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Synthesis and biological activity of new quinolone derivatives

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Summary — A series of new quinolone derivatives bearing covalent modifications at the piperazine ring was synthesized and investigated for their biological properties. Two different types of substitutions at the level of the nitrogen at the 4' position were considered: introduction of a di- or tri-oxymethylene chain to modify steric hindrance and improve solubility in aqueous media or formation of a tertiary amide ending with a carboxylate group. In the latter case the net charge on the piperazine moiety changes from positive to negative at physiological conditions. In addition, a 'bis-quinolone' compound was examined, which lacks the piperazine ring and is also negatively charged at neutral pH. The new derivatives, except one, exhibited drug uptake, inhibition of DNA-gyrase activity and anti-bacterial potencies comparable to those of norfloxacin, and were modulated by the nature of the N4'-substituent. Besides indicating possible new modifications of the quinolone basic structure, the observation that substantially different substitution patterns at the same position did not cause impairment of biological activity suggests that the steric and electronic properties of this part of the molecule are not crucial for the recognition of DNA-gyrase.

quinolone / DNA-gyrase / uptake / anti-bacterial potency / piperazine modification

Introduction

Quinolones are important chemotherapeutic agents widely prescribed for the treatment of numerous bacterial infections [1]. Their mechanism of action is believed to involve an inhibition of bacterial replication *via* a poisoning of the DNA-gyrase complex [1–4]. Although some of the details are known, it is still unclear what the function of the piperazine moiety is. The latter has been proposed to participate in drug–gyrase interaction [2], but clear-cut evidence in this sense is still lacking. Thus it appeared interesting to us to synthesize and investigate quinolone derivatives bearing covalent modifications at the piperazine level. In addition to a mechanistic relevance, *N*-substituted quinolones, in particular those not bearing a positively charged nitrogen at neutral pH, could exhibit modified uptake properties. In fact, quinolones are usually zwitterionic at physiological conditions, whereas the modified ones would behave as carboxylic acids. Hence modifications at the level of the amino group could provide compounds that were more effective in crossing the biological barrier. Derivatization of the amino-piperazinyl moiety to give tertiary amine, amide or urethane functions has been reported in recent papers [4–12]. *N*-Alkylation seems to be compatible with activity at least for ethyl, hydroxy-ethyl, propenyl and benzyl substituents [5]. Amido derivatives, such as formyl or acetyl, were also active against *Staphylococcus aureus* and *Escherichia coli,* whereas any kind of *N*-substitution appeared to reduce the drug's response against *Pseudomonas aeruginosa* [5]. Derivatization of the amino-piperazinyl moiety to give amido or urethane derivatives has also been considered in terms of production of quinolone prodrugs in a number of recent papers [7–12]. In particular formyl and (acyloxy)alkyl carbamate derivatives have been examined. In this case, rapid regeneration of the original quinolone is observed in the presence of serum or intestinal homogenate. Amide bond formation should, however, lead to less readily hydrolyzable compounds, perhaps exhibiting

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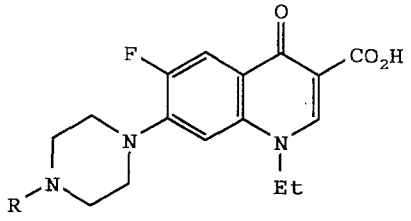
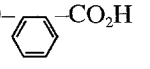
inherent *in vivo* biological activity. The case of formyl derivatives is special, since such compounds are spontaneously metabolized into the corresponding carboxyl group by oxidative enzymes [13]. Nonetheless, even succinyl norfloxacin is claimed to release norfloxacin after oral administration [14].

To gain better insight into the mechanism of action of quinolone drugs and possibly develop new pharmacologically interesting compounds, we prepared and investigated a number of derivatives of norfloxacin **5** modified at the piperazine nitrogen. Two of them



compd	R
5	–H
6	$-(CH_2)_2-O-(CH_2)_2-OH$
7	$-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-OH$
8	$-CO-(CH_2)_2-CO_2H$
9	

(**6** and **7**) contain 2 or 3 oxyethylene residues, which are expected to improve the solubility of the quinolone in aqueous media and considerably change the steric properties at the piperazine level. Two others (**8** and **9**), in addition to bulky substituents at the 4' nitrogen, exhibit a net negative charge at neutral pH. Finally, a 'bis'-quinolone compound has been considered (**4,** scheme 1), which preserves the basic quinolone moiety and is also negatively charged under physiological conditions.

