then to DMF (15 mL) and K_2CO_3 (978 mg, 7.08 mmol), led to **20a2** (611 mg, 1.55 mmol, 83%) as a yellow solid. $R_f = 0.65$ (97:3 $CHCl_3$/MeOH, UV); mp = 186–187 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz), 3.46 (d, 3H, OCH_3, $^5J_{H-F} = 1.5$ Hz), 4.26 (qd, 2H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz, $^9J_{H-F} = 1.3$ Hz), 7.00–7.07 [m, 2H, H(Ar)], 7.73 [td, 1H, H(Ar), $J = 8.6$ Hz, $J = 5.7$ Hz], 7.87 (dd, 1H, H_5, $^3J_{H-F} = 10.2$ Hz, $^4J_{H-F} = 8.5$ Hz), 8.16 (s, 1H, H_2); ^{19}F NMR ($CDCl_3$) δ −106.5 and −117.2 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F} = 8.2$ Hz), −136.4 and −144.8 (2d, 2F, F_6 and F_7, $^3J_{F-F} = 21.3$ Hz); ^{13}C NMR ($CDCl_3$) δ 14.3 (OCH_2CH_3), 61.1 (OCH_2CH_3), 62.0 (d, OCH_3, $^4J_{C-F} = 7.0$ Hz), 104.7 (dd, $C_{3'}$, $^2J_{C-F} = 26.7$ Hz, $^2J_{C-F} = 23.1$ Hz), 108.4 (d, C_5, $^2J_{C-F} = 19.0$ Hz), 110.9 (C_3), 112.0 (dd, $C_{5'}$, $^2J_{C-F} = 22.7$ Hz, $^4J_{C-F} = 4.0$ Hz), 125.0 (dd, C_{10}, $^3J_{C-F} = 6.2$ Hz, $^4J_{C-F} = 2.2$ Hz), 128.7 (dd, $C_{1'}$, $^2J_{C-F} = 13.5$ Hz, $^4J_{C-F} = 4.4$ Hz), 128.9 (d, $C_{6'}$, $^3J_{C-F} = 10.6$ Hz), 130.9 (dd, C_9, $^3J_{C-F} = 4.0$ Hz, $^4J_{C-F} = 2.2$ Hz), 139.2 (dd, C_8, $^2J_{C-F} = 12.4$ Hz, $^3J_{C-F} = 1.1$ Hz), 148.0 (dd, C_7, $^1J_{C-F} = 254.9$ Hz, $^2J_{C-F} = 15.5$ Hz), 149.2 (dd, C_6, $^1J_{C-F} = 252.3$ Hz, $^2J_{C-F} = 12.3$ Hz), 151.2 (C_2), 157.6 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 254.0$ Hz, $^3J_{C-F} = 12.8$ Hz), 162.8 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 253.2$ Hz, $^3J_{C-F} = 11.0$ Hz), 164.2 [C(O)O], 172.1 (br s, C_4).

6.2.3.3. Ethyl 1-cyclopropyl-8-ethyl-6,7-difluoro-1,4-dihydro-4-oxo-3-quinoline-carboxylate, ***20c1***. The procedure described for **20a1** when applied to **18c** (195 mg, 0.55 mmol), cyclopropylamine (0.10 mL, 1.42 mmol), and K_2CO_3 (300 mg, 2.17 mmol) gave after work-up **20c1** (148 mg, 0.46 mmol, 83%) as a yellow solid. $R_f = 0.65$ (96:4 $CHCl_3$/MeOH, UV); mp = 202–204 °C; ^{1}H NMR ($CDCl_3$) δ 1.01 and 1.22 [2m, 4H, CH_2(cPr)], 1.20 (br t, 3H, CH_3(Ar), $^3J_{H-H} = 7.5$ Hz), 1.37 (t, 3H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz), 3.39 (qd, 2H, CH_2(Ar), $^3J_{H-H} = 7.5$ Hz, $^4J_{H-F} = 2.8$ Hz), 3.88 (tt, 1H, CH(cPr), $^3J_{H-H} = 7.0$ Hz, $^3J_{H-H} = 3.5$ Hz), 4.35 (q, 2H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz), 8.09 (t, 1H, H_5, $^3J_{H-F} = {}^4J_{H-F} = 9.6$ Hz), 8.64 (s, 1H, H_2); ^{19}F NMR ($CDCl_3$) δ −131.0 and −138.5 (2d, 1F, F_6 and F_7, $^3J_{F-F} = 22.7$ Hz); ^{13}C NMR ($CDCl_3$) δ 11.1 [2 CH_2(cPr)], 14.4 (OCH_2CH_3), 15.3 [CH_3(Ar)], 19.3 (dd, CH_2(Ar), $^3J_{C-F} = 7.2$ Hz, $^4J_{C-F} = 1.7$ Hz), 38.9 [CH(cPr)], 60.9 (OCH_2CH_3), 110.6 (C_3), 112.4 (dd, C_5, $^2J_{C-F} = 18.9$ Hz, $^3J_{C-F} = 2.8$ Hz), 124.1 (d, C_8, $^2J_{C-F} = 15.4$ Hz), 127.2 (dd, C_{10}, $^3J_{C-F} = 5.1$ Hz, $^4J_{C-F} = 2.2$ Hz), 136.9 (dd, C_9, $^3J_{C-F} = 5.3$ Hz, $^4J_{C-F} = 2.0$ Hz), 148.6 (dd, C_6, $^1J_{C-F} = 250.7$ Hz, $^2J_{C-F} = 15.9$ Hz), 152.2 (C_2), 152.3 (dd, C_7, $^1J_{C-F} = 250.3$ Hz, $^2J_{C-F} = 14.5$ Hz), 165.0 [C(O)O], 173.0 (C_4).