Chemistry

Compound **4** was prepared following the usual method for constructing the heterocyclic ring of oxoquinolines from the relevant aromatic primary amine [5]. 2,6-Diaminopyridine was treated with diethyl ethoxymethylene malonate to obtain the dimalonate **1,** which, upon heating, cyclized to give the corresponding triazaanthracene diester **2**. Alkylation of **2** with ethyl iodide in dimethylformamide in the presence of potassium carbonate then gave then dialkyl dioxo triazaanthracene **3**, which was finally hydrolyzed in an acid–alcohol–water medium to afford the diacid **4** (scheme 1).

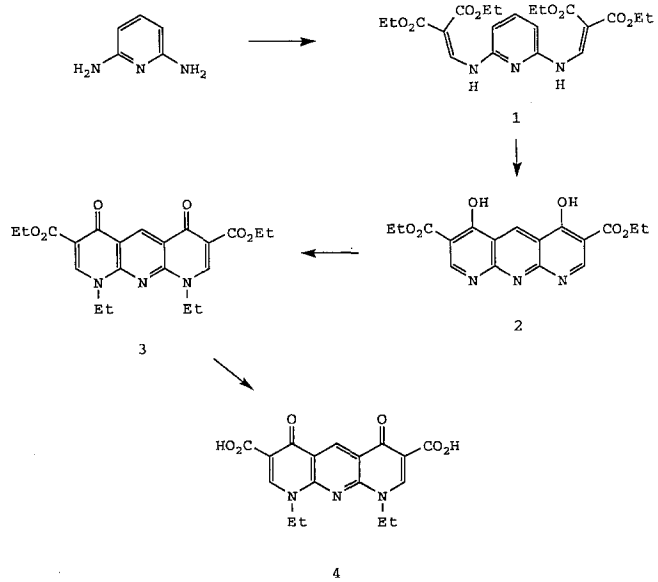
Products in which the nitrogen atom at position 4 of the norfloxacin piperazinyl moiety was alkylated (**6** and **7**) or acylated (**8** and **9**) were readily obtained by treating norfloxacin **5** in an alkaline medium with the corresponding alkyl halide (for **6** and **7**), succinic anhydride (for **8**) or phthalic anhydride (for **9**).

Anti-bacterial potency

Quinolone compounds **6** and **7** behaved like norfloxacin in terms of spectrum and potency of the antibiotic activity (table I). In no case were the former substances more or less effective than the parent compound by a value higher than one dilution. Compounds **8** and **9** were significantly less active than norfloxacin against *E coli, S aureus, Staphylococcus epidermidis* and *P aeruginosa,* but instead, comparably effective against *Proteus mirabilis, Streptococcus faecalis* and *Enterobacter* spp (table I). The bis-quinolone derivative **4**, on the other hand, was practically inactive.

Drug uptake

The technique used to study drug uptake enables measurement of the phenomenon at its initial velocity, thereby avoiding artifacts connected with drug redistribution within intra- and extracellular compartments [15]. Together with the reference drug norfloxacin,



Scheme 1.

Table I. Anti-bacterial potency of quinolone compounds.

Strain and drug tested	*No*	*Range (μg/ml)*	*Mean ± SEM (μg/ml)*
E coli			
Norfloxacin	14	0.09 – 1.56	0.38 ± 0.13
4	3	> 100	ND
6	13	0.09 – 0.78	0.32 ± 0.07
6	12	0.09 – 1.56	0.36 ± 0.12
8	10	0.19 – 3.12	1.93 ± 0.62
9	9	0.19 – 6.25	2.07 ± 0.84
S aureus			
Norfloxacin	12	0.39 – 3.12	0.94 ± 0.21
4	4	> 100	ND
6	13	0.39 – 3.12	1.35 ± 0.29
6	13	0.39 – 1.56	0.87 ± 0.14
8	7	1.56 – 12.50	6.47 ± 1.68
9	7	0.19 – 25.00	9.26 ± 3.15
S epidermidis			
Norfloxacin	15	0.09 – 0.78	0.38 ± 0.07
4	3	> 100	ND
6	11	0.09 – 1.56	0.66 ± 0.15
6	12	0.19 – 0.78	0.58 ± 0.07
8	4	1.56 – 6.25	3.51 ± 0.98
9	5	0.39 – 12.50	3.82 ± 2.21
P mirabilis			
Norfloxacin	12	0.09 – 3.12	1.38 ± 0.33
4	2	> 100	ND
6	9	0.78 – 0.39	0.60 ± 0.06
6	11	0.19 – 3.12	1.05 ± 0.33
8	4	0.39 – 1.56	0.97 ± 0.33
9	4	0.39 – 1.56	0.78 ± 0.27
P aeruginosa			
Norfloxacin	11	0.39 – 6.25	1.88 ± 0.55
4	3	> 100	ND
6	11	0.39 – 3.12	1.06 ± 0.25
6	10	1.56 – 3.12	2.34 ± 0.26
8	9	1.56 – 12.50	6.94 ± 1.52
9	10	0.39 – 12.50	4.10 ± 1.17
S faecalis			
Norfloxacin	13	1.56 – 12.50	3.36 ± 0.84
4	4	> 100	ND
6	12	1.56 – 6.25	2.34 ± 0.40
7	13	1.56 – 6.25	2.52 ± 0.37
8	3	1.56 – 3.12	3.64 ± 1.37
9	4	0.78 – 12.50	4.88 ± 2.59
Enterobacter spp			
Norfloxacin	9	0.39 – 0.78	0.73 ± 0.16
4	3	> 100	ND
6	10	0.39 – 3.12	1.28 ± 0.24
7	9	0.39 – 1.56	0.95 ± 0.19
8	4	0.39 – 6.25	1.95 ± 1.43
9	5	0.78 – 3.12	1.48 ± 0.46

ND : not done.

compounds **4** and **7** were used in this assay as the representative active (**7**) and inactive (**4**) molecules. Figure 1 illustrates drug uptake into *E coli:* the process takes place with similar kinetics for **7** and **5**, *ie*, uptake was linear for 30–40 s, after which a plateau phase was reached. The amount of drug incorporated did not differ significantly among the above substances either in the linear or in the stationary phase of uptake. Clearly, compound **4** was unable to enter the intracellular compartment, since the measurable fluorescence never exceeded the background level. A similar uptake pattern was observed for the molecules tested against *S aureus,* with the exception that drug influx was generally reduced as compared to *E coli* (not shown).

Inhibition of DNA-gyrase-directed supercoiling activity

DNA-gyrase inhibition of supercoiling by compound **7** is shown in figure 2. ID_{50} values obtained for the test derivatives are reported in table II. Compounds **7** and **9** (ID_{50} ~ 5 μM) inhibited DNA supercoiling to an extent highly comparable to that of norfloxacin . Nalidixic acid was less effective by approximately one order of magnitude (ID_{50} = 25μM). Inhibitory values obtained for norfloxacin and nalidixic acid were otherwise quite close to those already reported in the literature [16].

Compound **4** was quite inactive, since effective inhibitory concentrations were attained at drug mo-