6.2.3.4. Ethyl 8-ethyl-1-(2,4-difluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxo-3-quinoline-carboxylate, ***20c2***. The procedure described for **20a1** when applied to **11** (438 mg, 1.23 mmol), 2,4-difluoraniline (0.40 mL, 3.97 mmol), and K_2CO_3 (606 mg, 4.38 mmol) gave after work-up **20c2** (388 mg, 0.99 mmol, 80%) as an orange solid. $R_f = 0.65$ (97:3 $CHCl_3$/MeOH, UV); mp = 172–174 °C; ^{1}H NMR ($CDCl_3$) δ 0.79 (t, 3H, CH_3(Ar), $^3J_{H-H} = 7.4$ Hz), 1.28 (t, 3H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz), 2.17 [m, 2H, CH_2(Ar)], 4.26 (q, 2H, OCH_2CH_3, $^3J_{H-H} = 7.1$ Hz), 7.04–7.10 [m, 2H, H(Ar)], 7.70 [m, 1H, H(Ar)], 8.03 (t, 1H, H_5, $^3J_{H-F} = {}^4J_{H-F} = 9.6$ Hz), 8.18 (s, 1H, H_2); ^{19}F NMR ($CDCl_3$) δ −104.5 and −116.0 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F} = 8.5$ Hz), −130.0 and −137.8 (2d, 2F, F_6 and F_7, $^3J_{F-F} = 22.7$ Hz); ^{13}C NMR ($CDCl_3$) δ 13.8 [CH_3(Ar)], 14.2 (OCH_2CH_3), 18.2 (dd, CH_2(Ar), $^3J_{C-F} = 6.6$ Hz, $^4J_{C-F} = 1.1$ Hz), 61.0 (OCH_2CH_3), 105.9 (dd, $C_{3'}$, $^2J_{C-F} = 26.5$ Hz, $^2J_{C-F} = 22.9$ Hz), 111.0 (C_3), 112.4 (dd, C_5, $^2J_{C-F} = 18.7$ Hz, $^3J_{C-F} = 2.6$ Hz), 113.2 (dd, $C_{5'}$, $^2J_{C-F} = 22.7$ Hz, $^4J_{C-F} = 4.0$ Hz), 123.5 (d, C_8, $^2J_{C-F} = 16.1$ Hz), 126.6 (dd, C_{10}, $^3J_{C-F} = 5.1$ Hz, $^4J_{C-F} = 2.2$ Hz), 128.1 (dd, $C_{1'}$, $^2J_{C-F} = 12.8$ Hz, $^4J_{C-F} = 4.4$ Hz), 129.9 (d, $C_{6'}$, $^3J_{C-F} = 10.2$ Hz), 135.8 (dd, C_9, $^3J_{C-F} = 5.7$ Hz, $^4J_{C-F} = 2.0$ Hz), 148.7 (dd, C_6, $^1J_{C-F} = 251.4$ Hz, $^2J_{C-F} = 15.7$ Hz), 152.2 (C_2), 152.8 (dd, C_7, $^1J_{C-F} = 251.4$ Hz, $^2J_{C-F} = 14.3$ Hz), 157.5 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 255.4$ Hz, $^3J_{C-F} = 12.6$ Hz), 163.3 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 255.4$ Hz, $^3J_{C-F} = 11.0$ Hz), 164.2 [C(O)O], 172.8 (C_4).

6.2.4. Synthesis of boron complexes

6.2.4.1. (1-Cyclopropyl-6,7-difluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinoline-carboxylato-O3,O4)difluoro-boron, ***21a1***. $BF_3 \cdot Et_2O$ (1.5 mL, 11.8 mmol) was added to **20a1** (321 mg, 0.99 mmol) in suspension in THF (20 mL). After refluxing for 48 h, the clear reaction mixture was evaporated under reduced pressure. The crude oily residue was successively washed with Et_2O, $CHCl_3$, and water affording **21a1** as a white solid (265 mg, 0.77 mmol, 78%). $R_f = 0.40$ (97:3 $CHCl_3$/MeOH, UV); mp = 221–223 °C; ESI-MS (positive mode): $(M + H)^+ = 344.2$ (calcd for $C_{14}H_{10}BF_4NO_4$ 343.06); ^{1}H NMR (CD_3CN) δ 1.25–1.37 [m, 4H, $2CH_2$(cPr)], 4.19 (d, 3H, OCH_3, $^5J_{H-F} = 2.4$ Hz), 4.48 (tt, 1H, CH(cPr), $^3J_{H-H} = 7.3$ Hz,

$^3J_{H-H}$ = 3.8 Hz), 8.17 (dd, 1H, H_5, $^3J_{H-F}$ = 9.8 Hz, $^4J_{H-F}$ = 8.1 Hz), 9.17 (s, 1H, H_2); ^{19}F NMR (CD_3CN) δ −131.7 and −139.0 (2d, 2F, F_6 and F_7, $^3J_{F-F}$ = 19.9 Hz), −144.0 (s, 0.5F, $^{10}BF_2$), −144.1 (s, 2.4F, $^{11}BF_2$).

6.2.4.2. (1-(2,4-Difluorophenyl)-6,7-difluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylato-O3,O4)difluoro-boron, ***21a2***. Similarly, **20a2** (499 mg, 1.26 mmol) when reacted with $BF_3 \cdot Et_2O$ (2.0 mL, 15.80 mmol) in THF (25 mL) afforded after work-up **21a2** (407 mg, 0.98 mmol, 78%) as a white solid. R_f = 0.50 (97:3 $CHCl_3$/MeOH, UV); mp = 227–228 °C; ^{1}H NMR (CD_3CN) δ 3.54 (d, 3H, OCH_3, $^5J_{H-F}$ = 2.0 Hz), 7.26 [m, 2H, H(Ar)], 7.70 [m, 1H, H(Ar)], 8.30 (dd, 1H, H_5, $^3J_{H-F}$ = 9.7 Hz, $^4J_{H-F}$ = 8.0 Hz), 9.06 (s, 1H, H_2); ^{19}F NMR (DMSO-d_6) δ −105.6 and −116.9 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F}$ = 8.9 Hz), −128.5 and −135.9 (2d, 2F, F_6 and F_7, $^3J_{F-F}$ = 22.7 Hz), 139.0 and 140.2 (AB system, 0.6F, $^{10}BF_2$, J_{F-F} = 71.5 Hz), 139.1 and 140.3 (AB system, 2.0F, $^{11}BF_2$, J_{F-F} = 71.5 Hz).

6.2.4.3. (1-Cyclopropyl-8-ethyl-6,7-difluoro-1,4-dihydro-4-oxo-3-quinoline-carboxylato-O3,O4)difluoro-boron, ***21c1***. Similarly, **20c1** (117 mg, 0.364 mmol) and $BF_3 \cdot Et_2O$ (0.60 mL, 4.74 mmol) in THF (5 mL) afforded after work-up **21c1** (97 mg, 0.28 mmol, 78%) as a white solid. R_f = 0.50 (96:4 $CHCl_3$/MeOH, UV); mp = 274–276 °C; ^{1}H NMR (CD_3CN) δ 1.16 (br t, 5H, CH_2(cPr) and CH_3(Ar), $^3J_{H-H}$ = 7.5 Hz), 1.38 [m, 2H, CH_2(cPr)], 3.60 (qd, 2H, CH_2(Ar), $^3J_{H-H}$ = 7.5 Hz, $^4J_{H-F}$ = 3.3 Hz), 4.49 (tt, 1H, CH(cPr), $^3J_{H-H}$ = 7.0 Hz, $^3J_{H-H}$ = 3.5 Hz), 8.23 (t, 1H, H_5, $^3J_{H-F}$ = $^4J_{H-F}$ = 9.1 Hz), 9.24 (s, 1H, H_2); ^{19}F NMR (CD_3CN) δ −121.8 and −131.8 (2d, 2F, F_6 and F_7, $^3J_{F-F}$ = 21.3 Hz), −142.2 (s, 0.4F, $^{10}BF_2$), −142.3 (s, 1.9F, $^{11}BF_2$).

6.2.4.4. (8-Ethyl-1-(2,4-difluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylato-O3,O4) difluoro-boron, ***21c2***. Similarly, **20c2** (285 mg, 0.725 mmol) and $BF_3 \cdot Et_2O$ (1.20 mL, 9.44 mmol) in THF (25 mL) afforded **21c2** (152 mg, 0.37 mmol, 51%) as a white solid. R_f = 0.50 (92:8 $CHCl_3$/MeOH, UV); mp = 279–281 °C; ^{1}H NMR (CD_3CN) δ 0.87 (t, 3H, CH_3(Ar), $^3J_{H-H}$ = 7.5 Hz), 2.35 [m, 2H, CH_2(Ar)], 7.30 [m, 2H, H(Ar)], 7.80 [m, 1H, H(Ar)], 8.48 (t, 1H, H_5, $^3J_{H-F}$ = $^4J_{H-F}$ = 9.1 Hz), 9.09 (s, 1H, H_2); ^{19}F NMR (DMSO-d_6) δ −103.2 and −116.3 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F}$ = 9.3 Hz), −119.4 and −130.4 (d, 1F, F_6 and F_7, $^3J_{F-F}$ = 21.3 Hz), 141.6 and 142.4 (AB system, 0.5F, $^{10}BF_2$, J_{F-F} = 72.9 Hz), 141.7 and 142.5 (AB system, 2.0F, $^{11}BF_2$, J_{F-F} = 72.9 Hz).

6.2.5. Synthesis of 7-amino-derived-6-fluoro-8-methoxy-quinolinecarboxylic acids, ***1–4***

6.2.5.1. 7-[(1α,5α,6α)-6-Amino-3-azabicyclo[3.1.0]hex-3-yl]-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***1***

6.2.5.1.1. 1-Cyclopropyl-7-[(1α,5α,6α)-6-[[(1,1-dimethylethoxy)carbonyl]amino]-3-azabicyclo[3.1.0]hex-3-yl]-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***1-Boc***. A solution of **23** (78 mg, 0.39 mmol) and **21a1** (52 mg, 0.15 mmol) in CH_3CN (2 mL) was refluxed for one week. After evaporation under reduced pressure, the crude residue was partitioned in 1:1 $CHCl_3/H_2O$. The organic layer was evaporated leading to a yellow oil (97 mg) consisting of a mixture of **1′-Boc** (difluoro-boron complex of **1-Boc**) and **21a1** in 75:25 ratio (TLC and ^{19}F NMR monitoring). This oily residue dissolved in EtOH (2 mL) and triethylamine (0.9 mL, 6.4 mmol) was stirred at 80 °C for 48 h. After evaporation, the crude residue obtained was poured into 2 N NaOH and filtered. The precipitate was dissolved in $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4, filtered and evaporated to afford **1-Boc** (52 mg, 0.10 mmol, 72%) as a yellow oil. R_f = 0.50 (94:6 $CHCl_3$/MeOH, UV); ^{1}H NMR ($CDCl_3$) δ 0.95 and 1.18 [m, 4H, CH_2(cPr)], 1.42 [s, 9H, $C(CH_3)_3$], 1.78 (s, 2H, $H_{1''}$ and $H_{5''}$), 2.52 (s, 1H, $H_{6''}$), 3.55 (s, 3H, OCH_3), 3.62 and 3.89 (AB system, 4H, $H_{2''}$ and $H_{4''}$, $^2J_{H-H}$ = 11.1 Hz), 3.99 [m, 1H, CH(cPr)], 4.87 (br s, 1H, NHBoc), 7.72 (d, 1H, H_5, $^3J_{H-F}$ = 13.3 Hz), 8.74 (s, 1H, H_2), 14.89 (br s, 1H, COOH); ^{19}F NMR ($CDCl_3$) δ −118.4 (s, 1F, F_6); ^{13}C NMR ($CDCl_3$) δ 9.6 [CH_2(cPr)], 24.5 ($C_{1''}$ and $C_{5''}$), 28.4 [$C(CH_3)_3$], 31.8 ($C_{6''}$), 40.6 [CH(cPr)], 51.8 (d, $C_{2''}$ and $C_{4''}$, $^4J_{C-F}$ = 7.3 Hz), 61.7 (OCH_3), 79.8 [$C(CH_3)_3$], 107.7 (C_3), 107.9 (d, C_5, $^2J_{C-F}$ = 24.2 Hz), 120.3 (d, C_{10}, $^3J_{C-F}$ = 9.2 Hz), 134.2 (C_9), 136.5 (d, C_7, $^2J_{C-F}$ = 11.3 Hz), 143.8 (d, C_8, $^3J_{C-F}$ = 6.6 Hz), 149.9 (C_2), 155.1 (d, C_6, $^1J_{C-F}$ = 252.1 Hz), 156.4 [NHC(O)O], 166.9 [C(O)O], 176.9 (d, C_4, $^4J_{C-F}$ = 2.9 Hz).