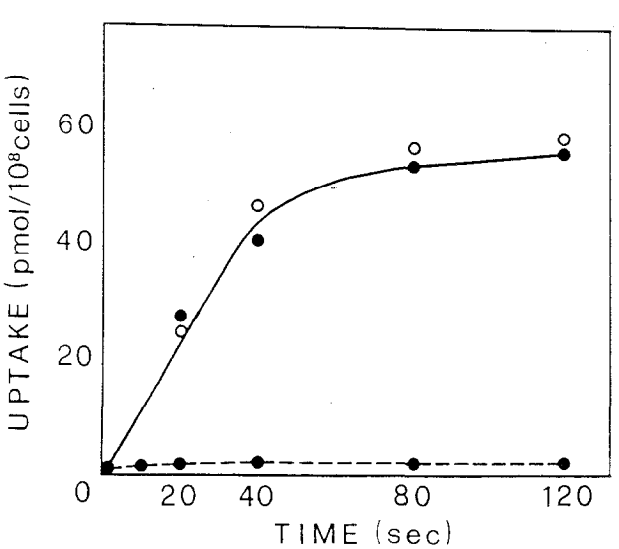


Fig 1. Kinetics of quinolone uptake in *E coli* strain wt 05 by norfloxacin (solid line, ●), compound **7** (○) and compound **4** (dashed line, ●). The same trend was observed for the 3 compounds using *E coli* reference strain KpO5124.

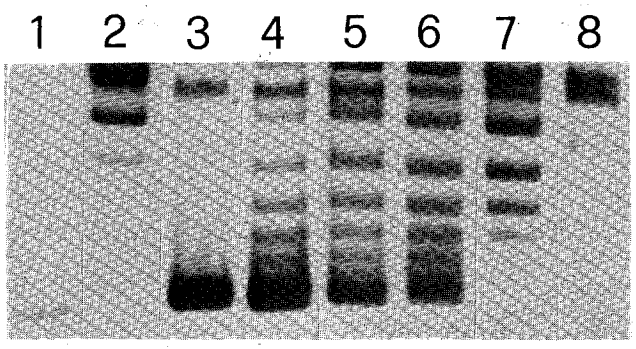


Fig 2. Inhibition of DNA-gyrase-directed DNA supercoiling by compound **7.** Lane 1: form 1 pUC8 DNA obtained after double-gradient purification as described in the *Experimental protocols;* lane 2: form 1 pUC8 DNA after treatment with topoisomerase I (DNA is unknotted and mostly in the nicked circular form); lane 3: topoisomerase I-treated DNA after incubation with DNA-gyrase (mostly form 1 is produced); lanes 4–8: topoisomerase I-treated DNA after incubation with DNA-gyrase and 1, 2, 5, 10 and 50 μM compound **7** respectively.

Table II. ID_{50} values for the inhibition of DNA-gyrase-directed supercoiling activity.

Compounds	*μM*
Norfloxacin	2
Nalidixic acid	25
4	200
7	5
9	6

larities (ID_{50}=200 μM) higher than those exhibited not only by all quinolones tested but also by a number of DNA ligands, such as doxorubicin, ethidium bromide, 4',6-diamidino-2-phenylindole (both intercalating and non-intercalating compounds) which are not considered to be specific inhibitors of DNA-gyrase.

Discussion and conclusions

The data on anti-bacterial potency show that the introduction of 2 or 3 oxymethylene groups at the *N*-piperazine level into the norfloxacin analogues presently examined did not impair activity against a number of bacterial strains. This observation was confirmed by the gyrase inhibition experiments, and is supported by literature data on similar systems [5, 6]. On the other hand, the amido derivatives **8** and **9** were as active as the reference drug against *P mirabilis, S faecalis and Enterobacter* spp, whereas they were relatively less effective against *E coli, S aureus and P aeruginosa.* As a possible explanation, it could be inferred that the latter derivatives behave as prodrugs, and thus are hydrolyzed to free **5** inside the cell. Then only norfloxacin activity would have to be monitored, the modulation effects observed being associated with drug uptake and rate of hydrolysis. This, however, cannot hold true for the gyrase-assay experiments in cell-free systems, where no drug hydrolysis occurred, as shown by the absence of the norfloxacin peak in the high-performance liquid chromatography (HPLC) elution profile of the test compounds after 1 h of incubation at 37°C in the gyrase-assay buffer. As reported in table II, compound **9** exhibits enzymatic inhibition properties quite close to **5**.