6.2.5.1.2. Synthesis of ***1*** *(as its 2.6 TFA salt).* The Boc deprotection of **1-Boc** (22 mg, 0.0465 mmol) was achieved in 1:1 CH_2Cl_2/TFA mixture (4 mL) at 0 °C for 2 h. After evaporation, several additions of hexane and evaporations were performed to eliminate excess TFA. The crude residue was successively washed with Et_2O and CH_2Cl_2. Recrystallization (9:1 H_2O/CH_3CN) followed by lyophilization afforded **1** (21 mg, 0.037 mmol, 79%) as a white solid. R_f = 0.20 (98:2 $CHCl_3$/MeOH, UV); HPLC: R_t = 5.2 min (solvent A) and 11.8 min (solvent B); ^{1}H NMR (1:4 CD_3CN/D_2O) δ 0.93 and 1.13 [m, 4H, CH_2(cPr)], 2.04 (s, 2H, $H_{1''}$ and $H_{5''}$), 2.54 (s, 1H, $H_{6''}$), 3.52 (s, 3H, OCH_3), 3.59 and 3.82 (AB system, 4H, $H_{2''}$ and $H_{4''}$, $^2J_{H-H}$ = 9.7 Hz), 4.10 [m, 1H, CH(cPr)], 7.63 (d, 1H, H_5, $^3J_{H-F}$ = 13.1 Hz), 8.75 (s, 1H, H_2); ^{19}F NMR (1:4 CD_3CN/D_2O) δ −74.4 (s, 4.8F, TFA), −117.4 (s, 1F, F_6); ^{13}C NMR (1:4 CD_3CN/D_2O) δ 9.3 [CH_2(cPr)], 20.9 ($C_{1''}$ and $C_{5''}$), 29.9 ($C_{6''}$), 41.1 [CH(cPr)], 51.2 (d, $C_{2''}$ and $C_{4''}$, $^4J_{C-F}$ = 5.6 Hz), 62.4 (OCH_3), 106.6 (C_3), 106.7 (d, C_5, $^2J_{C-F}$ = 24.1 Hz), 119.9 (d, C_{10}, $^3J_{C-F}$ = 8.0 Hz), 134.5 (C_9), 136.1 (d, C_7, $^2J_{C-F}$ = 11.4 Hz), 144.8 (d, C_8, $^3J_{C-F}$ = 6.9 Hz), 150.8 (C_2), 154.9 (d, C_6, $^1J_{C-F}$ = 250.1 Hz), 158.8 (q, CF_3CO_2H, $^2J_{C-F}$ = 31.7 Hz), 166.3 [C(O)O], 176.5 (C_4). ESI-MS (positive mode): $(M + H)^+$ = 374.2, $(M + Na)^+$ = 396.2, in agreement with the mass calculated for $M = C_{19}H_{20}FN_3O_4$ (373.14). ESI-HRMS

(positive mode): 374.1516 is in agreement with the mass calculated for $(M+H)=C_{19}H_{21}FN_3O_4$ (374.1516).

6.2.5.2. 7-[(1α,5α,6α)-6-(Aminomethyl)-3-azabicyclo[3.1.0]-hex-3-yl]-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***2***

6.2.5.2.1. 1-Cyclopropyl-7-[(1α,5α,6α)-6-[[[(1,1-dimethylethoxy)carbonyl]amino]-methyl]-3-azabicyclo[3.1.0]hex-3-yl]-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***2*****-Boc**. A solution of **24** (445 mg, 2.10 mmol) and **21a1** (157 mg, 0.46 mmol) in CH_3CN (14 mL) was refluxed for one week. After evaporation, the crude residue was dissolved in a 1:1 $CHCl_3/H_2O$ mixture. The organic layer was extracted, dried over Na_2SO_4, filtered and evaporated. The yellow oily residue was purified by flash chromatography on silica gel (100:0 to 96:4 $CHCl_3$/MeOH) affording **2-Boc** (180 mg, 0.37 mmol, 81%) as a colourless oil. $R_f=0.85$ (9:1 $CHCl_3$/MeOH, UV); ^{1}H NMR ($CDCl_3$) δ 0.70 (tt, 1H, $H_{6''}$, $^3J_{H-H}=3.4$ Hz, $^3J_{H-H}=6.9$ Hz), 0.90 and 1.13 [2m, 4H, CH_2(cPr)], 1.33 [s, 9H, $C(CH_3)_3$], 1.47 (s, 2H, $H_{1''}$ and $H_{5''}$), 3.02 (m, 2H, C*H*$_2$NHBoc), 3.49 (s, 3H, OCH_3), 3.53 (m, 2H, $H_{2''}$ and $H_{4''}$ exo or endo), 3.71 (d, 2H, $H_{2''}$ and $H_{4''}$ exo or endo, $^2J_{H-H}=10.2$ Hz), 3.95 (tt, 1H, CH(cPr), $^3J_{H-H}=3.6$ Hz, $^3J_{H-H}=7.0$ Hz), 4.93 (br s, 1H, NHBoc), 7.59 (d, 1H, H_5, $^3J_{H-F}=13.4$ Hz), 8.65 (s, 1H, H_2), 14.86 (br s, 1H, COOH); ^{19}F NMR ($CDCl_3$) δ −118.7 (s, 1F, F6); ^{13}C NMR ($CDCl_3$) δ 9.2 [CH_2(cPr)], 20.7 ($C_{6''}$), 21.0 ($C_{1''}$ and $C_{5''}$), 28.1 [$C(CH_3)_3$], 40.3 (CH_2NHBoc), 41.9 [CH(cPr)], 51.9 (d, $C_{2''}$ and $C_{4''}$, $^4J_{C-F}=7.3$ Hz), 61.3 (OCH_3), 78.9 [$C(CH_3)_3$], 107.1 (C_3), 107.3 (d, C_5, $^2J_{C-F}=24.2$ Hz), 119.4 (d, C_{10}, $^4J_{C-F}=9.2$ Hz), 133.9 (C_9), 136.7 (d, C_7, $^2J_{C-F}=11.0$ Hz), 143.3 (d, C_8, $^3J_{C-F}=6.6$ Hz), 149.4 (C_2), 154.6 (d, C_6, $^1J_{C-F}=252.1$ Hz), 155.7 [NHC(O)O], 166.6 [C(O)O], 176.5 (d, C_4, $J_{C-F}=3.3$ Hz).