The conclusion should then be drawn that even substantial (and persistent) modifications at the piperazinyl moiety are still compatible with drug activity. Thus the stereochemical and electronic requirements of this part of the molecule do not appear to be as strict as would be expected if specific recognition by DNA-gyrase were to take place at the position. Interestingly, derivative **4,** which exhibits a double quinolone ring, is devoid of activity. At first, this finding could be attributed to impaired uptake of this compound (fig 1). However, even in the *in vitro* assays, no activity could be detected. It must be concluded that, notwithstanding the presence of the essential pharmacophore, its structure is not compatible with enzyme inhibition.

As far as solubility properties are concerned, compounds **6** and **7** were indeed considerably more soluble than norfloxacin in aqueous media and could therefore represent useful substitutes for the parent drug. Compounds **8** and **9** exhibited solubility values close to that of norfloxacin. On the other hand, compound **4** was very sparingly soluble in aqueous solution, which could in part explain its very low biological activity. We are presently trying to obtain more soluble derivatives having the same basic structure.

In conclusion, derivatization of quinolones at the piperazine 4'–NH group, besides being convenient means of generating prodrugs that release their active species inside the cell, represents a valid way to introduce permanent modifications into the drug's structure without diminishing activity against some bacterial strains. In addition to providing valuable information on the mechanism of action of the quinolone family, the new compounds could possibly lead to anti-bacterial agents with improved pharmacological properties.

Experimental protocols

Chemistry

Melting points were measured on a Buchi 510 apparatus and are uncorrected. The IR spectra were recorded on a Perkin–Elmer 781 spectrometer (KBr discs). The ^{1}H-NMR spectra were obtained by means of a Varian CFT-20 instrument (80 MHz), using tetramethylsilane as the internal standard (chemical shifts in δ values, J in Hz). Mass spectra were obtained with a VG micromass 16F at 70 eV *m/e* (relative intensity and positive-ion fast atom bombardment (FAB) mass spectra on a VG 7070D mass spectrometer, equipped with a standard FAB ion source with a thioglycerol matrix. Elemental analyses were determined with a Perkin–Elmer 240B instrument (C, H, N). When indicated by the symbols of the elements, they were within ± 0.4% of theoretical values. Merck silica gel 60 (70–230 mesh) was used for column chromatography. Analytical thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F254 (0.25 mm).

*2,6-Bis[2,2-di(ethoxycarbonyl)ethenylamino]pyridine **1***
A mixture of 2,6-diaminopyridine (5 g, 45.87 mmol) and diethyl ethoxymethylenemalonate (20 g, 91.74 mmol) was heated for 5 h at 130°C. The resulting EtOH was evaporated off and the residue was recrystallized from toluene–ligroin to give 17.4 g (84.5%) of **1**: mp: 132–133°C; ^{1}H-NMR ($CDCl_3$): 11.05(2H, d, 2(NH), J = 12.9), 9.09 (2H, d, –CH–N, J = 12.9), 7.61 (lH, t, H4 pyridine, J = 7.9), 6.59(2H, d, H3–H5 pyridine, J = 7.9), 4.32 and 4.28 (4H + 4H, 2q, 4(–CH_2–CH_3), J = 3.1), 1.38 and 1.35 (6H + 6H, 2t, 4(–CH_3), J = 7.1). Anal $C_{21}H_{27}N_3O_8$ (C, H, N).

*Diethyl-4,5-dihydroxy-1,8,9-triazaanthracene-3,6-dicarboxylate **2***
A solution of **1** (750 mg, 1.67 mmol) in diphenyl ether (8 ml) was refluxed for 1 h. After the solution cooled, EtOH (25 ml) was added and it was left overnight at 0°C. The resulting precipitate was filtered off, washed with EtOH and dried. Yield: 316 mg (53%) of **2**: mp > 300°C. ^{1}H-NMR (CF_3–CO_2D): 10.29 (lH, s, H10), 9.60(2H, s, H2–H7), 4.64 (4H, q, 2(–CH_2– CH_3), J = 7.1), 1.48 (6H, t, 2(–CH_3), J = 7.1). Anal $C_{17}H_{15}O_6N_3$ (C, H, N).