6.2.5.2.2. Synthesis of ***2*** *(as its 0.6 TFA salt).* Likewise, the Boc deprotection procedure when applied to **2-Boc** (114 mg, 0.234 mmol) afforded, after recrystallization (9:1 H_2O/CH_3CN) and lyophilization, **2** as a white solid (103 mg, 0.148 mmol, 63%). $R_f=0.35$ (80:20 $CHCl_3$/MeOH, UV); HPLC: $R_t=7.4$ min (solvent A) and 13.2 min (solvent B); ^{1}H NMR (1:9 CD_3CN/D_2O) δ 0.93 [m, 2H, CH_2(cPr)], 1.13 [m, 3H, CH_2(cPr) and $H_{6''}$], 1.66 (s, 2H, $H_{1''}$ and $H_{5''}$), 2.88 (d, 2H, C*H*$_2$NHBoc, $^3J_{H-H}=7.5$ Hz), 3.49 (s, 3H, OCH_3), 3.53 and 3.75 (AB system, 4H, $H_{2''}$ and $H_{4''}$, $^2J_{H-H}=10.4$ Hz), 4.13 (tt, 1H, CH(cPr), $^3J_{H-H}=7.2$ Hz, $^3J_{H-H}=3.6$ Hz), 7.45 (d, 1H, H_5, $^3J_{H-F}=13.4$ Hz), 8.74 (s, 1H, H_2); ^{19}F NMR (1:9 CD_3CN/D_2O) δ −74.4 (s, 1.8F, TFA), −117.2 (s, 1F, F_6); ^{13}C NMR (1:9 CD_3CN/D_2O) δ 9.9 [CH_2(cPr)], 18.6 (s, $C_{6''}$), 22.6 ($C_{1''}$ and $C_{5''}$), 42.2 and 42.5 [CH(cPr) and CH_2NH_2], 52.7 (d, $C_{2''}$ and $C_{4''}$, $^4J_{C-F}=7.0$ Hz), 62.7 (OCH_3), 107.0 (C_3), 107.5 (d, C_5, $^2J_{C-F}=24.2$ Hz), 119.9 (d, C_{10}, $^3J_{C-F}=8.0$ Hz), 135.7 (C_9), 138.4 (d, C_7, $^2J_{C-F}=11.4$ Hz), 145.1 (d, C_8, $^3J_{C-F}=7.0$ Hz), 151.8 (C_2), 156.1 (d, C_6, $^1J_{C-F}=250.7$ Hz), 169.5 [C(O)O], 177.6 (C_4). ESI-MS (positive mode): $(M+H)^+=388.2$ is in agreement with the mass calculated for $M=C_{20}H_{22}FN_3O_4$ (387.16). ESI-HRMS (positive mode): 388.1656 is in agreement with the mass calculated for $(M+H)=C_{20}H_{23}FN_3O_4$ (386.1673).

6.2.5.3. 7-[(1α,5α,6α)-6-Amino-3-azabicyclo[3.1.0]hex-3-yl]-1-(2,4-difluoro-phenyl)-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***3***

6.2.5.3.1. 1-(2,4-Difluorophenyl)-7-[(1α,5α,6α)-6-[[(1,1-dimethylethoxy)carbonyl]-amino]-3-azabicyclo[3.1.0]hex-3-yl]-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***3*****-Boc**. The procedure, as described for **2-Boc**, when applied to **23** (189 mg, 0.95 mmol) and **21a1** (157 mg, 0.38 mmol) in CH_3CN (8 mL) gave, after work-up and flash chromatography, **3-Boc** (151 mg, 0.277 mmol, 73%) as a colourless oil. $R_f=0.70$ (9:1 $CHCl_3$/MeOH, UV); ^{1}H NMR ($CDCl_3$) δ 1.42 [s, 9H, $C(CH_3)_3$], 1.72 (s, 2H, $H_{1''}$ and $H_{5''}$), 2.45 (s, 1H, $H_{6''}$), 3.07 (s, 3H, OCH_3), 3.38 [d, 1H, $H_{2''}$ or $H_{4''}$ endo or exo, $^2J_{H-H}=10.1$ Hz], 3.64 (s, 2H, $H_{2''}$ or $H_{4''}$ endo or exo), 3.94 [d, 1H, $H_{2''}$ or $H_{4''}$ endo or exo, $^2J_{H-H}=10.4$ Hz], 4.76 (br s, 1H, NHBoc), 7.03 [m, 2H, H(Ar)], 7.45 (td, 1H, H(Ar), $J=8.7$ Hz, $J=5.6$ Hz), 7.85 (d, 1H, H_5, $^3J_{H-F}=13.3$ Hz), 8.45 (s, 1H, H_2), 14.73 (br s, 1H, COOH); ^{19}F NMR ($CDCl_3$) δ −106.7 and −117.3 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F}=8.2$ Hz), −117.8 (br s, 1F, F_6); ^{13}C NMR ($CDCl_3$) δ 24.4 ($C_{1''}$ and $C_{5''}$), 28.4 [$C(CH_3)_3$], 31.8 ($C_{6''}$), 51.4 (d, $C_{2''}$ or $C_{4''}$, $^4J_{C-F}=7.0$ Hz), 52.1 (d, $C_{4''}$ or $C_{2''}$, $^4J_{C-F}=7.7$ Hz), 60.8 (OCH_3), 79.9 [$C(CH_3)_3$], 104.9 (dd, $C_{3'}$, $^2J_{C-F}=26.7$ Hz, $^2J_{C-F}=23.1$ Hz), 108.1 (d, C_5, $^2J_{C-F}=24.2$ Hz), 108.5 (C_3), 111.9 (dd, $C_{5'}$, $^2J_{C-F}=22.9$ Hz, $^4J_{C-F}=3.8$ Hz), 119.8 (d, C_{10}, $^3J_{C-F}=9.2$ Hz), 127.3 (d, $C_{6'}$, $^3J_{C-F}=10.3$ Hz), 129.2 (dd, $C_{1'}$, $^2J_{C-F}=13.5$ Hz, $^4J_{C-F}=4.4$ Hz), 133.0 (C_9), 136.5 (d, C_7, $^2J_{C-F}=11.3$ Hz), 142.5 (d, C_8, $^3J_{C-F}=7.0$ Hz), 150.6 (C_2), 155.4 (d, C_6, $^1J_{C-F}=252.5$ Hz), 156.4 [NHC(O)O], 157.3 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F}=254.3$ Hz, $^3J_{C-F}=12.4$ Hz), 162.8 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F}=253.6$ Hz, $^3J_{C-F}=11.2$ Hz), 166.6 [C(O)O], 177.4 (d, C_4, $J_{C-F}=2.9$ Hz).