*Diethyl-1,8-diethyl-4(1H),5(8H)-dioxo-1,8,9-triazaanthracene-3,6-dicarboxylate **3***
A mixture of **2** (700 mg, 1.96 mmol), K_2CO_3 (1.25 g, 10.07 mmol), EtI (2 ml, 25 mmol) and dimethylformamide (DMF) (10 ml) was heated at 80°C with stirring. After 20 h, the mixture was concentrated to dryness and the residue was partitioned between H_20 and $HCCl_3$. The organic layer was washed with H_20, dried and evaporated. Yield: 502 mg (61.9%) of **3**: mp > 300°C. IR: 3070, 2980, 1740, 1730, 1640, 1585, 1450, 1390, 1300, 1220, 1185, 1070, 805 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 9.38 (1H, s, H10), 8.49 (2H, s, H2–H7), 4.43 and 4.38 (4H + 4H, 2q, 4(–CH_2-CH_3), J = 7.1), 1.56 and 1.41 (6H + 6H, 2t, 4(–CH_3), J = 7.1). MS, *m/e* (%): 413 (M^+, 12.3), 368 (M^+–OEt, 11.8), 341(M^+–CO_2Et, 100), 267 (M^+–2(CO_2Et), 32.8). Anal $C_{21}H_{23}N_3O_6$ (C, H, N).

*1,8-Diethyl-4(1H),5(8H)-dioxo-1,8,9-triazaanthracene-3,6-dicarboxylic acid **4***
A mixture of **3** (200 mg, 0.48 mmol), 2 N HCl (20 ml) and EtOH (20 ml) was refluxed for 1 h. After cooling, the resulting precipitate was filtered off, washed with H_2O: and dried. Yield: 152 mg (87.9%) of **4**: mp > 300°C. IR: 3460, 3050, 2980, 1730 (br), 1625, 1595, 1460, 1390, 1315, 1260, 1215, 1125, 810 cm^{-1}. ^{1}H-NMR (CF_3CO_2D): 10.08 (lH, s, H10), 9.50 (2H, s, H2–H7), 4.90 (4H, q, 2(–CH_2–CH_3), J = 7.0), 1.68 (6H, t, 2(–CH_3), J = 7.0). Anal $C_{17}H_{15}N_3O_6$ (C, H, N).

*1-Ethyl-6-fluoro-1,4-dihydroxy-4(1H)-oxo-7{4-[2-(2-hydroxyethoxy)ethyl]-1-piperazinyl}-quinoline-3-carboxylic acid **6***
A mixture of **5** (100 mg, 0.31 mmol), Et_3N (0.1 ml, 0.72 mmol), 2-(2-chloroethoxy)ethanol (0.05 ml, 0.47 mmol) and CH_3CN (15 ml) was refluxed with stirring. After 48 h, the mixture was concentrated to dryness. The residue was recrystallized from toluene to give 89 mg (69.7%) of **6**: mp: 184–185°C. IR: 3350, 1715, 1625, 1480, 1380, 1270 cm^{-1}. ^{1}H-NMR (CF_3–$C0_2D$): 9.33 (lH, s, H2), 8.34 (1H, d, H5, J = 12.4), 7.53 (lH, d, H8, J = 6.5), 4.89 (2H, q,–CH_2–CH_3, J = 7.1), 4.15–3.60 (16H, m, 8(–CH_2–)), 1.79 (3H, t, –CH_3, J = 7.1). MS-FAB, *m/e* 408 (M^+ + H). Anal $C_{20}H_{26}N_3O_5$ (C, H, N).

*1-Ethyl-6-fluoro-1,4-dihydroxy-4(1H)-oxo-7-[4-(8-hydroxy-3,6-dioxaoctyl)-1-piperazinyl]-quinoline-3-carboxylic acid **7***

Norfloxacin **5** (100 mg, 0.31 mmol) was alkylated with 2-[2-(2-chloroethoxy)ethoxy]ethanol (0.07 mg, 0.48 mmol) by the above procedure to give 90 mg (63.6%) of **7**: mp: 166–167°C. IR: 3425, 1740, 1625, 1475, 1270, 1255 cm^{-1}. ^{1}H-NMR (CF_3–CO_2D): 9.33(lH, s, H2), 8.34 (lH, d, H5, J = 12.5), 7.53 (lH, d, H8, J = 6.8), 4.90(2H, q, –CH_2–CH_3, J = 6.9), 4.15–3.60 (20H, m, 10 (–CH_2–)), 1.79 (3H, t, –CH_3, J = 6.9). MS-FAB, *m/e* 452 (M^+ + H). Anal $C_{22}H_{30}N_3O_6$ (C, H, N).