6.2.5.3.2. Synthesis of ***3*** *(as its 1.6 TFA salt).* Likewise, **3-Boc** (41 mg, 0.0753 mmol) gave, after Boc deprotection, recrystallization (9:1 H_2O/CH_3CN) and lyophilization, **3** (33 mg, 0.053 mmol, 70%) as a pale yellow solid. $R_f=0.30$ (84:14:2 $CHCl_3$/MeOH/H_2O, UV); HPLC: $R_t=9.0$ min (solvent A) and 14.6 min (solvent B); ^{1}H NMR (1:4 CD_3CN/D_2O) δ 1.98 (m, 2H, $H_{1''}$ and $H_{5''}$), 2.46 (br s, 1H, $H_{6''}$), 2.97 (s, 3H, OCH_3), 3.28 and 3.57 (2d, 1H each, $H_{2''}$ or $H_{4''}$ endo or exo, $J_{H-H}=7.8$ Hz), 3.45 and 3.82 (2d, 1H each, $H_{2''}$ or $H_{4''}$ endo or exo, $J_{H-H}=9.1$ Hz), 7.11 [m, 2H, H(Ar)], 7.50 (d, 1H, H_5, $^3J_{H-F}=13.0$ Hz), 7.63 [m, 1H, H(Ar)], 8.84 (s, 1H, H_2); ^{19}F NMR (1:4 CD_3CN/D_2O) δ −74.4 (s, 4.8F, TFA), −106.7 and −117.9 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F}=7.9$ Hz), −117.5 (s, 1F, F_6); ^{13}C NMR (1:4 CD_3CN/D_2O) δ 21.5 and 21.6 ($C_{1''}$ and $C_{5''}$), 30.7 ($C_{6''}$), 51.5 (d, $C_{2''}$ or $C_{4''}$, $^4J_{C-F}=6.9$ Hz), 52.2 (d, $C_{4''}$ or $C_{2''}$, $^4J_{C-F}=6.9$ Hz), 61.8 (s, OCH_3), 105.1 (dd, $C_{3'}$, $^2J_{C-F}=27.0$ Hz, $^2J_{C-F}=23.5$ Hz), 107.6 (d, C_5, $^2J_{C-F}=24.1$ Hz), 107.8 (C_3), 112.7 (d, $C_{5'}$, $^2J_{C-F}=21.8$ Hz), 120.0 (d, C_{10}, $^3J_{C-F}=7.4$ Hz), 128.7 (d, $C_{6'}$, $^3J_{C-F}=10.7$ Hz), 129.5 (dd, $C_{1'}$, $^2J_{C-F}=13.2$ Hz, $^4J_{C-F}=4.0$ Hz), 133.8 (C_9), 137.3 (d, C_7, $^2J_{C-F}=11.5$ Hz), 143.7 (d, C_8, $^3J_{C-F}=6.9$ Hz), 152.1 (C_2), 156.1 (d, C_6, $^1J_{C-F}=251.0$ Hz), 157.7 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F}=251.3$ Hz, $^3J_{C-F}=12.6$ Hz), 162.5 (dd, $C_{2'}$ or

$C_{4'}$, $^1J_{C-F} = 251.3$ Hz, $^3J_{C-F} = 10.9$ Hz), 168.2 [C(O)O], 178.0 (C_4). ESI-MS (positive mode): $(M + H)^+ = 446.1$ is in agreement with the mass calculated for $M = C_{22}H_{18}F_3N_3O_4$ (445.12). ESI-HRMS (positive mode): 446.1313 is in agreement with the mass calculated for $(M + H) = C_{22}H_{19}F_3N_3O_4$ (446.1328).