*1-Ethyl-6-fluoro-1,4-dihydroxy-4(1H)-oxo-7-[4-(3-carboxy-1-oxopropyl)-1-piperazinyl]-quinoline-3-carboxylic acid **8***
A mixture of **5** (100 mg, 0.31 mmol), Et_3N (0.1 ml, 0.72 mmol), succinic anhydride (50 mg, 0.50 mmol) and toluene (40 ml) was refluxed for 1 h with stirring. After cooling, the resulting precipitate was filtered off and recrystallized from toluene to give 116 mg (88.3%) of **8**: mp: 253°C (lit 244–246°C [8]). IR: 3420, 3200, 3060, 1715, 1650, 1625, 1480, 1445, 1245, 1165, 1020 cm^{-1}. ^{1}H-NMR (CF_3–CO_2D): 9.28 (1H, s, H2), 8.28 (1H, d, H5, J = 12.4), 7.37 (lH, d, H8, J = 6.6), 4.81 (2H, q,–CH_2–CH_3, J = 6.9), 4.10–3.70 (8H, m, (–CH_2–), piperidine), 3.20–2.90 (4H, m, –CH_2–CH_2–CO_2H), 1.79 (3H, t,–CH_3, J = 6.9). MS-FAB, *m/e* 420 (M^+ + H). Anal $C_{20}H_{22}N_3O_6$ (C, H, N).

*1-Ethyl-6-fluoro-1,4-dihydroxy-4(1H)-oxo-7-[4-(2-carboxybenzoyl)-1-piperazinyl]-quinoline-3-carboxylic acid **9***
Norfloxacin (**5**) (100 mg, 0.31 mmol) was acylated with phthalic anhydride (74 mg, 0.50 mmol) by the same procedure as above (with 3 h of refluxing). Yield: 118 mg (80.6%) of **9**: mp: 257°C. IR: 3430, 3060, 1720, 1625, 1475, 1450, 1250, 1010 cm^{-1}; ^{1}H-NMR (CF_3–CO_2D): 9.30 (lH, s, H2), 8.45–7.45 (6H, m, aromatics), 4.90 (2H, q, –CH_2–CH_3, J = 7.0), 4.10–3.70 (8H, m, (–CH_2–), piperidine), 1.79 (3H, t, –CH_3, J = 7.0). MS-FAB, *m/e* 468 (M^+ + H). Anal $C_{24}H_{22}N_3O_6$ (C, H, N).

Pharmacology

Drug susceptibility testing
Minimal inhibitory concentrations (MICs) were assayed using a conventional broth dilution assay [17]. Essentially, bacteria were inoculated at a seeding concentration of 1 x 10^6 bacteria/ml in Mueller–Hinton broth containing different dilutions of the tested compounds. Cultures were then incubated

at 37 °C for 16 h and growth was recorded according to optical turbidity. The MIC represented the highest dilution at which no visible bacterial growth was apparent.

Uptake studies

For these studies 2 strains of *E coli* and 1 strain of *S aureus* were used namely the *E coli* reference strain Kp05124 (a kind gift from Glaxo, Verona, Italy), the *E coli* clinical isolate wt 05 obtained from a patient with cystitis and the *S aureus* strain wt 115. Experiments were basically performed according to our previously published method [15]. Briefly, bacteria, harvested from exponentially growing cultures, were washed and resuspended in a buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl and 50 mM glucose. The relative Mg^{2+} and Ca^{2+} ion concentrations in the buffer, as calculated by atomic absorption measurements, were generally less than 10 μM. The following drugs were used: norfloxacin and compounds **4** and **7**. Equal volumes (100 μl) of quinolone solutions and of cell suspensions (2 x 10^9cells/ml) were mixed simultaneously with a dual syringe device and distributed into a series of 500-μl microhemocytometer tubes placed in an Eppendorf centrifuge 5414 containing 50 μl of 20% (w/v) trichloroacetic acid (TCA) overlaid with 100 μl of a dense (1.13 g/ml) paraffin/silicon oil mixture [15]. After laying on top of the oil-paraffin for varying periods of time (generally from 0 to 120 s), cell suspensions were sedimented by centrifugation at 12 000 *g* for 30 s. The microfuge tubes were frozen in dry ice and ethanol, the tube bottom containing the cell pellet in TCA was cut away and the pellet extracted with 10 volumes of methanol. The drug methanolic extract was subjected to fluorometric determinations in a Perkin–Elmer MPF66 apparatus equipped with a computerized data station. The excitation wavelength was set at 330 nm and the emission at 420 nm. In some experiments, [^{14}C]-norfloxacin (spec act 45.6 μCi/mg) was also used.

Inhibition of DNA supercoiling induced by DNA-gyrase

Enzyme purification. The A and B subunits of *E coli* DNA-gyrase were purified from overexpression vector strains N4186 and MK47, respectively, kindly provided by M Gellert (NIH, Bethesda, MD). The purification steps involved were essentialy those previously reported by Mizuuchi *et al* [18], *ie*, polymin P fractionation, DEAE–Sepharose chromatography and valine–Sepharose chromatography for purification of DNA–gyrase A protein; streptomycin–ammonium sulfate fractionation, heparin–agarose chromatography, hydroxyapatite chromatography and leucine–agarose chromatography for purification of gyrase B protein. The 2 subunits were stored separately at –80°C, in a 40% glycerol solution until use.

Preparation of substrates. pUC8 was prepared from *E coli* strain K12JM 83 (ara, Δlac–pro, Stra, Thi, Δ80 lacZ M15) as described in [19]. Supercoiled DNA was further separated by sucrose density-gradient centrifugation [20]. Relaxed closed-circular pUC8 was then obtained by treating the supercoiled form with topoisomerase I (BRL, Bethesda, MD) according to the manufacturer's instructions. Finally, the substrate was phenol-extracted twice, ethanol-precipitated, resuspended in distilled water and stored at –20 °C.

Assay of DNA supercoiling. This assay measures the conversion of relaxed closed-circular pUC8 into the supercoiled form by the intervention of DNA-gyrase (a topoisomerase II) [3]. 500–100 U of the A and B subunits were first incubated at 21°C in a buffer containing 0.05 M Tris–HCl (pH 7.5), 10% glycerol, 0.2 mM EDTA, 5 mM dithiothreitol. The standard reaction mixture (70 μl) contained 300–400 ng of DNA substrate, 35 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 1.8 mM spermidine–HCl, 24 mM KCl, 0.14 mM EDTA, 5 mM dithiothreitol, 6.5% glycerol, 1.4 mM ATP, 9 μg/ml of *E coli* tRNA (Boehringher, Mannheim, Germany), 0.36 mg/ml of bovine serum albumin (Sigma) and 2 U of reconstituted enzyme, previously diluted in a buffer containing 50 mM Tris–HCl (pH 7.5), 0.2 M KCl, 1 mM EDTA, 5 mM dithiothreitol, 50% glycerol, 3.6 mg/ml bovine serum albumin and different drug concentrations. The solution was incubated at 37°C for 60 min and then extracted twice with an equal volume of chloroform–isoamyl alcohol (24:1, v/v). After a brief centrifugation, to the aqueous phase (50 μl) were added 12.5 μl of a mixture of 5% SDS, 25% glycerol and 0.25 mg/ml of bromophenol blue and the samples were loaded onto 1% agarose gels. Samples were electrophoresed with Tris–borate–EDTA buffer [3] at 40 V for 16 h. Gels were then stained with ethidium bromide and photographed with a Polaroid MP4 land camera under UV illumination. Negative films were scanned with a scanning densitometer to evaluate the amount of supercoiled DNA produced.

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