6.2.5.4. 7-[(1α,5α,6α)-6-(Aminomethyl)-3-azabicyclo[3.1.0]-hex-3-yl]-1-(2,4-difluorophenyl)-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***4***

6.2.5.4.1. 1-(2,4-Difluorophenyl)-7-[(1α,5α,6α)-6-[[[(1,1-dimethylethoxy)carbonyl]-amino]methyl]-3-azabicyclo[3.1.0]-hex-3-yl]-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, ***4-Boc***. The procedure, as described for **2-Boc**, when applied to **24** (211 mg, 0.99 mmol) and **21a2** (145 mg, 0.35 mmol) gave after work-up and flash chromatography **4-Boc** (173 mg, 0.31 mmol, 89%) as a colourless oil. $R_f = 0.75$ (9:1 $CHCl_3$/MeOH, UV); ^{1}H NMR ($CDCl_3$) δ 0.94 (tt, 1H, $H_{6''}$, $^3J_{H-H} = 3.4$ Hz, $^3J_{H-H} = 6.8$ Hz), 1.37 [s, 9H, $C(CH_3)_3$], 1.56 (s, 2H, $H_{1''}$ and $H_{5''}$), 3.03 (s, 3H, OCH_3), 3.40–3.80 (m, 6H, $H_{2''}$, $H_{4''}$ and CH_2NHBoc), 4.79 (br s, 1H, NHBoc), 6.95 [m, 2H, H(Ar)], 7.49 [m, 1H, H(Ar)], 7.73 (d, 1H, H_5, $^3J_{H-F} = 13.4$ Hz), 8.38 (s, 1H, H_2), 14.69 (br s, 1H, COOH); ^{19}F NMR ($CDCl_3$) δ −106.8 and −117.4 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F} = 8.3$ Hz), −117.6 (br s, 1F, F_6); ^{13}C NMR ($CDCl_3$) δ 20.9 and 21.1 ($C_{1''}$ and $C_{5''}$), 21.2 ($C_{6''}$), 28.4 [$C(CH_3)_3$], 42.2 (CH_2NHBoc), 51.7 (d, $C_{2''}$ or $C_{4''}$, $^4J_{C-F} = 7.3$ Hz), 52.5 (d, $C_{4''}$ or $C_{2''}$, $^4J_{C-F} = 7.2$ Hz), 60.7 (OCH_3), 79.3 [$C(CH_3)_3$], 104.7 (dd, $C_{3'}$, $^2J_{C-F} = 26.7$ Hz, $^2J_{C-F} = 22.7$ Hz), 107.8 (d, C_5, $^2J_{C-F} = 24.5$ Hz), 108.2 (C_3), 111.7 (dd, $C_{5'}$, $^2J_{C-F} = 22.9$ Hz, $^4J_{C-F} = 3.8$ Hz), 119.8 (d, C_{10}, $^3J_{C-F} = 9.2$ Hz), 127.3 (d, $C_{6'}$, $^3J_{C-F} = 9.5$ Hz), 129.1 (dd, $C_{1'}$, $^2J_{C-F} = 13.4$ Hz, $^4J_{C-F} = 4.2$ Hz), 132.9 (C_9), 137.0 (d, C_7, $^2J_{C-F} = 11.0$ Hz), 142.2 (d, C_8, $^3J_{C-F} = 7.0$ Hz), 150.4 (C_2), 155.2 (d, C_6, $^1J_{C-F} = 252.5$ Hz), 155.9 [NHC(O)O], 157.1 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 254.3$ Hz, $^3J_{C-F} = 12.4$ Hz), 162.7 (dd, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 252.9$ Hz, $^3J_{C-F} = 11.0$ Hz), 166.4 [C(O)O], 177.1 (d, C_4, $J_{C-F} = 3.3$ Hz).

6.2.5.4.2. Synthesis of ***4*** *(as its 1.2 TFA salt).* Likewise, compound **4-Boc** (63 mg, 0.113 mmol) gave, after Boc deprotection, recrystallization (9:1 H_2O/CH_3CN) and lyophilization, **4** (43 mg, 0.072 mmol, 64%) as a pale yellow solid. $R_f = 0.20$ (84:14:2 $CHCl_3$/MeOH/H_2O, UV); HPLC: $R_t = 14.0$ min (solvent A) and 16.1 min (solvent B); mp = 126–130 °C; ^{1}H NMR (1:4 CD_3CN/D_2O) δ 1.00 (m, 1H, $H_{6''}$), 1.59 (s, 2H, $H_{1''}$ and $H_{5''}$), 2.83 (d, 2H, CH_2NH_2, $^3J_{H-H} = 5.8$ Hz), 2.98 (s, 3H, OCH_3), 3.27 and 3.59 (2d, 1H each, $H_{2''}$ or $H_{4''}$ endo or exo, $J_{H-H} = 9.5$ Hz), 3.38 and 3.82 (2d, 1H each, $H_{2''}$ or $H_{4''}$ endo or exo, $J_{H-H} = 10.0$ Hz), 7.13 [m, 2H, H (Ar)], 7.66 [m, 1H, H (Ar)], 7.73 (d, 1H, H_5, $^3J_{H-F} = 13.4$ Hz), 8.48 (s, 1H, H_2); ^{19}F NMR (1:4 CD_3CN/D_2O) δ −74.4 (s, 3.6F, TFA), −107.3 and −118.1 (2d, 2F, $F_{2'}$ and $F_{4'}$, $^4J_{F-F} = 7.6$ Hz), −116.2 (s, 1F, F_6); ^{13}C NMR (1:4 CD_3CN/D_2O) δ 18.4 ($C_{6''}$), 22.3 and 22.4 ($C_{1''}$ and $C_{5''}$), 42.2 (CH_2NH_2), 52.0 (d, $C_{2''}$ or $C_{4''}$, $^4J_{C-F} = 5.7$ Hz), 52.9 (br s, $C_{4''}$ or $C_{2''}$), 61.7 (OCH_3), 105.2 (t, $C_{3'}$, $^2J_{C-F} = 25.2$ Hz), 107.9 (d, C_5, $^2J_{C-F} = 21.8$ Hz), 108.1 (C_3), 112.8 (dd, $C_{5'}$, $^2J_{C-F} = 20.7$ Hz, $^4J_{C-F} = 4.4$ Hz), 119.9 (d, C_{10}, $^3J_{C-F} = 3.3$ Hz), 128.8 (d, $C_{6'}$, $^3J_{C-F} = 8.0$ Hz), 129.8 (dd, $C_{1'}$, $^2J_{C-F} = 13.5$ Hz, $^4J_{C-F} = 4.8$ Hz), 134.2 (C_9), 138.2 (d, C_7, $^2J_{C-F} = 12.6$ Hz), 143.4 (d, C_8, $^3J_{C-F} = 6.6$ Hz), 152.3 (C_2), 156.2 (d, C_6, $^1J_{C-F} = 253.5$ Hz), 157.9 (d, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 251.0$ Hz), 165.6 (d, $C_{2'}$ or $C_{4'}$, $^1J_{C-F} = 250.0$ Hz), 168.7 [C(O)O], 178.1 (C_4). ESI-MS (positive mode): $(M + H)^+ = 460.2$, $(M + Na)^+ = 482.2$ is in agreement with the mass calculated for $M = C_{23}H_{20}F_3N_3O_4$ (459.14). ESI-HRMS (positive mode): 460.1488 is in agreement with the mass calculated for $(M + H) = C_{23}H_{21}F_3N_3O_4$ (460.1484).

6.3. Biological assays

6.3.1. In vitro antiparasitical activities

6.3.1.1. Anti-T. gondii activity. Briefly, following a previously described method [22], the virulent RH strain of *T. gondii* was maintained in mice by intraperitoneal passage every two days. For each experiment, tachyzoites were collected from the peritoneal cavity of infected mice then resuspended in physiological saline. Tissue cultures and drug tests were carried out using MRC5 fibroblast tissue cultures. Confluent monolayers prepared in 96-well tissue culture plates were inoculated with 2000 fresh tachyzoites. After 4 h, drugs at various concentrations were added into the culture medium and culture plates were incubated for an additional 72 h. Each culture plate comprised eight negative control (without *T. gondii*) and eight positive control wells (without drug). After incubation, the plates were examined microscopically for cytopathic effects and thereafter fixed with cold methanol for 5 min. *Toxoplasma* growth was assessed by enzyme linked immunoassay (ELISA) performed directly on the fixed cultures using a peroxidase labeled monoclonal antibody directed against the SAG-1 surface protein of *T. gondii*. After addition of the substrate, spectrophotometric readings were recorded at a wavelength of 405 nm with blank on the negative control well. For each well, the results were expressed as optical density (OD) values. The effect of each drug at various concentrations was described by plotting the OD values as a function of the logarithm of the concentration and a linear regression model was used to summarize the concentration–effect relationship and to determine the IC_{50}.

6.3.1.2. Anti-P. falciparum activity. Cultures of the NF54-R chloroquine-resistant derived from the NF54 strain were maintained in continuous culture according to Ref. [64]. The in vitro activities of the drugs were evaluated by using the methods described in Ref. [65]. Two hundred microliters of ring stage parasitized erythrocytes (parasitemia, 0.5%; hematocrit, 1.8%) were distributed in 96-well plates preloaded with nine concentrations (0.025–166 μg/mL) of each drug in triplicate and with serial dilutions of chloroquine in positive control wells. After 72 h, [^{3}H] hypoxanthine was added to each well and then plates were incubated for an additional 24 h. Parasites were harvested, and incorporation of radioactivity was

determined by liquid scintillation counting. Experiments were repeated twice.

6.3.1.3. Anti-P. yoelii yoelii activity. Mouse hepatocyte cultures were prepared in Lab-Tek slides as described in Ref. [66] and then incubated for 24 h before sporozoite inoculation. Sporozoites were obtained by dissection of the salivary glands of *Anopheles stephensi* mosquitoes infected with *P. yoelii yoelii* (265 By). Dilutions of drugs were made in culture medium (1–100 μg/mL), and 8×10^4 sporozoites of *P. yoelii yoelii* were added to hepatocyte cultures. Each drug was tested in four replicates. The culture medium containing the drug was renewed every 24 h, thus maintaining a correct concentration. Cultures were incubated for 48 h for *P. yoelii yoelii* and then fixed with cold methanol. Schizonts were evaluated using an immunofluorescence test with an anti-HSP-i72 polyclonal antibody. Both the numbers and size of schizonts in the culture were taken into account to assess drug activity. Experiments were repeated twice.

6.3.2. Inhibitory activity against DNA gyrase of M. tuberculosis

A reaction mixture containing 2U of purified *M. tuberculosis* DNA gyrase, DNA gyrase assay buffer (40 mM Tris–HCl, pH 7.5), 25 mM KCl, 6 mM magnesium acetate, 2 mM spermidine, 4 mM dithiothreitol, 0.1 mg of *Escherichia coli* tRNA per mL, bovine serum albumin (0.36 mg/mL), 100 mM potassium glutamate, 1 mM ATP (pH 8) and relaxed pBR322 DNA (0.4 μg) as the substrate were incubated with or without increasing concentrations of quinolones at 37 °C for 1 h. Reactions were terminated by the addition of 50% glycerol containing 0.25% bromophenol blue, and the total reaction mixture was subjected to electrophoresis in a 1% agarose gel in 0.5× TBE buffer. After running for 5.5 h at 50 V, the gel was stained with ethidium bromide (0.7 μg/mL). One unit of enzyme activity was defined as the amount of DNA gyrase that converted 400 ng of relaxed pBR322 to the supercoiled form in 1 h at 37 °C. DNA gyrase of *E. coli* was used as a positive control for the assay procedures and buffer. Inhibition of the supercoiling activity of the recombinant DNA gyrase was performed by the following method. A reaction mixture containing 1 U of purified DNA gyrase and increasing concentrations of quinolones was incubated as describe above. The inhibitory effect of quinolones on DNA gyrase was assayed by determining the concentration of drug required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}).

6.3.3. In vitro antibacterial activity against M. tuberculosis – determination of MICs

MICs were determined by the 1% standard proportion method on 7H11 agar supplemented with 10% oleic acid–albumin–dextrose–catalase (OSI) [67]. Bacterial suspensions were prepared by diluting *M. tuberculosis* H37Rv grown in 7H9 medium at 37 °C for 21–36 days in normal saline to match that of a standard 1 mg/mL suspension of *Mycobacterium bovis* BCG. Those suspensions were further diluted for MIC determination. MIC values were defined as the lowest concentration of quinolone that inhibited more than 99% of the bacterial growth.

Acknowledgements

We thank the Centre National de la Recherche Scientifique (CNRS) for financial support, and MNERT (G.A.) for grant.

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